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IRF6 Screening of Syndromic and a priori Non-Syndromic Cleft Lip and Palate Patients: Identification of a New Type of Minor VWS Sign

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Key Words
Cleft palate · Genetic · *IRF6* · Non-syndromic cleft lip · PPS · VWS

Abstract
Van der Woude syndrome (VWS), caused by dominant *IRF6* mutation, is the most common cleft syndrome. In 15% of the patients, lip pits are absent and the phenotype mimics isolated clefts. Therefore, we hypothesized that some of the families classified as having non-syndromic inherited cleft lip and palate could have an *IRF6* mutation. We screened in total 170 patients with cleft lip with or without cleft palate (CL/P): 75 were syndromic and 95 were a priori part of multiplex non-syndromic families. A mutation was identified in 62.7 and 3.3% of the patients, respectively. In one of the 95 a priori non-syndromic families with an autosomal dominant inheritance (family B), new insights into the family history revealed the presence, at birth, of lower lip pits in two members and the diagnosis was revised as VWS. A novel lower lip sign was observed in one individual in this family. Interestingly, a similar lower lip sign was also observed in one indi-

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vidual from a 2nd family (family A). This consists of 2 nodules below the lower lip on the external side. In a 3rd multiplex family (family C), a de novo mutation was identified in an a priori non-syndromic CL/P patient. Re-examination after mutation screening revealed the presence of a tiny pit-looking lesion on the inner side of the lower lip leading to a revised diagnosis of VWS. On the basis of this data, we conclude that *IRF6* should be screened when any doubt rises about the normality of the lower lip and also if a non-syndromic cleft lip patient (with or without cleft palate) has a family history suggestive of autosomal dominant inheritance.

Orofacial clefts are the most frequent congenital craniofacial malformations (MIM#119530) with a prevalence of approximatively 1/700, varying with ethnicity and cleft type. Seventy percent of clefts occur isolated (i.e. without any other associated malformation) whereas 30% are secondary to chromosomal aberrations, Mendelian syndromes or result from known teratogens [Tolarova and Cervenka, 1998]. The aetiology of isolated cleft is likely to be multifactorial; some result from genetic mutations while others may be due to environmental factors or a combination thereof. The mode of inheritance of non-syndromic cleft lip with or without palate (NSCL/P) remains controversial.

The most common cleft syndrome (1/100,000–1/35,000) is Van der Woude syndrome [Gorlin et al., 2001] (VWS, MIM#119300). It is a dominantly inherited disorder characterized by the presence of pits and/or sinuses on the lower lip in 85% of cases, cleft lip and/or cleft palate (CL/P) in 50% of the patients and hypodontia in 20% of them [Van Der Woude, 1954; Lacombe et al., 1995]. The Popliteal Pterygium syndrome (PPS, OMIM #119500) is a less frequent allelic orofacial clefting disorder. In addition to the signs of VWS, PPS includes webbing of the knee, syndactyly (or absence) of the toes and digits, ankyloblepharon, syngnathia, and genital abnormalities [Froster-Iskenius, 1990].

The interferon regulatory factor-6 *(IRF6)* gene, localized on 1q32.2, has been shown to be mutated in patients with VWS and/or PPS in several populations [Kondo et al., 2002; Kayano et al., 2003; Kim et al., 2003; Ghassibe et al., 2004; Item et al., 2005; Du et al., 2006; Brosch et al., 2007]. *IRF6* belongs to a family of nine transcription factors with a conserved DNA binding domain and a less conserved protein binding domain. It has been implicated in the differentiation of primary superficial epithelia in *Danio rerio* and in *Xenopus* embryos, in keratinocyte proliferation-differentiation switch in mice and cell cycle arrest in mammary epithelial cells in vitro [Richardson et al., 2006; Bailey et al., 2008; Sabel et al., 2009].

In addition to the cleft lip and palate, lower lip pits are the major criterion to diagnose VWS. However, their shape and signs/symptoms range from clear bilateral pits with salivary leakage, pain, swelling, discharge, and inflammation, to weak elevations on the lower lip [Gorlin et al., 2001]. In 15% of the VWS patients, lip pits are absent and the phenotype becomes indistinguishable from a NSCL/P. Interestingly, NSCL/P has been associated with the *IRF6* locus [Zucchero et al., 2004; Blanton et al., 2005; Ghassibe et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005; Park et al., 2007; Vieira et al., 2007, 2008; Suazo et al., 2008]. Therefore, we hypothesized that some of the families classified as non-syndromic cleft lip and palate could have an *IRF6* mutation.

