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Novel IRF6 mutations in Honduran Van Der Woude syndrome patients

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Abstract. Van der Woude syndrome (VWS) is an autosomal dominant inherited disease characterized by lower lip pits, cleft lip and/or cleft palate. Missense, nonsense and frameshift mutations in *IRF6* have been revealed to be responsible for VWS in European, Asian, North American and Brazilian populations. However, the mutations responsible for VWS have not been studied in Central American populations. Here, we investigated the role of *IRF6* in patients with VWS in a previously unstudied Honduran population. *IRF6* mutations were identified in four out of five VWS families examined, which strongly suggests that mutations in *IRF6* are responsible for VWS in this population. We reported three novel mutations and one previously described mutation. In the first family, a mother and daughter both exhibited a p.N88I mutation in the DNA-binding region of *IRF6* that was not present in unaffected family members. In the second, we found a unique p.K101QfsX15 mutation in the affected patient, leading to a frameshift and early stop codon. In the third, we identified a p.Q208X mutation occurring in exon 6. In the fourth, we found a nonsense mutation in exon 9 (p.R412X), previously described in Brazilian and Northern European populations. In the fifth, we did not identify any unique exonic missense, nonsense or frameshift mutations. This study reports the first cases of *IRF6* mutations in VWS patients in a Central American population, further confirming that the causal link between *IRF6* and VWS is consistent across multiple populations.

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Introduction

Cleft lip with or without cleft palate (CL/P) is a common congenital malformation, presenting in 1/500 to 1/2000 births, with increased prevalence in Hispanic, Native American and Chinese populations. CL/P occurs in non-syndromic or syndromic forms, with non-syndromic forms constituting the majority (~70%) of cases. Of the syndromic forms of CL/P, Van der Woude syndrome (VWS; OMIM 119300) is the most common. It is responsible for 2% of cases of clefting overall, with an incidence of 1/35,000 to 1/100,000 births (1). VWS is an autosomal dominant disease with greater than 90% penetrance, characterized by lower lip pits, cleft lip, cleft palate and hypodontia (2). Popliteal pterygium syndrome (PPS; OMIM 119500) is a related disorder and is characterized by cleft lip, cleft palate, lower lip pits, syndactyly, pterygia in popliteal fossa and digital and genital anomalies, among other malformations (3). Families have been reported to have members expressing both VWS and PPS (4), suggesting that these syndromes may exist as a spectrum. Kondo *et al* (5) found that missense, nonsense and frameshift mutations in *IRF6* were causal for VWS and PPS in North American, European and Brazilian patients. Since then, there have been further validating reports of *IRF6* mutations in VWS in Asian, European and Brazilian populations (4,6,7). In addition, single nucleotide polymorphisms in and around *IRF6* have also been revealed to be associated with non-syndromic forms of CL/P (8), highlighting the importance of this gene in orofacial development.

The function of the *IRF6* gene is poorly understood. It is part of the interferon regulatory factor family, which consists of 9 members. While other members are involved in infection response pathways, *IRF6* does not share this role. *IRF6* contains 10 exons and codes for a purported transcription factor, with exons 3-9 being the coding exons. Exons 3 and 4 constitute a tryptophan pentad repeat DNA-binding domain, while exons 7 and 8 form a Smad-interferon regulatory factor-binding domain. The DNA-binding domain, and to a lesser extent the protein-binding domain, are highly conserved.

We have previously reported an association between *IRF6* and non-syndromic CL/P in a Honduran population (9). Since previous studies in other populations demonstrated

