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# Three Novel Mutations of the *IRF6* Gene With One Associated With an Unusual Feature in Van der Woude Syndrome

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Van der Woude syndrome (VWS) is a dominantly inherited disorder characterized by cleft lip with or without cleft palate and lip pits. It remains the most common syndromic form of oral clefts. Mutations in the *interferon regulatory factor 6* (*IRF6*) gene have been identified in patients with VWS. We reported three unrelated families with lower lip anomalies. Two had lower lip pits, a cardinal sign of VWS, but the other had a heart-shaped mass on lower lip without pits, oral clefts, or hypodontia. This isolated anomaly has not been previously observed in VWS. We performed mutation analysis by PCR-sequencing the entire coding region of the *IRF6* gene. Three potentially pathogenic mutations, c.145C>T (p.Q49X), c.171T>G (p.F57L), and 1306C>G (p.L436V) were successfully identified. All the missense mutations were not detected in 100 unaffected ethnic-matched control chromosomes and have never been previously reported. The p.Q49X and p.F57L mutations were located in the highly conserved DNA binding domain while the p.L436V was located at the carboxy-terminal region. This study reported an undescribed clinical feature of VWS and three novel mutations, expanding the phenotypic spectrum of VWS and mutational spectrum of *IRF6*.


**Key words:** Van der Woude syndrome; *IRF6*; Thai; novel mutations

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## INTRODUCTION

Van der Woude syndrome (VWS, OMIM 119300) is the most common oral cleft syndrome characterized by pits and/or sinuses of the lower lip, cleft lip with or without cleft palate, and hypodontia [Van Der Woude, 1954; Schinzel and Klausler, 1986]. It is inherited in an autosomal dominant manner with a high degree of penetrance and variable expressivity [Shprintzen et al., 1980; Lacombe et al., 1995]. VWS is caused by mutations in the *interferon regulatory factor 6* (*IRF6*) gene [Kondo et al., 2002]. This gene is also responsible for the popliteal pterygium syndrome (PPS), a disorder sharing the clinical features of VWS with the addition of popliteal and oral webs and genital anomalies (OMIM 119500) [Kondo et al., 2002]. It encodes a protein product which belongs to a family of nine transcription factors sharing a highly conserved helix-turn-helix DNA-binding domain (amino acids 13–113) and a less conserved protein-binding domain (amino acids 226–394) termed SMIR (Smad-interferon regulatory factor-binding domain) [Eroshkin and Mushegian, 1999]. Most IRFs are regulators of host defense after viral infection [Taniguchi et al., 2001]. However, little is known about the function of IRF6.

At least 200 different mutations in the *IRF6* gene have been described with the majority being missense/nonsense mutations [de Lima et al., 2009] (http://www.hgmd.cf.ac.uk, accessed March 2009). Most of the mutations are located in the regions encoding the conserved DNA-binding and SMIR domains suggesting a critical role of each domain for IRF6 function.

In this study, we performed mutation analysis of all the coding region of the *IRF6* gene in three Thai unrelated individuals with VWS. A novel mutation was identified in each case.

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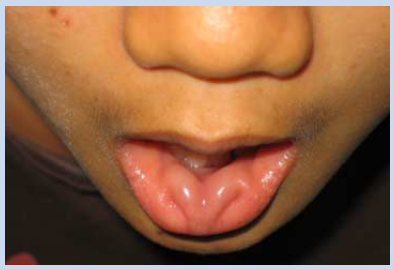

**FIG. 1. A heart-shaped mass at the midline of lower lip found in Patient 1.**

## MATERIALS AND METHODS

### Patients

Patient 1 was a 16-year-old female with bilateral conical elevations without pit joining at the midline of the lower lip. It looked like a heart-shaped mass (Fig. 1). No other anomalies were found. Since her parents were divorced, she had been staying with her father. She was told that her mother and some other relatives had similar findings. None of them were available for examination. Patient 2 was a 23-year-old female with lower lip pits and surgically repaired bilateral cleft lips without cleft palate. Patient 3 was an 8-year-old girl with lower lip pits without other anomalies. Patients 2 and 3 were sporadic. Written informed consent was obtained from all patients or their parents included in the study.

### Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes according to standard protocols. Direct sequencing of PCR-amplified DNA representing the entire coding region of *IRF6* was performed as previously described [Kondo et al., 2002; Shotelersuk et al., 2003]. The PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH), according to the manufacturer's recommendations, and sent for direct sequencing in both directions at the Macrogen, Inc. (Seoul, Korea). The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI). For each novel missense mutation, restriction enzyme digestion was used to confirm its presence in the patient as well as to screen in 100 control chromosomes from unaffected ethnic-matched individuals. The c.171T>G (p.F57L) and the c.1306C>G (p.L436V) mutations eliminate the *Mse*I and the *Eco57*I sites, respectively. The *Mse*I and the *Eco57*I restriction enzymes were therefore used to screen for the presence of each mutation.

