ONLINE MUTATION REPORT

Six families with van der Woude and/or popliteal pterygium syndrome: all with a mutation in the *IRF6* gene

M Ghassibé, N Revencu, B Bayet, Y Gillerot, R Vanwijck, C Verellen-Dumoulin, and M Vikkula

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Van der Woude syndrome (VWS, OMIM #119300) is a dominantly inherited developmental disorder characterised by pits and/or sinuses of the lower lip, cleft lip with or without cleft palate (CL/P), isolated cleft palate (CP), bifid uvula (BU), and hypodontia (H).[1–3] Cleft lip deformity is established during the first 6 weeks of life due to failure of fusion of maxillary and medial nasal processes or to incomplete mesodermal ingrowth into the processes. Palatal clefts result from failure of fusion of the palatal shelves that normally change from a vertical to horizontal position and fuse during the sixth to ninth weeks of gestation.

The severity of VWS varies widely, even within families. About 25% of individuals with the syndrome have minimal findings, such as absent teeth or trivial indentations in the lower lips. Clefting of the lip or palate is present in ~50% of cases. Lip pits and/or sinuses are the cardinal features of this syndrome, present in 70–80% of VWS patients.[2] They are often associated with accessory salivary glands that empty into the pits, sometimes leading to embarrassing visible discharge.[4 5] Another syndrome closely mimicking VWS is popliteal pterygium syndrome (PPS, OMIM #119500). It combines VWS signs and symptoms with popliteal and oral webs, unusual nails, syndactyly, ankyloblepharon, and genital anomalies[6] (fig 1).

VWS is the most common cleft syndrome, affecting 1 in 35 000–100 000 people (~1/70 000).[7] About 1–2% of patients with cleft lip or palate have VWS. The VWS pedigrees suggest an autosomal dominant inheritance pattern, yet, in 30–50% of cases, mutation may rise de novo.[8] Penetrance is estimated to be about 75%, but is actually closer to 100% when supposedly unaffected carriers are closely examined for minor expressions of the syndrome.[2 4] PPS is a rarer malformation, with an incidence of 1/300 000.[9]

Most reported cases of VWS have been linked to chromosome 1q32-q41 (VWS1),[10–12] but a second VWS locus (VWS2) has been mapped to 1p34.[13] Recently, the *interferon regulatory factor-6* (*IRF6*) gene, localised to the VWS1 locus on chromosome 1q32.2, was shown to harbour mutations in patients with Van der Woude and/or popliteal pterygium syndrome.[14]

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 called SMIR.[15 16] In the only report so far on mutations in the *IRF6* gene in VWS and PPS patients, not all families harboured an alteration in *IRF6*.[14] Thus, analysis of novel families, with or without PPS, is interesting in order to determine the prevalence of *IRF6* as a disease causative gene and to identify additional mutations responsible for VWS with or without PPS. In this study, we screened six VWS and PPS families followed at the Centre Labiopalatin for mutations. A novel mutation was identified in *IRF6* for all the cases.

Key points

- Van der Woude syndrome is a dominantly inherited developmental disorder characterised by pits and/or sinuses of the lower lip, cleft lip with or without cleft palate, isolated cleft palate, bifid uvula, and hypodontia.
- Its severity varies widely, even within families, with about a quarter having minimal findings, and about half having cleft lip or palate.
- Lip pits or sinuses are the cardinal features.

MATERIALS AND METHODS

Family assessment

Patients diagnosed as VWS were recalled from the registries of the Cleft Lip and Palate Centre and the Centre for Human Genetics. All the families had members with clefts of the lip and/or palate, lip pits, or bifid uvula, and were thus considered VWS patients. In family VWS5, individuals IV:1 and IV:3 had, in addition to VWS features, oral synechiae and syndactyly, which are signs of PPS. The clinical data for each individual are shown in figure 2. Each participant gave informed consent prior to his/her participation in the study, as approved by the ethics committee of the medical faculty at the Université catholique de Louvain. The status of the unaffected individuals has been ascertained in order to identify all the microforms of VWS, especially small lip sinuses and hypodontia.

For genomic DNA extraction, venous blood samples were drawn for all participants other than VWS11-II:1, for whom a buccal cell sample was obtained. DNA was extracted by isopropanol precipitation or, from the buffy coats, using QIAamp DNA Blood Mini Kit (Qiagen, Westburg, Leusden, The Netherlands). A second blood sample was obtained from some individuals for lymphocytic transformation with Epstein-Barr virus.

