

Short Report

Epidemiology of Van der Woude syndrome from mutational analyses in affected patients from Pakistan

Malik S, Kakar N, Hasnain S, Ahmad J, Wilcox ER, Naz S. Epidemiology of Van der Woude syndrome from mutational analyses in affected patients from Pakistan.

Clin Genet 2010; 78: 247–256. © John Wiley & Sons A/S, 2010

Mutations in *IRF6* cause Van der Woude syndrome (VWS), one of the most common syndromes associated with cleft lip (CL) with or without cleft palate (CP). The presence of pits on the lower lip of patients is the most characteristic feature of the syndrome. We have identified three novel and seven previously reported *IRF6* mutations in 12 of 16 unrelated families segregating VWS from Pakistan. The three newly identified mutations include a frameshift (c.568delG) and two missense mutations c.295G>A (p.G99S) and c.1219T>C (p.S407P). Recent functional studies on *IRF6* and the three-dimensional structure of *IRF5* carboxy (C) terminus, a protein encoded by a paralog of *IRF6*, shed light on the p.S407P substitution. Additionally, the identification of the same mutations responsible for VWS in Pakistan, as reported in other global populations worldwide, marks these residues as mutational hotspots and indicates their essential role in structural stability or function of *IRF6*. This is the first study of VWS in Pakistan and we estimate that 1 in 100 patients with CL with or without CP (CL/P) are affected in the Pakistani population predominantly from the Punjab area.

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Key words: cleft lip – cleft palate – *IRF6* mutations – Pakistan – Van der Woude syndrome

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Received 21 November 2009, revised
and accepted for publication 4 January
2010

Orofacial clefts including cleft lip and cleft palate (CL/P) are a major structural birth defect with the worldwide incidence roughly 1 in 500–1000 births (1, 2). More than 400 syndromes include cleft lip (CL) with or without cleft palate (CP) in their etiology. Van der Woude syndrome (VWS) (MIM# 119300) is one of the most common accounting for about 2% of all cases of CL/P worldwide (3).

VWS is inherited in an autosomal dominant manner and has a high-penetrance rate (96.7%) with intrafamilial and interfamilial variation of the associated phenotype. The distinctive feature of VWS is the presence of lower lip pits and/or sinuses, which are present in approximately 85%

of cases. In some rare cases, a single barely visible pit might be the only distinguishable feature of VWS. Other anomalies that are frequently associated with VWS include hypodontia, submucous CP and bifid uvula (4). Infrequent anomalies include congenital adhesion of the jaw, narrow arched palate and ankyloglossia (tongue tie) (5).

The clinical features of VWS closely resemble Popliteal Pterygium Syndrome (PPS; MIM# 119500). PPS is also inherited in a dominant fashion but lip pits are less common (45.6%). Additional features include skin webbing, genital abnormalities, syndactyly of fingers and/or toes, and abnormal skin around the nails. Both VWS and PPS are caused by mutations in interferon

regulatory factor 6 (*IRF6*), located on chromosome 1 (6). Also, up to 18% of non-syndromic cases of CL/P may be accounted for by variants in *IRF6* (7, 8). *IRF6* has two non-coding and seven coding exons. *IRF6* belongs to a family of nine transcription factors that share a highly conserved helix-turn-helix DNA-binding domain and a less conserved protein-binding domain termed Smad-interferon regulatory factor-binding domain (SMIR).

The incidence of VWS has been reported as 1 in 75,000–1:100,000 live births (9, 10). Although Pakistan is reported to have the fourth largest population of individuals with CL with or without CP [Unpublished data from cleft lip and palate association of Pakistan (CLAPP)], no data are available on the incidence of VWS in the Pakistani population. However, no formal epidemiological studies have been conducted in Pakistan for this disorder. A small study on the incidence of CL/P was conducted in the North-West Frontier province (NWFP) in Pakistan. A total of 106 patients with CL/P and associated syndromes were examined and 1 patient with CL/P was reported to have VWS (11). No molecular characterization of *IRF6* in patients with VWS has been previously performed in Pakistan.

Mutations in any of the coding or non-coding exons of *IRF6* result in VWS, whereas mutations predominantly affecting one residue encoded by exon 4 and a few other mutations in exons 3, 4, 6 and 9 have been reported to cause PPS (4, 6, 12–28). These mutations have been reported in patients of diverse nationalities but there are no reports of *IRF6* mutations from South Asia.

Mutations, which cause PPS, are mostly confined to the DNA-binding domain and are thought to act via a dominant-negative effect in contrast to the mechanism of haploinsufficiency proposed for VWS (6). However, the finding of some cases in which the same mutation can cause either VWS or PPS demonstrates that there are exceptions and the genotype–phenotype correlation may also depend on genetic or environmental modifiers (29).

In order to identify the contribution of *IRF6* mutations to VWS in Pakistan, we performed sequencing analysis of *IRF6* on DNA from individuals affected with VWS from 16 unrelated families. We have identified three novel and seven known mutations segregating with the phenotype in 12 of these families. We have also identified two additional residues in *IRF6*, which are likely mutational hotspots.