### Protein Sequence Comparison

IRF6 orthologues were first identified through a BLAST search of the non-redundant database using Homo sapiens IRF6, accession NP_006138 as the reference sequence. All known and complete IRF6 sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by ClustalX 1.81 program. The human IRF6 was aligned with chimpanzee (Pan troglodytes; XP_514168), cow (*Bos taurus*; NP_001070402), mouse (*Mus musculus*; NP_058547), xenopus (*Xenopus tropicalis*; AAI58988) and zebrafish (*Danio rerio*; NP_956892). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

## RESULTS

Analysis of the *IRF6* gene by PCR-sequencing revealed three different sequence variants, one from each patient (Table I). To our knowledge, all these variants have never been previously reported. Patient 1 harbored a heterozygous nonsense mutation, a C → T transition at nucleotide position 145 (c.145C>T) in exon 3 expected to result in changing a glutamine at amino acid position 49 into a stop codon (p.Q49X) (Fig. 2, left upper panel). The mutation is located in the DNA-binding domain. Although she seemed to be a familial case, samples from other affected members were unavailable for analysis.

A heterozygous mutation for a 171T>G at nucleotide position 171 in exon 3 was identified in Patient 2 (Fig. 2, middle upper panel). This was expected to result in a phenylalanine to leucine substitution at codon 57 (p.F57L). This mutation is located at a highly conserved residue within the DNA-binding domain (Fig. 3). Patient 3 was found to harbor a single base transversion, c.1306C>G in exon 9 leading to the substitution of a leucine by a valine at position 436 (p.L436V) (Fig. 2, right upper panel). It locates in the C-terminus 3′ to all currently known functional domains. The mutation also occurs at the evolutionarily conserved residue found in other species (Fig. 3).

One hundred ethnic-matched unaffected control chromosomes were screened for the presence of both missense mutations by restriction enzyme digestion of the PCR products. Neither of them was observed (data not shown).

## DISCUSSION

We described three unrelated Thai patients who had lower lip anomalies. Two patients had lip pits which were the cardinal features of this syndrome. One of these two also had cleft lip. Interestingly, Patient 1 presented with bilateral conical elevations joining at the midline of the lower lip without any pit, cleft or

**TABLE I. *IRF6* Mutations Identified in Thai Patients With VWS**

| Patients | Characteristics of patients | Mutations | Amino acid change |
|---|---|---|---|
| 1 | Female, familial, a heart-shape mass on the lower lip | c.145C>T | p.Q49X |
| 2 | Female, sporadic, lip pits and cleft lip | c.171T>G | p.F57L |
| 3 | Female, sporadic, lip pits | c.1306C>G | p.L436V |

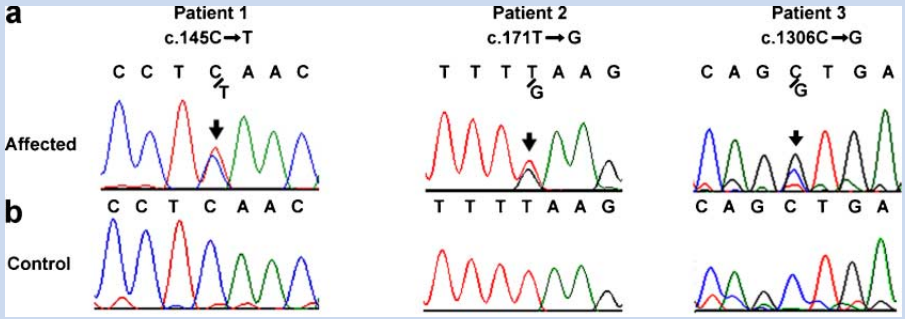


**FIG. 2. Mutation analysis. The left, middle, and right upper panels relate to c.145C>T, c.171T>G, and c.1306C>G mutations, respectively. a: Electropherograms of patients, showing the mutations (arrows). b: Electropherograms of controls showing normal genotypes.**

