Short Report

Novel lip pit phenotypes and mutations of \textit{IRF6} in Van der Woude syndrome patients from Pakistan


The role of interferon regulatory factor 6 (\textit{IRF6}) gene mutations in causing Van der Woude syndrome (VWS) and popliteal pterygium syndrome has been described in different populations worldwide. The former is one of the major syndromes of cleft lip and/or cleft palate (CL/P) with the distinct phenotype of presence of pits with or without sinuses on the lower lip. We identified seven probands with VWS from Punjab province of Pakistan and recognized two previously unreported lip pit phenotypes. The mutational analysis of \textit{IRF6} in this cohort revealed four novel and two previously reported mutations. The newly identified mutations include three frameshifts (c.635delG; c.21_33del13; c.627delC) and one transition mutation (c.2T>C) affecting the first codon of \textit{IRF6}. Together with a past epidemiological study on VWS in Pakistan, the frequency of this syndrome among CL/P individuals from Punjab was calculated to be 1.17%.

**Conflict of interest**

There is no conflict of interest declared by the authors.

Mutations of \textit{IRF6} cause congenital orofacial abnormalities including Van der Woude syndrome (VWS, MIM\#119300) and popliteal pterygium syndrome (PPS, MIM\#119500). The presence of lower lip pits and/or sinuses are considered a hallmark of VWS which are usually present in about 85% of VWS patients (1). Some of the other frequently associated anomalies of VWS include hypodontia, bifurcated uvula, and submucous cleft palate (2). VWS is reported to account for 2% of all cases of cleft palate (CL/P) in most world populations (1).

There is clinical overlap in the features of VWS and PPS. Skin webbing, genital abnormalities, syndactyly of fingers/toes, and abnormal skin around the nails are additional to the presence of lip pits/sinuses in PPS. Both syndromes are allelic variants of \textit{IRF6} (3). The coding exons of \textit{IRF6} include exons 3–9. Exon 3 and exon 4 encode the DNA-binding domain while exons 7 and 8 encode the protein-binding domain of IRF6. Different mutations in the DNA-binding domain are known to disrupt the function of IRF6 and therefore result in defects in orofacial development (2). Similarly, mutations in the protein-binding domain affect the role of IRF6 as a cooperative transcriptional activator (4).

Incidence of VWS is reported as 1:75,000–1:100,000 live births (5, 6). The incidence of VWS in Pakistan is not known but the frequency of VWS among individuals with CL/P was previously determined to be 1 in 100 (7). Here we extend our analyses and report new phenotypes and mutations in \textit{IRF6}.

**Materials and methods**

The approval for this study was obtained from the Institutional Review Board at the School of Biological Sciences, University of the Punjab, Lahore and Brigham Young University, Provo. Informed written consent was taken from all the participants.

Four probands with VWS were identified from Plastic Surgery hospital in Lahore and three probands were recruited from different camps held in Punjab by cleft lip and palate association of Pakistan.
criteria for the diagnosis of VWS were the presence of lip pits or lip bumps in the proband with or without orofacial clefts. Additional patients were identified in the extended families with or without lip pits. All patients and their available family members were examined for the presence of overt cleft lip, narrow or high arched palate and sub-mucous cleft palate. The details regarding the presence of oral clefts, lip pits, and presence of abnormal lip phenotype were ascertained from the proband and close relatives when additional members could not be recruited. DNA from 200 ethnically matched individuals with no family history of CL/P (7) were available as a control.

Blood samples were processed for DNA extraction (7). Polymerase chain reaction (PCR) products for all exons of IRF6 including the immediate intron–exon boundaries were sequenced bidirectionally for the participants. The mutations were also checked by Sanger sequencing or allele-specific PCR in the controls (7, 8).

Results

Four new and two previously described mutations were identified in IRF6. Two of the novel mutations were identified in exon 3. The proband of family VWS-SM17 (Fig. 1a) exhibited lip pits, unilateral complete cleft at the left side of the lip and cleft palate (Fig. 1b). The mother of the proband was phenotypically normal and no history of orofacial clefts or lip pits in other members of the family was reported. A 13 bp deletion, c.21_33del(AGTCCGGCTAAAG) leading to a frameshift and introducing a pre-mature stop codon p.S212fsX12.