Materials and methods

Subjects

Institution Review Board (IRB) approvals were obtained at School of Biological Sciences, University of the Punjab, Balochistan University of Information Technology and Management Sciences and Brigham Young University, Provo, UT, USA. Sampling was completed after getting written informed consent from all participants.

Fifteen probands with a phenotype consistent with VWS were recruited from the Plastic Surgery and Shalimar hospitals in Lahore and various locations in the Punjab province with the help of CLAPP from 2007 to 2009. Extended pedigrees were drawn and other members were recruited in the studies whenever possible. One family (VWS-NU1) was recruited from the Balochistan province of Pakistan.

Lip pits were present in all affected probands of the recruited families. Seven families had multiple individuals with CL/P, lip pits, bifid uvula, or hypodontia. The probands and some additional members of each family were clinically examined by doctors at the hospitals or those who were part of the CLAPP team. One of the authors (S. M.) was trained at a hospital for detection of submucous palate and other anomalies associated with CL with or without CP and she examined some family members and patients with VWS at their homes. As all families reside in remote areas of Punjab, Azad Kashmir and Balochistan, it was not possible to conduct clinical examinations for every individual. Moreover, affection status for many individuals related to the probands was assigned through oral histories.

Controls

Anonymous unaffected individuals with no history of orofacial clefts or lip pits in their families were recruited as normal controls. Blood was collected for DNA isolation purposes. Two hundred DNA samples were obtained from the Punjab province and another 80 from Quetta, Balochistan. We had very few control DNA samples from Azad Kashmir.

Frequency calculations

In order to calculate the frequency of VWS among CL/P patients in the Punjab province, we considered the number of all patients with CL/P who arrived at the camps or hospitals for treatment from which a patient with VWS was recruited. In total, there were 12 probands with

VWS among 966 individuals with CL/P from the Punjab province. The number of probands with VWS recruited from Azad Kashmir is 3 out of 313 individuals with CL/P. Family VWS-NU1 was recruited through personal contacts from Balochistan and was therefore excluded from the frequency calculations.

Mutation analyses

Genomic DNA was extracted from whole blood by a non-organic method involving lysis, proteinase K digestion and salting out of DNA with isopropanol precipitation (30, 31). In order to identify mutations in *IRF6*, PRIMER 3 software was used to design polymerase chain reaction (PCR) primers for the nine coding and non-coding exons, which included flanking upstream and downstream intron–exon boundaries (GenBank NM_006147). PCR was performed in a final volume of 50 μ l using 50-ng genomic DNA, KCl buffer (Fermentas, Glen Burnie, MD), 1–3 mM MgCl₂, 0.2 mM dNTPs, 0.24 μ M of each primer, and 0.5 units of *Taq* DNA polymerase (Fermentas). Unincorporated dNTP and primers were removed by precipitation with ethanol or purification through columns (Qiagen, GmbH, Germany). Sequencing reactions were performed using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA). The sequencing PCR reactions were precipitated with ethanol, dissolved in Hi-Di formamide (Applied Biosystems, Foster City, CA) and run on ABI PRISM 310 or ABI 3730 DNA analyzers. PCR products were sequenced bidirectionally for all affected individuals and all nine exons of *IRF6* were sequenced for the 16 families.

Each mutation was checked in the control population through allele-specific PCR [amplification refractory mutation system (ARMS)]. We also directly sequenced exons 4 and 9 from 200 control DNA samples. To perform Tetra primers ARMS PCR (32), we designed specific primers (sequences are available on request) for the transition and transversion mutations using a web-based program http://cedar.genetics.soton.ac.uk/public_html/primer1. Conditions for PCR amplification were as recommended (32) except for the reduction of the concentration of *Taq* DNA polymerase units by half. In some cases, the concentration of allele-specific primers was also reduced by half without changing the concentration of the outer primers. Additionally, we varied MgCl₂ concentration from 1.8 to 2.5 mM. Tetra primers ARMS PCR were performed using a touchdown PCR protocol, optimized for detection of each mutation. To detect the mutation c.568delG in

control samples, we designed primers and performed a competitive allele-specific PCR assay as described in Ref. 33 except for the reduction of final PCR volume from 50 to 10 μ l. As a positive control for each set of allele-specific PCR reactions, we included DNA from the affected individuals known to carry the respective mutation, whereas DNA from an individual known to lack the particular mutation was used as a negative control.

In order to confirm the deletion mutation, c.568delG, we ligated the PCR amplified product from VWS-SM2 patient into a T/A cloning vector (Fermentas) and transformed the ligation mixture into DH5 α competent bacterial cells. Colony PCR was performed on six randomly picked white bacterial colonies, obtained after blue/white screening on Ampicillin/X-gal/IPTG agar plates. The colony PCR products were sequenced after purification as described earlier.