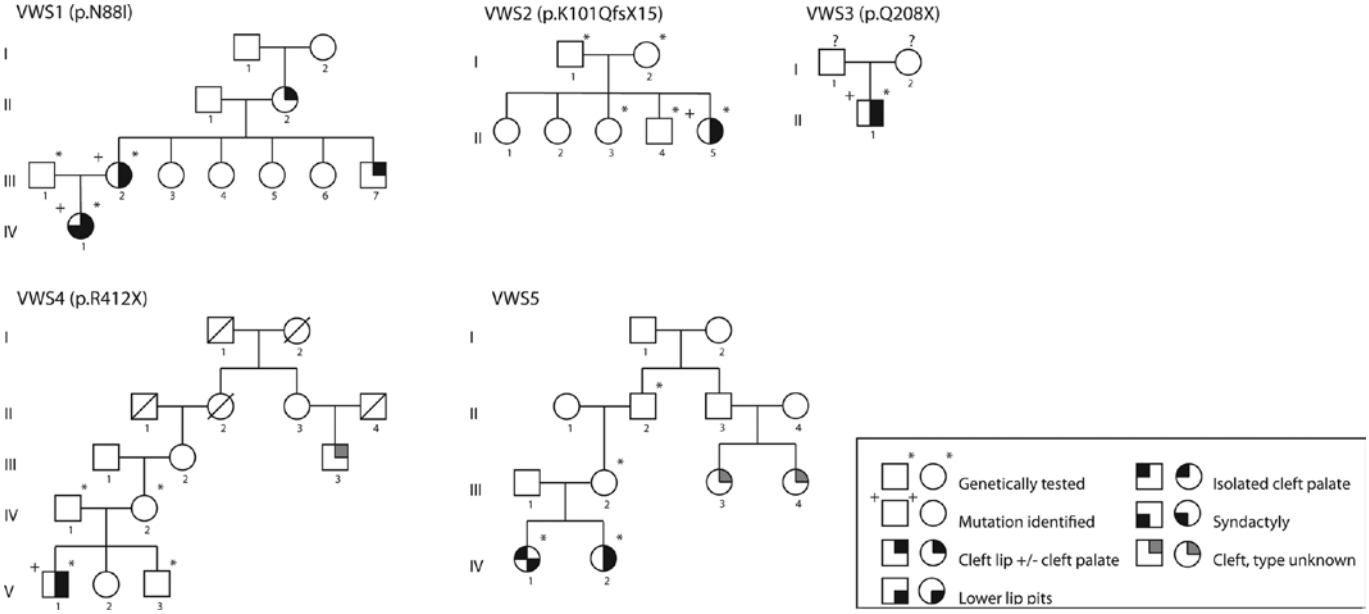

Figure 1. Family pedigrees of VWS patients. VWS1 reveals an autosomal dominant inheritance pattern. VWS2 reveals a *de novo* mutation in the patient, with no family history. VWS3 has an unknown family history. VWS4 reveals a *de novo* mutation in the patient and a distant relative exhibiting unknown cleft type. VWS5 reveals siblings with VWS. Notably, the mother's uncle has two daughters with clefts of unknown type.

that mutations in *IRF6* were associated with VWS, here we examined a potential pleiotropic effect of *IRF6* by extending our genetic study of clefting and *IRF6* to VWS in a Honduran population.

Materials and methods

Patients. The study was approved by the Columbia University Medical Center Institutional Review Board. We screened for patients with VWS in a pediatric cleft clinic affiliated with the Honduran Medical Institute and the Department of Plastic Surgery of the Hospital Escuela, Tegucigalpa, Honduras. The presence of VWS was verified by oropharyngeal inspection and identification of clefting and lower lip pits. Complete histories were obtained, physical examination was performed, and patients were screened to avoid environmental confounders, such as folate antagonists. When available, family members were also examined for VWS and PPS phenotypes. Family histories and pedigrees were constructed based on patient reports.

Control subjects (100) were selected from a pediatric population recruited from the emergency, orthopedic, plastic surgery and general surgery departments of the Hospital Escuela. Control subjects were screened for the absence of a family history of CL/P or other congenital anomalies.

Written informed consent was obtained from VWS patients and family members and the controls. Assent was obtained when applicable.

DNA samples. Venous blood was collected from patients with VWS and from available family members. Samples were shipped by overnight mail to the Columbia University Medical Center. Genomic DNA was isolated using the FlexiGene DNA-isolation kit (Qiagen, Valencia, CA, USA) per the manufacturer's protocol.