Materials and Methods

Family Assessment

Clinical data and samples from patients with NSCL/P and their family members were collected in collaboration with the 'Centre labiopalatin', Cliniques universitaires Saint-Luc, Brussels, Belgium. Families were from Belgium, which has a heterogeneous population with immigrants from various origins and from France. Overall, 95 families with at least two individuals affected with CL/P were included in this study. In addition, 75 VWS and PPS patients were screened. They originated from Belgium, The Netherlands, UK, Switzerland, Turkey, Spain and France. Families with at least one member with lower lip pits were considered as VWS. Families were diagnosed with PPS if at least one individual had PPS signs.

Each participant was asked to fill in a questionnaire recapitulating his/her malformations, familial history and conditions/exposure of the mother during pregnancy. Diagnosis was based on clinical examination and the questionnaire. Informed consent was obtained from each participant prior to participation in the study, as approved by the local ethics committee.

Venous blood samples were drawn for the majority of participants. Genomic DNAs were extracted from buffy coats using 'QIAamp DNA Blood Mini Kit' (Qiagen Inc., Valencia, Calif., USA) or from whole blood using 'DNA purification kit' (Gentra systems Inc., Minneapolis, Minn., USA). When whole-blood collection was not possible, DNA was extracted from buccal brush or swab sample, and DNA was purified by an adapted phenol-chloroform protocol [Haines et al., 2000].

Mutational Screening

IRF6 was screened by Denaturing High Performance Liquid Chromatography (DHPLC, Wave System 3500A, Transgenomic). The coding exons 2–8 and part of exon 9, including intron-exon boundaries, were amplified by standard PCR. Amplicons with an abnormal chromatogram elution profile were sequenced with

Table 1. Mutations identified in VWS patients

Family code	Exon	Inheritance	de novo/ familial mutation	Nucleotide change[a]	Amino acid change	Mutation	Same mutation reported in
VWS-68	3	F	F	c.31A>T	p.Lys11*	nonsense	–
VWS-6	3	S	Father -ve	c.47C>T	p.Ala16Val	missense	Ghassibe et al., 2004
VWS-33	3	F	NA	c.74G>T	p.Gly25Val	missense	de Lima et al., 2009
VWS-55	3	F	F	c.115C>T	p.Pro39Ser	missense	–
VWS-83	3	S	NA	c.119G>A	p.Trp40*	nonsense	–
VWS-73	4	S	Mother -ve	c.176C>T	p.Ala59Val	missense	–
VWS-2	4	F	F	c.191C>T	p.Thr64Ile	missense	Ghassibe et al., 2004
VWS-81	4	F	NA	c.206A>G	p.Glu69Gly	missense	–
VWS-80	4	F	F	c.245A>C	p.Gln82Pro	missense	–
VWS-35	4	S	NA	c.251G>C	p.Arg84Pro	missense	de Lima et al., 2009
VWS-78	4	S	de novo	c.263A>G	p.Asn88Ser	missense	de Lima et al., 2009
VWS-14	4	F	F	c.269G>T	p.Ser90Ile	missense	de Lima et al., 2009
VWS-53	4	F	NA	c.275A>T	p.Glu92Val	missense	–
VWS-7	4	F	F	c.298A>G	p.Thr100Ala	missense	Ghassibe et al., 2004
VWS-22	4	F	F	c.328A>T	p.Ile110Leu	missense	de Lima et al., 2009
VWS-13	6	F	F	c.526_529del	p.Ala176Valfs*47	frameshift	de Lima et al., 2009
VWS-67	6	F	F	c.601G>T	p.Glu201*	nonsense	–
VWS-38	7	S	NA	c.693_697del	p.Tyr232Trpfs*28	frameshift	–
VWS-24	7	F	F	c.748C>T	p.Arg250*	nonsense	de Lima et al., 2009
VWS-31	7	F	NA	c.748C>T	p.Arg250*	nonsense	de Lima et al., 2009
VWS-63	7	F	F	c.749G>A	p.Arg250Gln	missense	Kondo et al., 2002
VWS-1	7	F	F	c.752T>C	p.Leu251Pro	missense	Ghassibe et al., 2004
VWS-11	7	F	F	c.772C>T	p.Pro258Ser	missense	Ghassibe et al., 2004
VWS-65	7	NA	NA	c.799G>T	p.Gly267Cys	missense	–
VWS-42	7	F	NA	c.881T>C	p.Leu294Pro	missense	Kondo et al., 2002
VWS-34	7	F	F	c.961G>A	p.Val321Met	missense	Kondo et al., 2002
VWS-10	7	Adoption	/	c.974G>A	p.Gly325Glu	missense	de Lima et al., 2009
VWS-64	8	NA	NA	c.1135T>G	p.Trp379Gly	missense	–
VWS-59	8	F	F	c.1141G>T	p.Asp381Tyr	missense	–
VWS-26	9	F	F	c.1198C>T	p.R400W	missense	de Lima et al., 2009
VWS-86	9	NA	NA	c.1277_1280delins TTGATGATGTTAT	p.Pro426delins LeuAspAspValIle	missense	–
VWS-36	IVS3	F	F	c.174+1G>A	p. = ?	splicing	de Lima et al., 2009
VWS-74	IVS3	F	NA	c.174+1G>A	p. = ?	splicing	de Lima et al., 2009
VWS-47	IVS8	F	NA	c.1180-1G>C	p. = ?	splicing	–