Linkage analysis for VWS-SM4 was performed with microsatellite markers closely flanking *IRF6* in both upstream and downstream directions from the gene, using standard protocols. In brief, five markers D1S1663, D1S245, D1S491, D1S205 and D1S425 were amplified in a final volume of 10–20 μ l and resolved either on ABI 310 or on a 6% polyacrylamide, 8 M urea gel followed by visualization through a fast silver staining method (34).

Results

Twelve probands were identified among 966 individuals with CL/P, between 2007 and 2009, from hospitals and camps in the Punjab province, 3 probands out of 313 CL/P individuals were identified from Azad Kashmir, whereas one was recruited independently from Balochistan. Thus, the frequency of VWS in patients with CL/P from Punjab and Azad Kashmir was 1.24% and 1%, respectively. Patients with CL with or without CP, or their families who attended the camps were questioned about relationships to confirm that all cases were truly independent and represent random cases of CL with or without CP. However, it is possible that some patients with CL/P were unaware of their relationships or were excluded because they did not have visible lip pits and therefore the estimate of 1 in 100 VWS among CL/P patients is an underestimate as the worldwide incidence is 2% (3).

In total, 36 patients were clinically examined and found to have VWS in 16 families, whereas a further 30 individuals were reported by different members of the families to have phenotypes

consistent with VWS (Fig. 1). The phenotypic features of individuals with VWS in the clinically examined individuals are summarized in Table 1. There is inter- and intrafamilial variability of phenotype. Lip pits were present in 93.75% of all examined patients, whereas CL was present in 71.87% and CP in 59.37% of the patients. Only 9.37% of the affected individuals had a bifid uvula.

We identified 10 mutations in affected individuals in families with VWS patients, 3 of which were novel. The segregation of the mutations with the phenotype was verified for families with multiple affected individuals. However, for families VWS-SM1, VWS-SM2, VWS-SM6, VWS-SM8, VWS-SM10, VWS-SM11, VWS-SM12, VWS-SM13, VWS-SM14, and VWS-SM15 we had DNA samples from the probands only.

In family VWS-SM2, we identified a deletion of guanine at nucleotide 568 (Fig. 2a) in the sample from the proband. The parents of the proband did not exhibit any clinically detectable signs of VWS, however, because we did not have their DNA samples, we could not confirm if this was a *de novo* mutation.

A novel transition mutation c.295G>A in exon 4 was identified for the proband of family VWS-SM15 resulting in a missense substitution p.G99S (Fig. 2b), whereas in all patients in family VWS-SM3, we identified a transition of c.1219T>C in exon 9, leading to the substitution p.S407P (Fig. 2c).

In total, we identified seven known mutations in *IRF6* in VWS patients (Table 2). For patients in families VWS-SM9 and VWS-SM14, we identified a transition mutation of c.250C>T in exon 4 resulting in a missense substitution p.R84C in *IRF6*.

In family VWS-NU1 from Balochistan, we discovered the transition of c.749G>A. In family VWS-SM12, we identified a transition mutation c.961G>A. Both mutations are located in exon 7 of *IRF6* and result in missense substitutions (p.R250Q and p.V321M, respectively). In family VWS-SM6, we identified a transition mutation, c.1122C>G in exon 8 causing a missense substitution, p.C374W.

We also identified three known mutations in exon 9 in four families. For family VWS-SM5, we identified the transition mutation c.1198C>T resulting in the substitution p.R400W; and for family VWS-SM7, we identified the transition mutation c.1199G>A leading to amino acid substitution p.R400Q. In families VWS-SM1 and VWS-SM10, we identified the same transition mutation, c.1234C>T leading to a non-sense mutation p.R412X.

We screened a total of 200 control samples for both newly identified missense mutations, p.G99S and p.S407P, and did not detect them in any sample. All other mutations were absent in at least 100 DNA samples from ethnically matched controls as determined by sequencing or allele-specific PCR reactions (competitive or Tetra primers ARMS PCR). These mutations could be detected with corresponding allele-specific primers in respective patients' samples in each competitive or Tetra primer ARMS PCR, demonstrating the robustness of each assay (Fig. 2d,e).

We were unable to identify mutations in the coding and the non-coding exons for four of the families VWS-SM4, VWS-SM8, VWS-SM11 and VWS-SM13. There are only single affected individuals in families VWS-SM8, VWS-SM11 and VWS-SM13 and therefore we could not perform linkage analysis for these families. Only two of the five genotyped markers (D1S1663 and D1S425) were informative for family VWS-SM4. All 10 affected individuals (I:1, II:2, II:6, II:8, II:11, II:12, II:15, III:1, III:2, and III:4) shared the same haplotype on one of the chromosomes for these two markers, whereas the mother (I:2) and other unaffected individuals (II:4, II:9, II:14, II:16, II:17 and III:3) did not share the putative disease chromosome (Fig. 1). It seems likely that the phenotype in family VWS-SM4 is linked to *IRF6* and the mutation has been missed in our mutation screening strategy. Patients in family VWS-SM4 were heterozygous for single nucleotide polymorphisms (SNPs) in exons 4, 5 and 7 excluding the possibility of a complete deletion of *IRF6*. The mutation could lie in intronic or regulatory sequences or could involve a partial genomic deletion.