Mutation screening. Mutational analyses were performed using direct genomic sequencing. Primers were designed to cover the full exon and intron/exon boundaries for each exon individually, for exons 1-9 in *IRF6*. Primer sequences are available upon request. Polymerase chain reactions were performed to amplify each of the 9 exons individually from genomic DNA. Amplified DNA was then sent for DNA sequencing (Macrogen, Rockville, MD, USA). Sequences were analyzed using Sequence Scanner software (ABI, Foster City, CA, USA) and compared to consensus sequences using an NCBI BLAST search (http://blast.ncbi.nlm.nih.gov). We first sequenced all affected individuals to identify mutations and then genotyped all identified mutations in participating relatives and in controls. Sequences from patients with VWS were compared to control patient sequences to identify and exclude common population variants in *IRF6*. For all novel alterations, independent PCRs using both forward and reverse sequencing were performed to confirm a true mutation.

Results

Van der Woude syndrome families. Five families with VWS were identified from those attending the Honduran pediatric cleft clinic. One family (VWS1) had a history of three generations of CL/P, suggesting an autosomal dominant inheritance pattern (Fig. 1). Three other families (VWS2, VWS4 and VWS5) had no history of VWS in other generations, and physical inspection of available family members did not reveal signs of VWS (Fig. 1). One family (VWS5) consisted of a sibling pair affected by VWS with unaffected parents. One family (VWS3) consisted of a male patient, for whom family history was not obtained. An image of this patient's lower lip pits is depicted in Fig. 2. Pedigrees listing specific phenotypes are shown in Fig. 1.

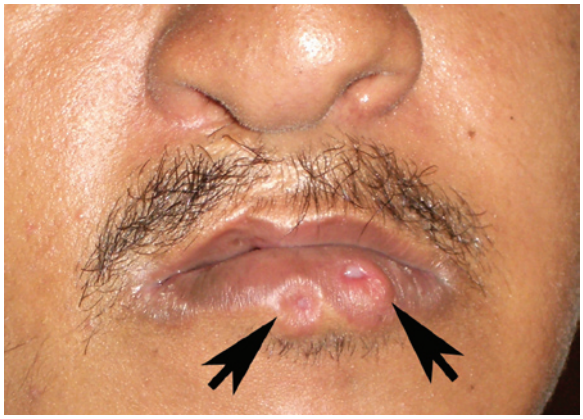

Figure 2. Patient with VWS from family VWS3 exhibiting lower lip pits (arrows) and repaired right cleft lip.

IRF6 mutations. Overall, four exonic *IRF6* mutations in five families (a total of 5/7 affected individuals) were identified. The four identified mutations consisted of missense, nonsense and frameshift mutations (Table I) and were absent in unaffected family members. Three of the four mutations (p.N88I, p.K101QfsX15 and p.Q208X) are novel and have not been reported previously.

A novel c.263A>T mutation causing an asparagine to isoleucine missense mutation was found in exon 4 (p.N88I) in both an affected mother and daughter (VWS1; Fig. 3A). A novel single base pair duplication mutation was identified (c.300dup C) in exon 4 of another proband (VWS2; Fig. 3A), resulting in a frame shift and an early stop codon 15 amino acids downstream in exon 4 (p.K101QfsX15). A novel c.622C>T mutation leading to a nonsense mutation (p.Q208X) was confirmed in exon 6 (VWS3; Fig. 3A). We also identified a c.1234C>T mutation leading to p.R412X, a nonsense mutation in exon 9 (VWS4; Fig. 3A) that has been previously described in Brazilian and Northern European populations (6). No unique exonic missense, nonsense or frameshift mutations were found in our fifth family (VWS5), although a c.921C>T mutation resulting in a conserved serine to serine mutation was found in exon 7 in the affected sibling pair (p.S307S). The unaffected mother had the same mutation.