Genotyping

Genotyping of nine individuals of the VWS2 family was performed as described elsewhere.[17] Linkage was tested using four polymorphic microsatellite markers: D1S245, D1S491, D1S205, and D1S414, on the basis of their genetic location given by the Genome DataBase (http://gdbwww.gdb.org/). These markers flank the VWS1 critical region and are spaced

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Abbreviations: PPS, popliteal pterygium syndrome; VWS, Van der Woude syndrome

at ~1 cM intervals. The radioactively labelled PCR products for each marker were resolved on polyacrylamide gels. In order to test linkage between the disease and the *IRF6* gene, two point analyses of disease status versus marker alleles were carried out manually, under the hypothesis of dominant inheritance. All individuals with CL/P, pits or sinuses, bifid uvula, hypodontia, or a combination of these symptoms were considered as affected. On the basis of the four markers, haplotypes were manually generated across the VWS region.

Mutational screening

The genomic sequences containing the *IRF6* gene were identified by performing a Blast search with the *IRF6* mRNA sequence (NM_006147) on the human genome sequence at the NCBI Blast server (http://www.genome.ucsc.edu/cgi-bin/). The results were confirmed by the genomic sequence retrieved. Eighteen intronic primers (sequences available on request) were synthesised from the genomic sequence to amplify the coding exons 2–9 of *IRF6*, including the intron–exon boundaries. The gene consists of 10 exons, the first and the last being non-coding. Genomic *IRF6* DNA was screened by radioactive SSCP and heteroduplex analysis for individuals VWS1-II:2; VWS1-III:1; WS2-IV:2; VWS2-V:1; VWS5-III:6; VWS5-III:1; VWS6-I:1; VWS6-II:2; VWS7-III:4; VWS7-IV:3; VWS11-II:1; VWS11-III:2 (fig 2), as described elsewhere.[18] Amplified fragments were also loaded onto denaturing 5% acrylamide sequencing gels to identify potential insertions or deletions. Fragments with abnormal migration were reamplified, purified, and sequenced on a CEQ2000 capillary sequencer (Beckman Coulter, Analis, Namur, Belgium), using the intronic primers mentioned above, with unaffected individuals from the same family as controls.