For the family VWS-SM13, we identified a mutation c.239A>G, leading to a conservative substitution p.K80R that was not observed in 200 normal controls from Pakistan. However, the samples were not ethnically matched and p.K80R might be a common polymorphism in Azad Kashmir, the city of origin of family VWS-SM13. p.K80R substitution is in the DNA-binding domain of *IRF6*. As it substitutes a positive amino acid with a similarly charged amino acid, it may not be pathogenic. On the other hand, it might be the causative mutation as similar conservative substitutions in the DNA-binding region of proteins (35, 36), or silent changes in the region containing exonic splicing enhancer (ESE) can disrupt splicing mechanism and/or RNA editing and can have far reaching effects on the resultant protein (37). Additional control samples from Azad Kashmir need to be analyzed to identify if this mutation is a polymorphism and functional

Mutational analysis of *IRF6* in Pakistani population

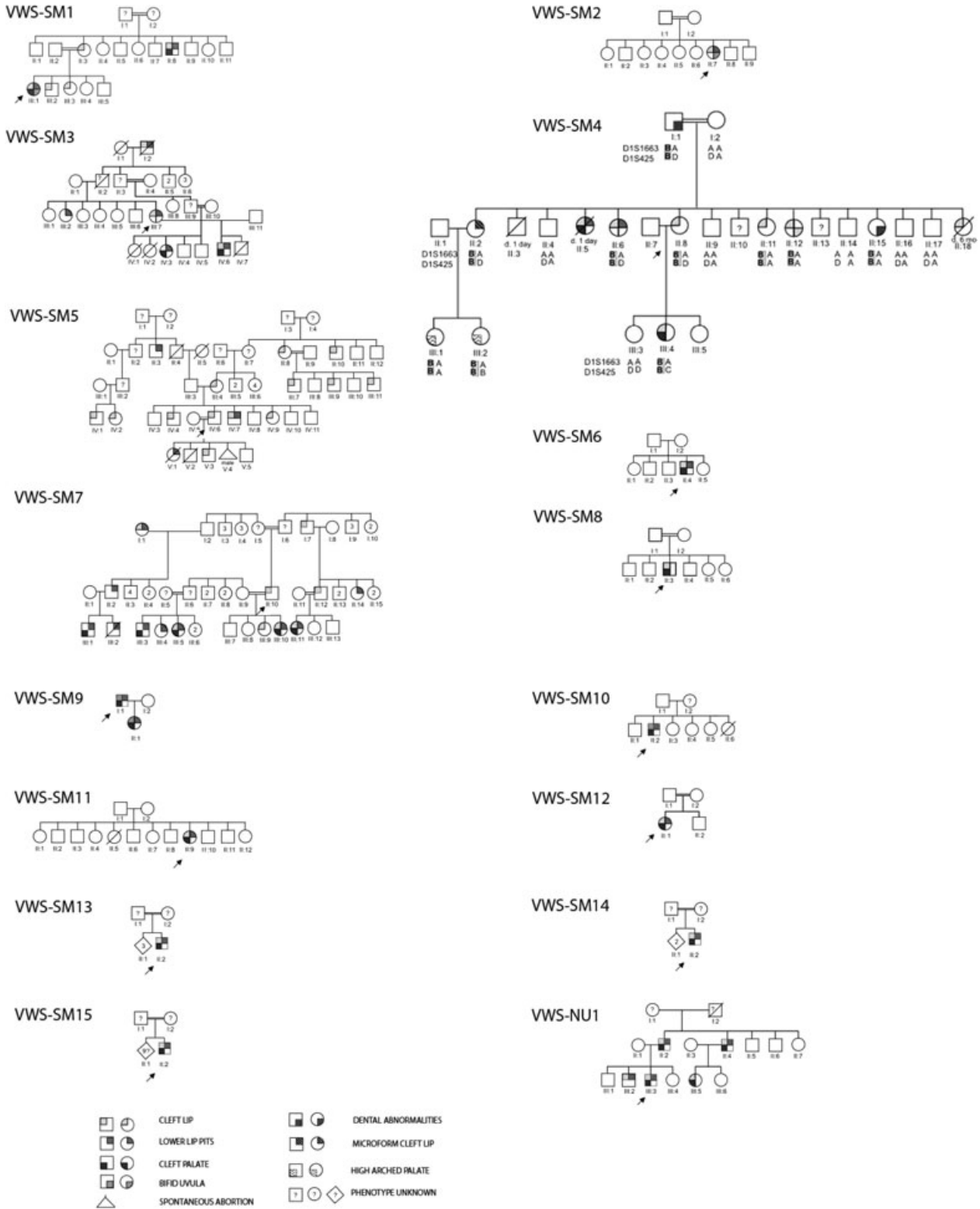


Fig. 1. Family trees for 16 pedigrees with patients affected by Van der Woude syndrome (VWS). Probands are indicated by an arrow. Haplotypes for the two informative chromosome 1 markers close to *IRF6* are shown for family VWS-SM4. The putative disease chromosome is shaded in gray.