In family VWS-SM19 (Fig. 2d), the proband presented a lip pit that was surgically repaired in childhood and was therefore only visible in the form of a healed blister with unilateral complete cleft lip at the left side (Fig. 2e). There was no apparent family history of orofacial clefts or lip pits. However, upon clinical examination of the proband’s father, an overt cleft lip was suspected which could not be explored further by ultrasonography. Sequencing revealed a deletion in exon 6, c.627delC (Fig. 2f) causing a frameshift p.209fsX15.

Two known mutations c.610 C>T (p.R204X) and c.1234 C>T (p.R412X) were identified in VWS-SM18 and VWS-SM21, respectively (data not shown). Both probands presented classical bilateral lip pits, placed symmetrically on either side of the midline of the lower lip in addition to cleft lip and cleft palate (data not shown). The identified mutations in IRF6 were absent in 200 normal controls as assessed by tetra-primer allele specific PCR or sequencing.

In VWS-SM20 (Fig. 1d) there was no reported history of orofacial clefts or lip pits in the family and only the phenotypically normal mother I:2 and the proband were available for clinical examinations. The patient’s lips were apparently normal but a thorough examination revealed the presence of a single lower lip pit that was present on the inner side of the lip, below the vermilion border (Fig. 1e). A bumpy mass was present on the other side of the pit. The proband had an incomplete cleft palate. On sequencing IRF6, a new mutation was found in exon 3 in the proband’s sample. This was a transition mutation c.2T>C (Fig. 1f) affecting the codon of first methionine residue.

Two novel mutations in exon 6 of IRF6 were identified which segregated with the phenotype. In family VWS-SM16 (Fig. 2a), there were four affected individuals with variable manifestations of VWS related phenotypes. The proband presented lip pits, unilateral complete cleft lip at the right side and complete cleft palate (Fig. 2b). The manifestation of lip pit phenotype was different in the father I:1 as compared with that of the proband. He had a lower lip with irregular surface marked by undefined pits and elevations (Fig. 2b). This lip phenotype has not been previously described. One of the proband’s sister and brother also had elevations on the lower lip according to the history obtained from the parents. On DNA analysis, a heterozygous deletion in exon 6 (c.635delG) was identified (Fig. 2c) resulting in a frameshift p.S212fsX12.

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We were unable to identify a mutation in the known exons of IRF6 for VWS-SM22 (Fig. 2g). The affected individual presented a small blister-like bump on the lower lip in addition to cleft lip and cleft palate (Fig. 2h). The presence of blister-like mass was also reported in one paternal aunt of the proband in the absence of visible oral clefts.

Discussion

The role of IRF6 mutations and polymorphisms in VWS and non-syndromic CL/P, respectively, is well established in different world populations (3, 9). This study has extended the number of known mutations in IRF6 as well as provided new phenotypes associated with VWS. The finding of irregular surface of the lower lip in the presence of undefined pits and elevations in individual I:1 of family VWS-SM16, and the presence of an unusual lip bump in the form of a small solid blister in the proband of VWS-SM22, were the novel phenotypes. However, in the absence of a detected mutation in IRF6 in VWS-SM22, it is uncertain whether the phenotype observed in this family is truly a sign of VWS or an incidental finding. This will be clarified in future after analyses of IRF6 by copy-number analyses and sequencing of regulatory regions. Moreover, VWS is molecularly heterogeneous and it is possible that a mutation in a gene at another locus such as 1p34 cause the disorder in VWS-SM22.

The classical phenotypes of lip related to VWS include the presence of lip pits/sinuses or bumps on the lower lip. The association of unusual phenotypes of different lip phenotypes such as the presence of lip pits on the inner side of the lower lip is known to be present in few individuals with VWS. Similarly, pits on the upper lip and commissural pits are findings that can be found in association with VWS (10, 11).
Novel lip pit phenotypes and mutations of IRF6

Fig. 1. Families, phenotypes and mutations of IRF6. (a) Pedigree of family VWS-SM17 with presence of lower lip pits, cleft lip and cleft palate in the proband. Double line indicates a consanguineous union. (b) Photograph of the lower lip of the proband. The arrow indicates the presence of a prominent lip pit. (c) Electropherograms of part of exon 3 of IRF6. The 13 bp deletion (AGTCCGGCTAAAG) results in double sequence after the site of deletion. (d) Pedigree of family VWS-SM20 in which the proband had lip pits and cleft. (e) Photographs of the lower lip of the proband, in the left photo the arrow indicates the presence of a mass on the inner right side of the lip while in the right photo the arrow is pointing towards a lip pit. (f) Electropherogram from part of exon 3. The forward sequence of the proband’s DNA with a transition c.2T>C (indicated by an arrow) leads to an amino acid change p.M1T in the protein transcript. The wild-type DNA sequence is given above the mutant sequence. A key for the phenotypes of the pedigree is given below the chromatograms.