Co-segregation of mutations

Co-segregation of the substitutional mutations with the phenotype was studied by restriction fragment length assay. Numbering of nucleotides is based on the cDNA sequence (NM_0061147), with the adenosine of the ATG start codon marked as +1. The 65T→C substitution in family VWS5 created a new *Nla*IV restriction enzyme cutting site, whereas the 755T→C substitution in family VWS1 abolished the *Mbo*II restriction enzyme cutting site, and the 47C→T substitution in family VWS6 abolished the *Hae*III restriction enzyme cutting site. As the 191C→T substitution in family VWS2 and the 775C→T mutation in family VWS11 did not change any restriction site, allele specific PCR was used. For family VWS2, we synthetised wild type and mutant primers for the substitution site (5'-GCCTGGGCTGTAGAGAC/T-3'), and used them in combination with a reverse primer (3'-ACCTTCTCCCCAGCACCT-5'). For family VWS11, the wild-type and mutant primers (3'-TCCTGGTCAGGCAT GGG/A-5') were used in combination with a forward primer of exon 7 (5'-TCTCACTGTCATCTCT AACCTTGC-3'). The annealing temperature was 57.1 °C for family VWS2 and 62 °C for family VWS11. A reverse primer (3'-TTCTCATT GGTAATATGCTCAGGA-5') for family VWS11 was added to the reaction to serve as an internal positive control and as a competitor to improve the specificity of the reaction. For family VWS7, the co-segregation of the mutation was confirmed by SSCP. Fifty unrelated healthy controls were screened for all the mutations using the corresponding methods.
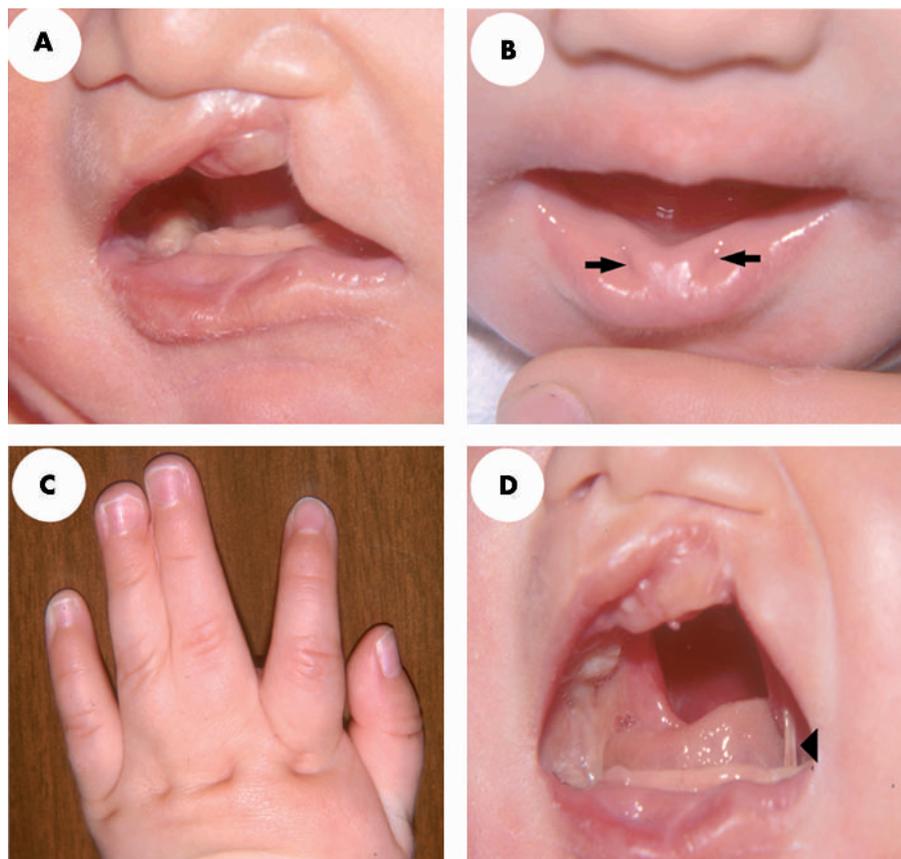
Figure 1 The major features of VWS and PPS. (A) Unilateral cleft lip in VWS5-IV:3; (B) lip pits in VWS5-IV:1; (C) syndactyly of fingers in VWS5-IV:1; (D) buccal synechiae in VWS5-IV:3. The position of the lip pits is shown by the arrow and the arrowhead indicates the buccal synechiae.
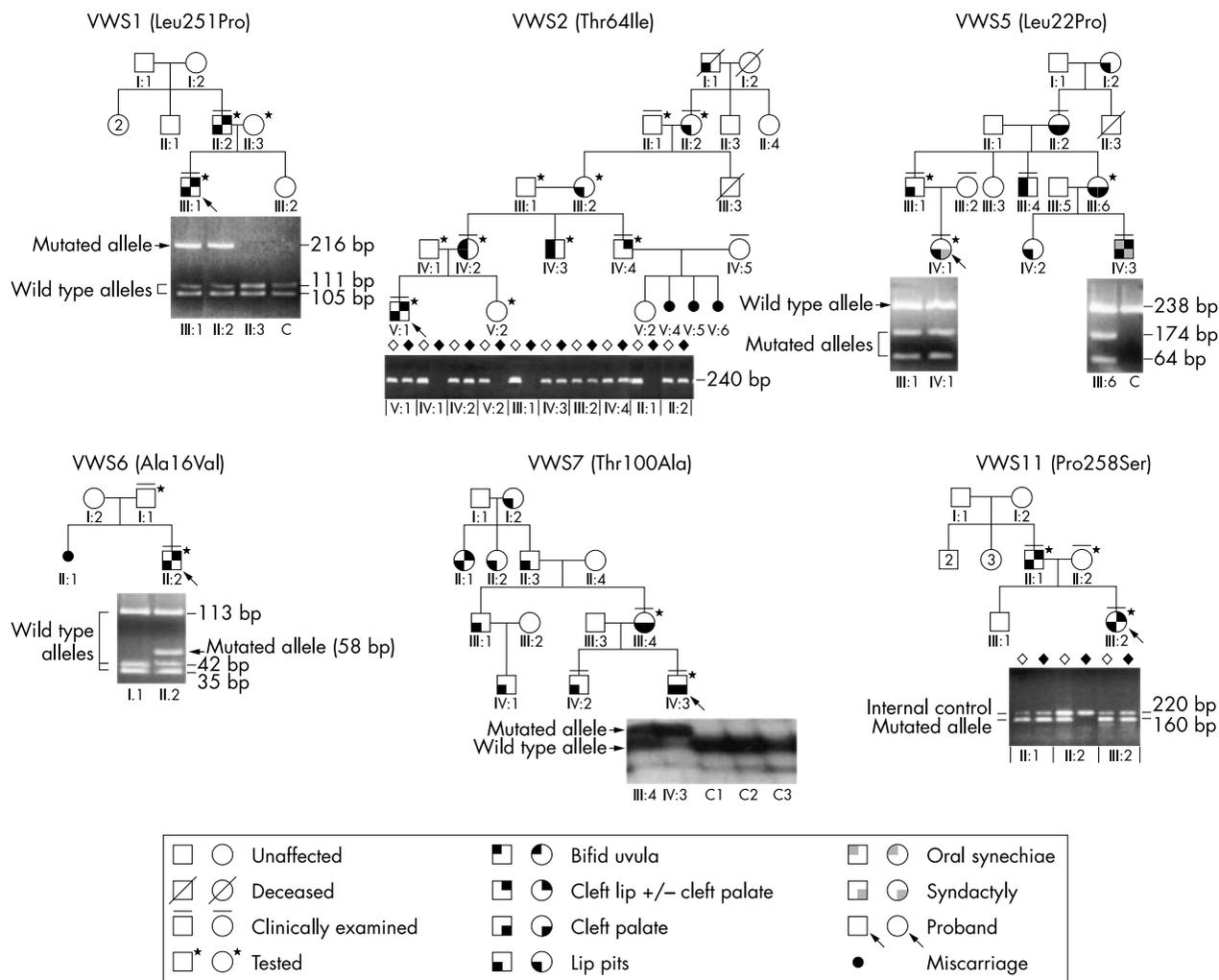
Figure 2 Pedigrees of six families with van der Woude and popliteal pterygium syndrome. A photograph of an agarose gel underneath each pedigree shows the segregation of the allele harbouring the substitution. VWS1, VWS5, VWS6: restriction enzyme digestion; VWS2, VWS11: allele specific PCR. VWS7: single strand conformational polymorphism (SSCP) analysis. ◇, wild type allele; ◆, mutant allele.