Table 1. Phenotypic features for patients with Van der Woude syndrome (VWS) who participated in this research^a

| Individuals | VWS-SM1 | | VWS-SM2 | | VWS-SM3 | | VWS-SM4 | | | | | | | |
|------------------------------------|---------|------|---------|------|---------|------|---------|------|-------|-------|-------|-------|-------|-------|
| | III:1 | II:7 | III:7 | IV:6 | I:1 | II:2 | II:6 | II:8 | II:11 | II:12 | II:15 | III:1 | III:2 | III:4 |
| Lip pits | + | + | + | + | - | - | + | + | + | + | - | - | - | + |
| Cleft lip | + | + | + | + | - | - | + | - | - | - | - | - | - | - |
| Microform cleft lip | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| Cleft palate | + | - | - | + | - | - | - | - | - | - | - | - | - | + |
| Bifid uvula | + | + | - | - | - | - | - | - | - | + | - | - | - | - |
| Submucous cleft palate | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Missing teeth/dental abnormalities | - | + | - | - | + | - | - | - | - | - | + | - | - | - |
| High-arched palate | - | - | - | - | - | - | - | - | - | - | - | + | + | - |

| Individuals | VWS-SM5 | | VWS-SM6 | | VWS-SM7 | | | | VWS-SM8 | | VWS-SM9 | | VWS-SM10 | |
|------------------------------------|---------|------|---------|-----|---------|-------|-------|-------|---------|------|---------|------|----------|---|
| | IV:6 | IV:7 | II:4 | I:1 | II:2 | II:10 | III:1 | III:9 | III:10 | II:3 | I:1 | II:1 | II:2 | |
| Lip pits | + | + | + | + | - | + | - | + | + | + | + | + | + | + |
| Cleft lip | - | + | + | + | + | - | + | - | + | - | + | + | + | + |
| Microform cleft lip | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cleft palate | - | - | + | - | - | - | + | - | + | + | + | + | + | + |
| Bifid uvula | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Submucous cleft palate | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Missing teeth/dental abnormalities | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| High-arched palate | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| Individuals | VWS-SM11 | | VWS-SM12 | | VWS-SM13 | | VWS-SM14 | | VWS-SM15 | | VWS-NU1 | | |
|------------------------------------|----------|------|----------|------|----------|------|----------|------|----------|------|---------|-------|-------|
| | II:9 | II:1 | II:1 | II:1 | II:2 | II:2 | II:2 | II:2 | II:2 | II:2 | II:4 | III:2 | III:3 |
| Lip pits | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Cleft lip | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Microform cleft lip | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Cleft palate | + | + | + | + | + | + | + | + | + | + | + | - | + |
| Bifid uvula | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Submucous cleft palate | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Missing teeth/dental abnormalities | - | - | - | - | - | - | - | - | - | - | - | - | - |
| High-arched palate | - | - | - | - | - | - | - | - | - | - | - | - | - |

^aInformation is provided for only those individuals who were clinically examined; +, phenotype present; -, phenotype absent.

assays performed to determine the pathogenicity of this mutation.

Discussion

VWS is a common syndrome, identified by the presence of lip pits in the majority of patients with CL with or without CP. Although it is a heritable syndrome, *de novo* mutations are quite frequent as evident by all mutational reports so far. This study describes the spectrum of mutations in *IRF6* in the Pakistani population, specifically from the Punjab province.

Three of the 10 mutations identified in our work have not been reported previously. The c.568delG mutation in exon 6 of *IRF6* is predicted to result in a change of the *IRF6* reading frame introducing a stop codon within exon 6 after misincorporation of 34 amino acids. As this mutation is in an internal exon of *IRF6*, it is possible that the mutation marks the mRNA for non-sense mediated

decay, and no protein is produced from the mutant allele, resulting in haploinsufficiency of *IRF6*. This mechanism for pathogenesis has been proposed for mutations that cause VWS (6).

The missense substitution, p.G99S, is within the DNA-binding domain of *IRF6*, but it is difficult to predict how it may affect its structure and function. However, it is noteworthy that missense substitutions of amino acid residues Asp 98 and Thr 100, upstream and downstream p.G99S substitution, respectively, also result in VWS (4, 6, 25).

The p.S407P substitution is located in the domain encoded by exon 9 of *IRF6*. As this mutation is present in DNA of all affected members of the family VWS-SM3 and is absent from DNA of 200 control individuals, the mutation is predicted to be disease causing. Previously, a frameshift mutation has been reported involving serine residue 407, which results in VWS (38). The novel missense substitution p.S407P pinpoints