Control population samples. To determine the frequencies of these mutations in the general Honduran population, 100 control subjects were sequenced. None of the unique

Table I. Exonic *IRF6* mutations in VWS families

Family	Mutation type	nt change	aa change	Exon
VWS1	Missense	c.263A>T	p.N88I	4
VWS2	Frameshift	c.300dup C	p.K101QfsX15	4
VWS3	Nonsense	c.622C>T	p.Q208X	6
VWS4	Nonsense	c.1234C>T	p.R412X	9
VWS5	Conserved	c.921C>T	p.S307S	7

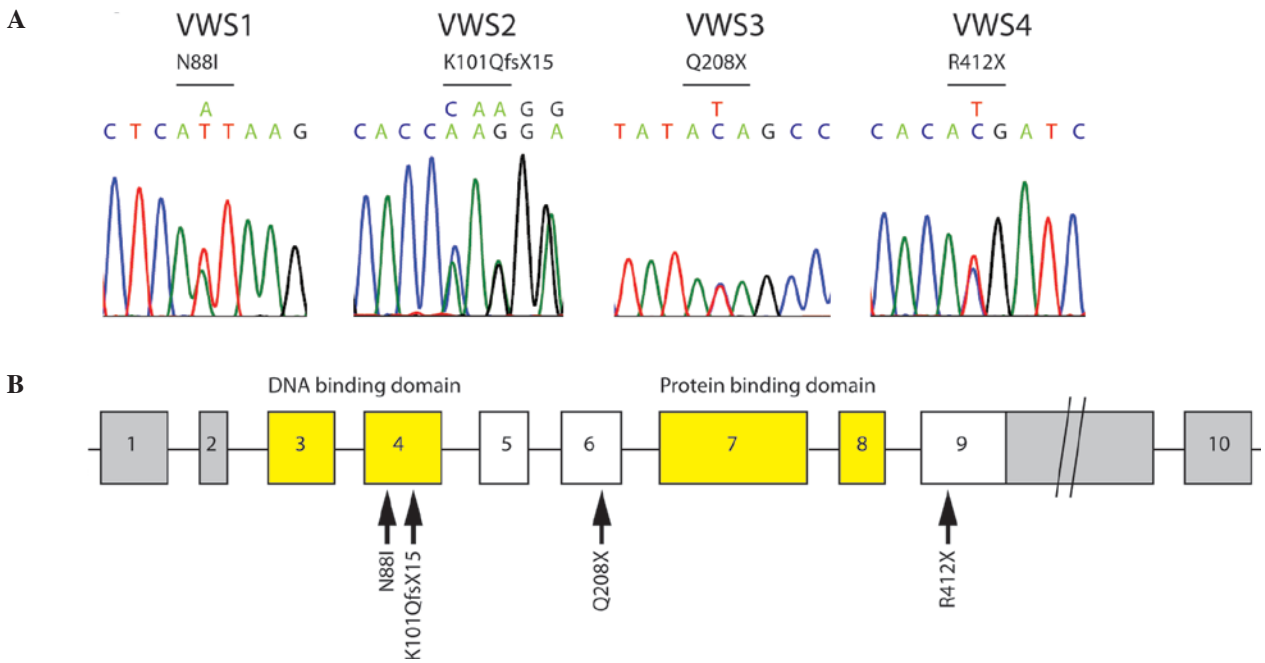

Figure 3. (A) Chromatogram sequences of mutations in *IRF6*. VWS1, Asn (AAT) to Ile (ATT) in exon 4; VWS2, C duplication leading to Lys (AAG) to Gln (CAA) frameshift in exon 4; VWS3, Gln (CAG) to Stop (TAG) in exon 6; VWS4, Arg (CGA) to stop (TGA) in exon 9. (B) Diagram of the *IRF6* gene revealing mutation locations. Non-coding exons are in gray; DNA and protein binding domains are in yellow. Figure is drawn to scale except for exon 9, which is truncated.

mutations were identified in the control subjects. The previously described c.820G>A polymorphism (causing a p.V274I change) (8) was found in control subjects with a minor allele frequency of 0.37. This polymorphism was not found in any of the patients with VWS. No other unique missense, nonsense or frameshift mutations were found in the control patients.

Discussion

This is the first study to describe genetic mutations in VWS in a Central American population. Overall, we found four unique mutations in *IRF6* in five patients, with three novel mutations that have not previously been reported. In each case, one copy of *IRF6* was mutated and the other was normal, consistent with a haploinsufficiency model.