RESULTS

Clinical characterisation and linkage analysis to the 1q32-q41 VWS locus

Clinical examination of six recalled patients with VWS (VWS1-III:1; VWS2-V:1; VWS3-IV:1; VWS4-II:2; VWS5-IV:3 and VWS6-III:2) revealed that five had at least one other affected family member, and for patient VWS6-III:2, there was no known family history of the disease (fig 2). Lip pits were present in ~90% of affected individuals (28/29), confirming that it is a major VWS symptom. The number of affected males and females in the six families was 16 and 13, respectively, and 72% (23/32) of children of an affected parent had the disorder. Examination of the pedigrees revealed that the condition did not skip any generation. Thus, an autosomal dominant pattern of inheritance with very high penetrance and absence of sex bias was observed. In family VWS5, IV:1 and IV:3 were affected with VWS and syndactyly of fingers, and IV:3 had oral synechiae.

Linkage analysis in the VWS1 locus on 1q32-q41, performed for the nine members of the VWS2 family (three unaffected individuals and six individuals with VWS), demonstrated co-segregation of marker alleles and a defined haplotype with the phenotype (data not shown), considering as affected each individual who had one or more of the VWS features.

Identified mutations

Screening the *IRF6* gene revealed six heterozygous substitutions detected in the six families analysed (fig 3). All were missense mutations affecting either the DNA binding domain or the protein binding domain of the transcription factor IRF6 (fig 4). The missense mutations detected in families VWS2, VWS5, VWS6, and VWS7 were Thr64Ile (191T→C), Leu22Pro (65T→C), Ala16Val (47C→T), and Thr100Ala (298A→G), respectively. They all affected the DNA binding domain of IRF6 (fig 4). The substitutions in families VWS1 and VWS11, Leu251Pro (755T→C), and Pro258Ser (775C→T), respectively, affected the protein binding domain of IRF6. Co-segregation of the mutant alleles with the VWS phenotype was confirmed in all five familial cases (fig 2). There were no unaffected carriers, probably because all family members were examined for minor expressions of the syndrome. None of the mutations was observed in the 100 control alleles screened, and no other nucleotide changes were identified.