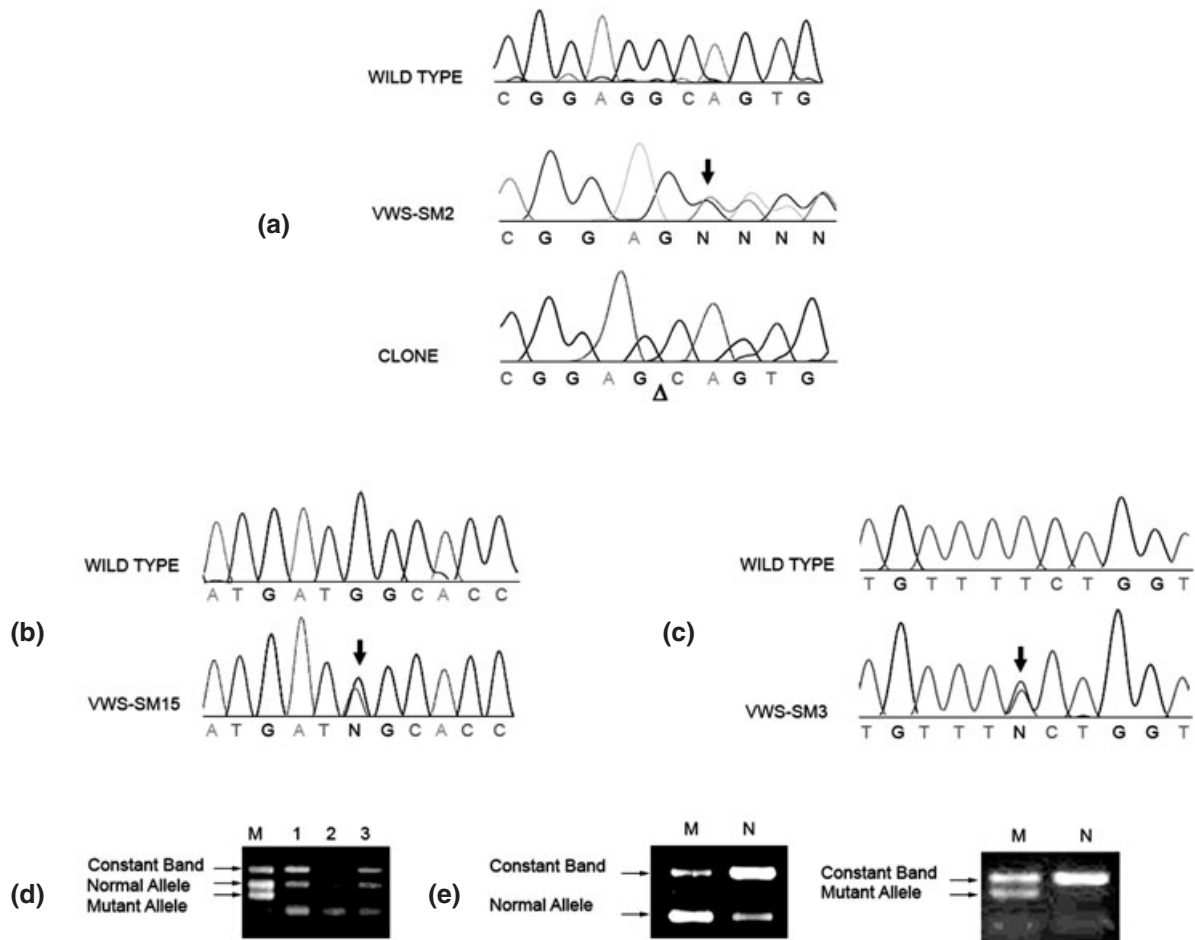


Fig. 2. Electropherograms for the three novel mutations identified in families VWS-SM2, VWS-SM15 and VWS-SM3. In each case, the wild-type sequence is uppermost and the arrows indicate the site of the mutations. **(a)** A heterozygous deletion of a guanine residue was observed in exon 6 of *IRF6*, resulting in indecipherable sequence after the mutation in the DNA of proband from family VWS-SM2. ‘ Δ ’ denotes the deletion of ‘G’ in the sequence trace obtained from a bacterial colony transformed with exon 6 polymerase chain reaction (PCR) product from the patient. **(b)** Transition mutation c.294 G>A in exon 4 was observed for family VWS-SM15. **(c)** c.1219 T>C transition mutation was identified on sequencing exon 9 PCR product in family VWS-SM3. **(d)** Results of Tetra primers amplification refractory mutation system (ARMS) PCR for mutation c.1234 C>T. PCR products were resolved on 2% agarose gel for detection of mutant and normal alleles in DNA from a patient in family VWS-SM1 (lane M) and normal control samples (lanes 1–3). The uppermost band is the constant band detected in the four lanes, whereas the lower band is detected due to the presence of mutant allele. The middle band is detected due to the wild-type allele. The mutant band is detected only in the sample known to carry the mutant allele, whereas the wild-type allele is detected in all samples. **(e)** Analysis of competitive allele-specific PCR for the c.568delG mutation. Lanes ‘M’ have DNA from the proband in family VWS-SM2, whereas lanes ‘N’ have DNA from a normal control. The first gel depicts the results when primers specific to the normal allele were added to the PCR mix, whereas in the last gel primers used were specific for the mutation. The mutant allele is detected in only the patient’s DNA as expected. Upper band in all lanes is the constant band showing robustness of the PCR assays.

a direct and significant role of this residue for *IRF6* structural stability or function. A three-dimensional crystal structure for *IRF6* has not been reported. However, a three-dimensional structure of the transactivation domain has been reported for the protein encoded by *IRF5*, (39), a paralog of *IRF6*. *IRF6* exhibits 49% amino acid identity and 63% overall similarity to *IRF5*. This similarity extends to 80% in the C-terminal domain (Fig. 3) thus suggesting that the two proteins may have similar three-dimensional structures. Clustal W

alignment of *IRF6* and *IRF5* reveals that this region is highly conserved across many vertebrate species (Fig. 3).