Additionally, irregular surface of the lower lip and swollen appearance of skin below the lower lip indicate the likely presence of underneath nodules and can be a sign for identification of VWS (12). The variable lower lip pit morphology as evident by the presence of heart-shaped mass around the pits or the presence of slit-like pits was described in different affected individuals within one family (13, 14). It is interesting to note that all mutations in IRF6 which result in unusual lip phenotypes are reported in VWS and not PPS. Secondly, the unusual lip phenotypes are found both inherited in large pedigrees and also due to de novo mutations. All previously reported mutations associated with the unusual phenotypes are either missense or nonsense mutations, in contrast to the deletion mutation in IRF6 which we identified in this work. Our research suggest that the type of mutation has no correlation in predicting the severity, or form of lip phenotype.

In this study, all novel mutations except one are predicted to mark the mRNA for nonsense-mediated decay with no protein production from the mutant alleles leading to haploinsufficiency of IRF6. We were unable to demonstrate this experimentally as we could not prepare cDNA specific to IRF6 from RNA derived from blood samples of the patients (data not shown), probably because of its low endogenous level. The c.635delG mutation is predicted to result in a change of the IRF6 reading frame and introduce a stop codon within exon 6. As this mutation is in an internal exon of IRF6 it is highly likely to invoke nonsense-mediated decay of the mutant mRNA. The other deletions, c.627delC and c.21_33del(AGTCCGGCTAAAG) may play similar role in pathogenesis.

A transition mutation c.2T>C leading to a codon change of methionine to threonine is also predicted to result in a functional null allele. Methionine is the first
Malik et al.

(a) Family pedigree of VWS-SM16 with multiple affected individuals manifesting varying phenotypes of Van der Woude syndrome (VWS). (b) Lip photograph (left) of I:1 showing the presence of uneven elevations and pits (indicated by arrows). The lip photograph at right shows large pits in the proband. (c) Bidirectional electropherograms from part of exon 6 showing the c.635delG mutation in VWS-SM16. The arrow marks the position of the deletion. Both forward and reverse wild-type DNA sequences are also shown at the top and under the patient’s chromatograms, respectively. (d) Pedigree of family VWS-SM19 showing lip pits, cleft lip and cleft palate in the proband. The father of the proband I:1 was suspected to have overt cleft lip. (e) The photograph of the lower lip in the proband showed the presence of a single lip pit (indicated by an arrow) which was surgically repaired in childhood and looked more like a healed blister. (f) Chromatogram showing a frameshift mutation caused by a deletion of ‘C’ (c.627delC). The position of deleted ‘C’ is indicated by an arrow both in the forward and the reverse sequence. The forward and reverse wild-type DNA sequences are also shown. (g) Family pedigree of VWS-SM22. (h) Arrow indicates an unusual lip phenotype in the form of a small solid blister present on the lower lip. This phenotype was observed in the proband, III:4. Similar lip phenotype was also reported to be present in her paternal aunt (II:2). A key for the phenotypes of the pedigree is given below the chromatograms.

The mutation p.R412X found in this study has been described in affected patients from many countries including Pakistan in a known mutational hotspot (7, 16). The mutation p.Q204X identified in VWS-SM18 has been reported in VWS patients of European, and Brazilian ancestry (16) and probably marks the mRNA for nonsense-mediated decay.

In summary, we have described novel lip phenotypes which can be helpful in the diagnosis of patients of
VWS. The different lower lip phenotypes observed in this study emphasizes the importance of careful examination for positive identification of individuals with VWS. These unusual features may also provide insights into embryonic development as they are probable remnants from early developmental stages (15). A total of 16 different mutations are now reported from Pakistani VWS individuals including those described earlier (7). Future studies will identify regulatory variants in IRF6 in families in which VWS cannot be explained by mutations in the coding regions of the gene. This will broaden our understanding of IRF6 role in normal orofacial development as well in individuals with VWS.

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