A unique mutation (p.N88I) was found in exon 4 of our first family (VWS1). The asparagine to isoleucine change is from a polar to non-polar amino acid. Using a protein structure damage predictor (Polyphen), this p.N88I mutation is thought to be damaging to the *IRF6* protein structure. Asparagine 88 to histidine, tyrosine and serine mutations were previously described in VWS patients (5,6), further suggesting that this is an important conserved residue. Mutations at this site may alter *IRF6* gene function, likely through DNA-binding ability. The syndactyly in the affected daughter is consistent with previous reports that mutations in the DNA-binding region of *IRF6* (exons 3 and 4) are associated with an increased likelihood of PPS findings (6), and that PPS and VWS findings are found in the same lineage (4).

The second pedigree (VWS2) contained an individual with a *de novo* insertion in exon 4 of *IRF6*, leading to a frame shift resulting in an early stop codon within exon 4. Insertion mutations in *IRF6* causing VWS have previously been described (6), but are less common than missense mutations. This mutation leads to both a disruption of the DNA-binding domain (exon 4) and to a complete loss of the protein-binding domain (exons 7 and 8).

A unique p.Q208X nonsense mutation was identified in exon 6 of the third patient (VWS3). This mutation is in a linker region between the DNA-binding and protein-binding domains of *IRF6* (Fig. 3B). It is a less common region for mutations in VWS, although cases in other populations have been described (6). This early stop codon prevents the transcription of the protein-binding domain, and may alter the final structure of the *IRF6* protein.

An arginine to stop mutation was found in exon 9 in the patient from the VWS4 family. The patient's mother, father and sibling all had normal *IRF6* sequences. De Lima *et al* (6) found multiple individuals with VWS with p.Arg412X mutations in Northern European and Brazilian populations. The p.R412X mutation is thought to be a 'hotspot' mutation because it contains a CpG dinucletotide that is easily methylated to a T (10), as in this case, where it converted arginine (CAG) into a stop (TAG) codon.

A c.921C>T mutation leading to a conserved serine residue was found in exon 7 in a sibling pair from the VWS5 family. The unaffected mother had the same mutation, suggesting that this is not the causal mutation for VWS. No other mutations were identified in the exons of these patients. It has been suggested that approximately 68% of mutations in *IRF6* causing VWS are in exonic regions (6), with the remainder likely in non-exonic *IRF6* regions. The lack of a causative exonic mutation in this sibling pair suggests an unidentified non-exonic mutation in *IRF6*, or possibly a mutation in another gene. Interestingly, the mother's uncle had two daughters with clefting of unknown type (Fig. 2, VWS5 III-3 and III-4), suggesting a family predisposition to clefting with other contributing variants.

In all families, the exons and bordering introns of available relatives of patients with VWS were sequenced. The results revealed that family members did not carry the mutations in *IRF6*, further supporting that these are indeed the causal mutations for VWS. A total of 100 control patients were sequenced, none of whom carried unique missense, nonsense, or frameshift mutations in *IRF6*, further supporting previous reports that mutations in *IRF6* are integral to the presence of VWS.

Much remains to be discovered in regards to the function of *IRF6*. It is thought to act as a transcription factor due to the presence of a DNA-binding domain and the observation that other members of the IRF family function as transcription factors (6). Mice deficient in *IRF6* reveal a defect in keratinocyte proliferation and differentiation, leading to abnormal skin, limb and craniofacial development, including CL/P and PPS features (11,12). The transcription factors p63 and AP-2α have been revealed to be upstream regulators of *IRF6* expression (13,14), and mutations in these genes lead to syndromes involving CL/P, suggesting a common pathway (15,16). Despite these recent advances, further studies are necessary to establish the function of *IRF6*.

A recent study in India did not find exonic *IRF6* mutations in a 13-patient cohort, and suggested that *IRF6* may not be responsible for VWS in a mixed Indian population (17), highlighting that various genes may be involved in different populations. Moreover, past groups have reported that the alternate chromosomal regions 1p34 (18) and 17p11 (19) may be associated with VWS. Thus, the verification of mutations in *IRF6* in VWS in a previously unstudied Central American population was crucial. Here we identified mutations in *IRF6* in patients with VWS in a population from Honduras, further strengthening results from other populations indicating that *IRF6* mutations cause VWS.

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