DISCUSSION

In this study, we describe six new families with autosomal dominantly inherited VWS caused by mutations in the *IRF6* gene localised at 1q32.2. In family VWS5, patient IV:1 had finger syndactyly, and IV:3 had toe syndactyly and oral
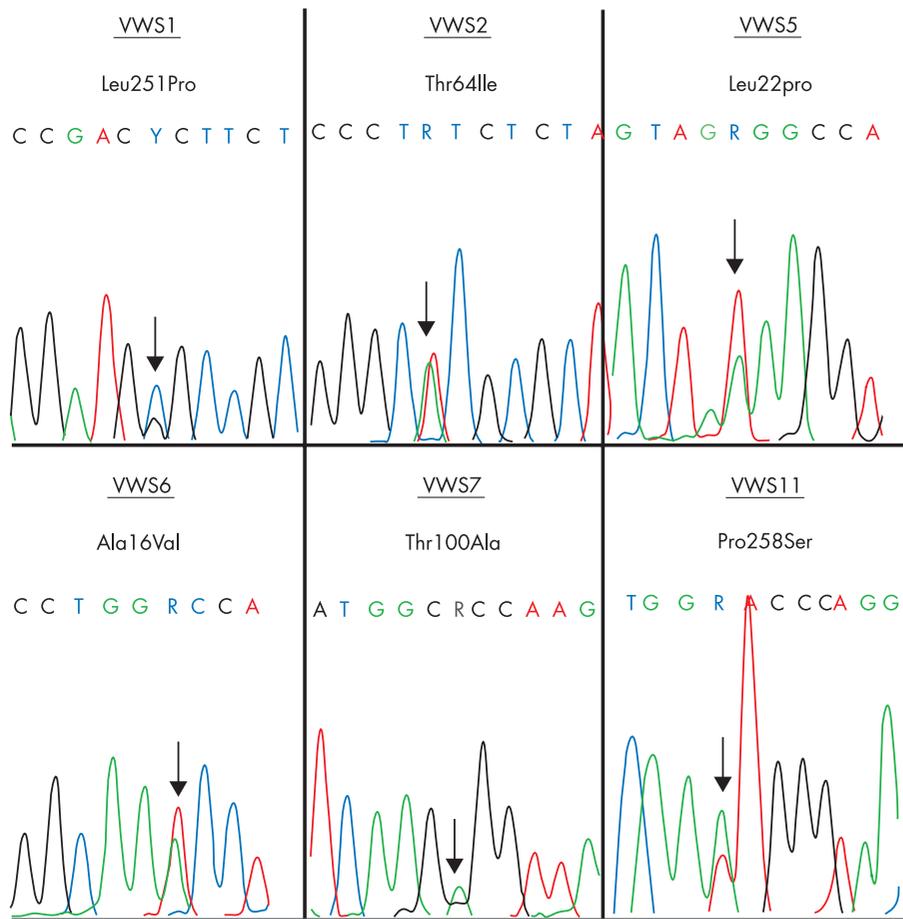
**Figure 3** Electropherograms of detected IRF6 heterozygous mutations. Arrow indicates site of mutation.

synechiae, in addition to signs of VWS. As these two features are considered the major PPS symptoms, these two individuals were taken as PPS patients. The other affected members of this family presented the VWS signs and were thus classified as VWS patients (fig 2). Another affected individual in this family, III:4, had a canine tooth in the palate. These data confirm the results of the previous study[14] that mutations in the *IRF6* gene are causative for VWS and PPS. It shows also that a single mutation in *IRF6* can be responsible for both syndromes. Both isolated cleft palate and clefts of the lip with or without cleft palate were noted within the same family, confirming that VWS, like the ectodermal dysplasia clefting syndromes, is one of the rare syndromes causing both cleft lip and palate, which, when isolated, are thought to be genetically and embryologically distinct.[19 20]

In the present study, all detected mutations were novel missense mutations affecting the well conserved DNA binding domain, or the less conserved protein binding domain that is needed for homo- and hetero-dimerisation of IRF6.[21 22] As two of the three residues mutated in the DNA binding domain are involved in interaction with DNA, the substitutions could cause VWS by loss of function. This may be caused by the mutations altering the three dimensional structure of IRF6, making it unable to bind DNA. Similarly, the protein binding domain substitutions could alter dimerisation of the transcription factor.