Members of the *IRF6* family exist as monomers in an inactive form in the cytoplasm (29). Activation through phosphorylation of serine residues in the C-terminal domain is important in overcoming this inhibition, leading to dimerization and subsequent localization to the nucleus (39) where they control DNA transcription of target genes after recruitment of other transcription factors (39). The

Table 2. List of mutations identified in *IRF6* after mutational analysis on DNA from participating patients affected with Van der Woude syndrome (VWS)

| Family | Type of mutation | Nucleotide change ^a | Amino acid | Exon | Domain | First report |
|----------|------------------|--------------------------------|-------------|------|--------------------|-----------------------|
| VWS-SM1 | Non-sense | c.1234C>T | p.R412X | 9 | Trans ^b | Kondo et al. (6) |
| VWS-SM2 | Frameshift | c.568delG | p.A190fsX34 | 6 | | This report |
| VWS-SM3 | Missense | c.1219T>C | p.S407P | 9 | Trans | This report |
| VWS-SM4 | | | | | | |
| VWS-SM5 | Missense | c.1198C>T | p.R400W | 9 | Trans | Wang et al. (18) |
| VWS-SM6 | Missense | c.1122C>G | p.C374W | 8 | SMIR ^c | Kondo et al. (6) |
| VWS-SM7 | Missense | c.1199G>A | p.R400Q | 9 | Trans | de Lima et al. (25) |
| VWS-SM8 | | | | | | |
| VWS-SM9 | Missense | c.250C>T | p.R84C | 4 | DNA binding | Matsuzawa et al. (16) |
| VWS-SM10 | Non-sense | c.1234C>T | p.R412X | 9 | Trans | Kondo et al. (6) |
| VWS-SM11 | | | | | | |
| VWS-SM12 | Missense | c.961G>A | p.V321M | 7 | SMIR | Kondo et al. (6) |
| VWS-SM13 | | | | | | |
| VWS-SM14 | Missense | c.250C>T | p.R84C | 4 | DNA binding | Matsuzawa et al. (16) |
| VWS-SM15 | Missense | c.295G>A | p.G99S | 4 | DNA binding | This report |
| VWS-NU1 | Missense | c.749G>A | p.R250Q | 7 | SMIR | Kondo et al. (6) |

^aNumbers with respect to the open reading frame of *IRF6*, corresponding to cDNA sequence from GenBank (AK312857) with the first nucleotide in the initiation codon designated as '+1'.

^bTransactivation domain.

^cSmad-interferon regulatory factor-binding domain.

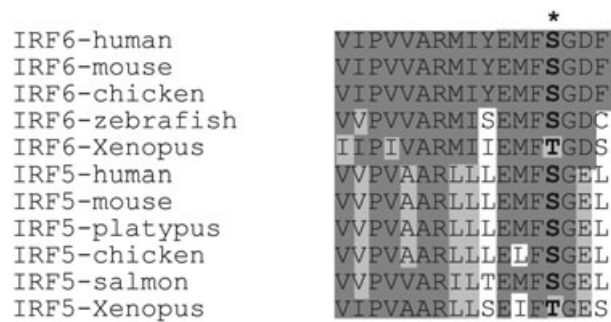


Fig. 3. Clustal W alignment of the carboxy end of pfam10401 showing the conservation of *IRF6* Ser 407 to *IRF5* Ser 421. The asterisk marks the position of all conserved residues corresponding to Ser 407 with respect to human *IRF6* and are shown in bold. Dark gray shading denotes identical amino acids, whereas light shading indicates similar amino acid residue substitutions. Only *IRF5* and *IRF6* are shown in this alignment because of the homology between these two proteins as seen in the Tree families database (<http://www.treefam.org>) (40), Accession No. TF328512, which also lists the GenBank accession numbers for all of the above proteins.

residue Ser 421 in human *IRF5* is homologous to the residue Ser 407 in *IRF6* (Fig. 3). The role of Ser 421 for *IRF5* activity was demonstrated by mutating Ser 421 to aspartic acid. The presence of aspartic acid in place of serine is equivalent to a phosphorylated serine (39). Functional analysis showed that p.S421D results in decreased binding of *IRF5* to other interacting proteins, indicating that Ser 421 is not directly involved in phosphorylation leading to activation of *IRF5*. However, Ser 421 is part of an α -helix (Helix 4) in

IRF5 (39). As Ser 421 is homologous to Ser 407 in *IRF6* and as proline can disrupt α -helices, the p.S407P missense mutation may destabilize the secondary structure of *IRF6*, resulting in instability of the mature protein.