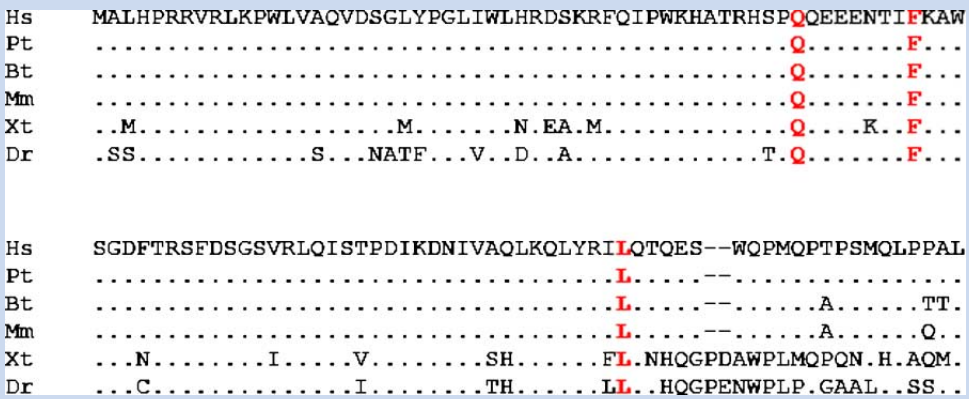


**FIG. 3. Sequence alignment of the DNA-binding domain (upper) and the carboxy-terminal region (lower) of IRF2 from different species. The site of the amino acid variant found in this study is indicated in bold red in all conserved species. Sites that are 100% conserved across all sequences are indicated by dots (.). Hs, Homo sapiens; Pt, Pan troglodytes; Bt, *Bos taurus*; Mm, *Mus musculus*; Xt, *Xenopus tropicalis*; Dr, *Danio rerio*.**

hypodontia. To our knowledge, this isolated feature has not been previously reported in VWS [Rizos and Spyropoulos, 2004]. Since this patient had only a heart-shaped mass, she had not been diagnosed with VWS. We however decided to perform mutation analysis of the *IRF6* gene in this patient along with Patients 2 and 3.

PCR-sequencing the entire coding region of *IRF6* successfully identified mutations in all three patients. All were novel missense/nonsense mutations affecting the well conserved DNA-binding domain or the carboxy-terminal region.

Patient 1 was heterozygous for a single base transition, c.145C>T in exon 3 resulting in changing a glutamine at amino acid position 49 into a stop codon (p.Q49X). The nonsense mutation (p.Q49X) presumably results in the formation of drastically truncated protein lacking parts of the DNA binding domain and the entire SMIR region. Since the patient with a heart-shaped mass on the lower lip without other features known to VWS was heterozygous for the p.Q49X mutation, she was then diagnosed with this syndrome. This unusual feature could be a rare entity of VWS. It would be interesting to study the histology of the mass. Unfortunately, the patient denied a biopsy of the mass.

The newly identified c.171T>G mutation affecting the DNA binding-domain was detected in Patient 2. This was expected to result in a phenylalanine to leucine substitution at codon 57 (p.F57L). The substitution occurred in the well-conserved DNA-binding domain (Fig. 3), possibly making the IRF6 protein unable to bind DNA. Other evidence also supports it as a disease-causing mutation. PolyPhen (http://coot.embl.de/PolyPhen/) and SIFT (http://blocks.fhcrc.org/sift/SIFT.html) predict it to be possibly damaging and deleterious, respectively. It was not detected in 100 ethnic-matched control chromosomes.

Patient 3 was heterozygous for a missense mutation in exon 9, c.1306C>G (p.L436V). Among presumably pathogenic missense mutations previously reported in the C terminus outside the protein-binding domain (amino acids 226–394) [Kondo et al., 2002; Kayano et al., 2003; Wang et al., 2003; de Lima et al., 2009], the p.L436V mutation identified in this study was located at the most 3′

position. Several lines of evidence support its possible etiologic role. First, the leucine at codon 436 is located at a highly conserved residue (Fig. 3). Second, this variant has not been reported to be a polymorphism in NCBI SNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), Ensembl (http://www.ensembl.org/index.html), or PupaSUITE/PupaSNP (http://pupasuite.bioinfo.cipf.es/) databases. And lastly, it was not detected in 100 ethnic-matched control chromosomes. However, there is other evidence suggesting that it is not pathogenic. Using PolyPhen and SIFT, this variant is predicted to be tolerated. In addition, this position is not located in a known functional domain. Functional characterization is required to elucidate the significance of this change.

Previous studies identifying deletions encompassing the VWS locus as well as mutations causing protein-truncation (nonsense and frameshift) suggested that the phenotype would be caused by haploinsufficiency [Bocian and Walker, 1987; Kondo et al., 2002; Ghassibe et al., 2004; Peyrard-Janvid et al., 2005; Ye et al., 2005]. This study has identified one potentially protein truncating mutation as well as two substitutions occurring in the highly conserved regions that could lead to loss of IRF6 function. Our observations further support that haploinsufficiency of IRF6 is the major mechanism underlying VWS and confirm the crucial role of IRF6 in orofacial development.

In summary, we reported three unrelated patients with lower lip anomalies. One had a heart-shaped mass on the lower lip, an unrecognized feature, expanding the phenotypic spectrum of VWS. Three potentially pathogenic mutations, c.145C>T (p.Q49X), c.171T>G (p.F57L), and 1306C>G (p.L436V) were identified. They have not been previously reported, expanding the mutational spectrum of *IRF6*.

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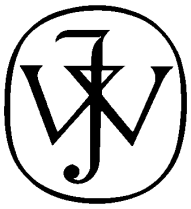

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