Loss of function is supported by the detection of a number of large deletions encompassing the VWS1 region in some patients in an earlier study.[10] Haploinsufficiency was also underscored by the identification of several protein truncating mutations in the *IRF6* gene that were more common in VWS than in PPS.[14]

The single PPS family studied (VWS5), harboured a Leu22Pro mutation. The Leu22 residue in IRF6 is comparable to Gln20 in IRF1, which belongs to helix-1 of the DNA binding domain, but does not interact with DNA.[23] In a previous study, the majority of PPS missense mutations was located in the DNA binding domain and was suggested to
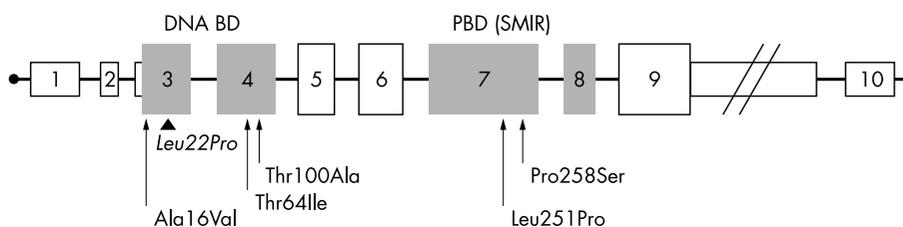
Figure 4 Structure of the *IRF6* gene. Exons (rectangles) are drawn to scale, except for exon 9, which is longer than shown. Line connecting exons represents introns. Untranslated regions are in grey. Exons 3–4 code for the DNA binding domain and exons 7–8 code for the protein binding domain (both in grey). The arrowhead indicates the relative position of the identified PPS mutation, and the arrows the relative positions of the other substitutions.

lead to an inactive form of the transcription complex in a dominant negative manner.[14] Individuals in our VWS5 family had either VWS or PPS caused by the same mutation. Therefore, it seems rather unlikely that the missense mutations in the DNA binding domain associated with VWS or PPS affect IRF6 function differently.[14]

The sequence variant Val274Ile, which is reported to be present in 3% of individuals of European descent and 22% of Asian population,[14] was not detected in any of the 22 white individuals analysed. Thus, it does not seem to be a phenotypic modifier in this subgroup. However, as wide variation is observed in the phenotypic effects of the detected mutations in the IRF6 gene, it is likely that other unknown stochastic factors or modifier genes influencing IRF6 function exist.

In the present study, 5/6 IRF6 mutations were hereditary, detected in familial VWS/PPS. None was a confirmed de novo mutation. However, in three families (VWS1, VWS6, and VWS11), we cannot exclude this possibility, owing to limited familial history and DNA samples. A mutation was detected in all the affected individuals of the families. This is an expected result for a PPS family, as a mutation in the IRF6 gene was previously detected in ~ 86.6% of PPS families (13/15).[14] In contrast, we did not expect to reveal mutations in all the VWS patients, as only ~43% (46/107) of VWS families had an IRF6 mutation in the previous study [154]. This may be due to our smaller sample size, or to the fact that the families were phenotypically more homogeneous.

VWS, one of the most common cleft syndromes in the world, is the best example of a single gene disorder that causes cleft lip and palate, and which can mimic a ''non-syndromic'' form. For this reason, the identification of the IRF6 gene as the cause of VWS and PPS can help in the complex puzzle of cleft lip and palate, but how does IRF6 cause clefts of palate and lips, and other variable craniofacial phenotypes? Very little is known about the function of IRF6. It is a transcription factor with two conserved domains, with the structure of an interferon regulatory factor gene, or IRF. It is not known which target genes it activates and when. They could be extracellular matrix genes, such as collagens that have been shown to be mutated in other syndromic forms of clefts, or genes acting in the TGFβ pathway, for example, which has been shown to be important for palate formation.[24–26] It could also act as a modifier, which by itself does not cause a cleft, but which affects the severity of the cleft, due to alterations in other proteins. Modifying effects could be due to expression level differences and thus, screening of, for example, the IRF6 promoter may unravel cleft lip/palate associated variants.

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Authors' affiliations

M Ghassibé, M Vikkula, Laboratory of Human Molecular Genetics, Christian de Duve Institute of Cellular Pathology and Universite catholique de Louvain, Avenue Hippocrate 74+4, Brussels, Belgium
N Revencu, Y Gillerot, C Verellen-Dumoulin, Centre for Human Genetics, Cliniques universitaires St Luc and Université catholique de Louvain, Brussels, Belgium

B Bayet, R Vanwijck, Centre Labiopalatin, Division of Plastic Surgery, Cliniques universitaires St Luc, Brussels, Belgium

Correspondence to: Professor M Vikkula, Laboratory of Human Molecular Genetics, Institute of Cellular Pathology and Universite catholique de Louvain, Avenue Hippocrate 74+4, Brussels B-1200, Belgium; vikkula@bchm.ucl.ac.be

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