Our inability to detect the disease-causing mutations in four families with VWS patients may be due to confining our analyses to exons of *IRF6* and the surrounding intron–exon boundaries. We did not screen for large deletions within the *IRF6* gene or regulatory sequences. Large deletions leading to VWS have been identified in other patients with VWS who were similarly negative for mutations in the exons of *IRF6* (12). Additionally, point mutations far into the introns may also lead to pathogenesis, as is true in other disorders such as retinitis pigmentosa (41). However, the lack of mutations in *IRF6* could also indicate the involvement of some other gene in the etiology of VWS in light of the finding that 32% of families with VWS do not have a mutation in *IRF6* (25).

The other seven mutations present in Pakistani families affected with VWS have been previously described from similarly affected patients around the world. Mutations p.R250Q, p.V321M and p.C374W have been described in Caucasian, Belgian and Brazilian populations, whereas the mutation p.R400W has been reported in families from China, Sweden, Belgium and Brazil.

The missense substitution p.R84C and the non-sense mutation c.1234C>T (p.R412X) have been most widely reported and are found in multiple

families from Brazil, Northern Europe, Germany and China (25). The presence of the same mutations in VWS patients from Pakistan as reported in patients of other nationalities is interesting because these are not likely to be founder mutations. Four of the mutations that we identified in families from Pakistan with VWS are known to involve nucleotide residues, which are hotspots for mutations and encode p.R84, p.R250, p.R400 and p.R412 (25). However, the nucleotide residues encoding amino acids p.V321 and p.C374 are not known mutational hotspots. Mutations in codons for p.V321 and p.C374 identified in two families from Pakistan have been previously reported twice in different patients of North European and Brazilian ancestries, thus bringing the total number of mutations at each residue to three with this report. The mutations affecting p.C374 involve two different substitutions, p.C374R and p.C374W, showing two independent mutational events involving the same codon thus indicating that they originated separately in the two families. The finding of these mutations in different populations can be thus interpreted in two ways. Either nucleotides encoding these residues are also hotspots for mutations or, alternatively, these mutations recur by chance due to the critical role of the two amino acids residues for IRF6 structural stability or function.

It is interesting to note that the p.R84C and p.R412X substitutions have been reported in patients diagnosed with either VWS or PPS. The patients in our study were diagnosed with VWS and no clinical signs of PPS were identified or reported by patients and their families. However, it is possible that genital hypoplasia, one of the subtle signs of PPS, may have been present that was not evaluated in this study.

Eight of the 10 mutations identified in our work are located in exons 6–9. Five of the 12 families with VWS in our study have a mutation in exon 9. Future work will show whether mutations in exon 9 are over-represented in a cohort of patients with VWS in Pakistan. Exon 9 encodes IRF6 amino acid residues 394–467. So far, 19 mutations have been reported in exon 9 of *IRF6*. All mutations in exon 9 are reported to result in VWS, except for three mutations associated with PPS (6, 25). The amino acids encoded by exon 9 in *IRF6* are homologous to those residues in IRF5, known to be involved in overcoming autoinhibition of IRF5 (39). This domain contains the serine residues important for phosphorylation-related activation of IRF5 (39). Recent work has also shown that residues 223–467 encode the transcription activation domain of IRF6 (29).

The calculated 1% frequency of VWS in Pakistan is lower than the 2% frequency of that reported in different world populations, which may be due to the fact that the patients in the current study were recruited from hospitals specifically treating orofacial clefts. Many individuals with VWS manifest only lip pits and therefore do not need orofacial surgery at the hospitals from which probands were recruited. Alternately, the finding of 1% frequency of VWS might reflect the true incidence of this syndrome because in a previously published study conducted in NWFP, Pakistan, only 1 of 106 patients with CL/P was found to have VWS (11).

Our research is the first report of *IRF6* mutations in VWS in a Pakistani population. The identification of previously identified and novel mutations in *IRF6* is useful information for genetic counselling of families affected with VWS. The known mutations highlight the importance of these amino acid residues for IRF6 function and identify two other residues as potential mutational hotspots, whereas the novel mutations add to the current repertoire of mutations in *IRF6*.

Acknowledgements

We are grateful to Dr Ghulam Qadir Fayyaz, Services Hospital, Lahore, President of Cleft Lip and Palate Association Pakistan (CLAPP), Mr Ejaz Ahmed, Mr Azam Shehzad, and Mr Shehzad Ahmed at Plastic Surgery Hospital, Lahore, and the administration of Shalimar Hospital, Lahore, for facilitating access to patients with CL/P. We thank Mr Zeddullah (School of Biological Sciences, University of the Punjab) for his untiring efforts throughout the project. We express our gratitude to all the patients and their families for participating in this research. This work was funded by an intramural Brigham Young University Mentoring Grant, USA. The generous financial support of Higher Education Commission, Pakistan, is also acknowledged.

Conflict of interest

The authors declare that they have no conflict of interest.

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