[a] Nucleotide positions relative to start codon in NM_006147.
F: Familial; NA: not available; S: sporadic; -ve: negative. * The mutation creates a translation termination site.

CEQ2000 capillary sequencer (Beckman Coulter, Analis, Namur, Belgium), using GenomeLab DTCS Quick Start Kit (Beckman Coulter, Namur, Belgium) and analyzed with Sequencher™ 4.5 software. Effects of amino acid changes on protein function were analyzed by Protein ANalysis THrough Evolutionary Relationships (Panther) (http://www.pantherdb.org/) [Thomas et al., 2003; Thomas and Kejariwal, 2004], Polymorphism Phenotyping (Polyphen) (http://genetics.bwh.harvard.edu/pph/) [Sunyaev et al., 2001; Ramensky et al., 2002] and Sorting Intolerant From Tolerant (SIFT) [Ng and Henikoff, 2003] (http://sift.jcvi.org/) prediction programs.

Results

Syndromic Patients

Of the 75 syndromic patients, 51 had a diagnosis of VWS, 17 had a diagnosis of PPS and in seven the phenotype was unclear. Among the patients with unclear phenotypes, one was reported to have unusual movement of the lower lip, one had a depressed lower lip without fistula and no data were available for five individuals. No mutation was identified in these seven patients and we

Table 2. Mutations identified in PPS patients

Family code	Exon	Inheritance	de novo/familial mutation	Nucleotide change[a]	Amino acid change	Mutation	Same mutation reported in
VWS-5	3	F	F	c.65T>C	p.Leu22Pro	missense	Ghassibe et al., 2004
VWS-23	4	S	NA	c.200A>G	p.Tyr67Cys	missense	de Lima et al., 2009
VWS-15	4	F	F	c.250C>T	p.Arg84Cys	missense	Kondo et al., 2002
VWS-28	4	S	NA	c.250C>T	p.Arg84Cys	missense	Kondo et al., 2002
VWS-46	4	S	de novo	c.250C>T	p.Arg84Cys	missense	Kondo et al., 2002
VWS-57	4	F	F	c.250C>T	p.Arg84Cys	missense	Kondo et al., 2002
VWS-84	4	S	NA	c.250C>T	p.Arg84Cys	missense	Kondo et al., 2002
VWS-29	4	NA	NA	c.251G>A	p.Arg84His	missense	Kondo et al., 2002
VWS-41	4	F	F	c.251G>A	p.Arg84His	missense	Kondo et al., 2002
VWS-62	4	F	NA	c.251G>A	p.Arg84His	missense	Kondo et al., 2002
VWS-69	4	S	de novo	c.251G>A	p.Arg84His	missense	Kondo et al., 2002
VWS-49	9	S	de novo	c.1271C>T	p.Ser424Leu	missense	–
VWS-25	IVS3	F	F	c.174+1G>T	p. = ?	splicing	de Lima et al., 2009

[a] Nucleotide positions relative to start codon in NM_006147.
F: Familial; NA: not available; S: sporadic; -ve: negative.

excluded them from the statistical analyses. We found an *IRF6* mutation in 66.7% (34/51) of the VWS patients and in 76.5% (13/17) of the PPS patients. Of the mutations in VWS patients, 94.1% (32/34) occurred in exons 3, 4, 7, 8 and 9 and the corresponding splice sites (table 1); exons 4 and 7 being mutated in 58.8% (20/34) of the patients. In patients with PPS, 76.9% (10/13) were located in exon 4 and 15.4% (2/13) in exon 3 or its corresponding splice sites (table 2). Fifteen out of the 38 mutations were previously undescribed mutations. Almost all of the PPS mutations were missense substitutions (12/13), whereas a significant proportion of truncating mutations, including the splice-site mutations, (10/34) was seen in VWS patients. Bioinformatic analysis predicted functional effects on IRF6 for all substitutions.

Non-Syndromic Patients

Within the group of 95 individuals with a priori non-syndromic familial CL/P, cleft palate only (CPO) was observed in 10 families, cleft lip only (CLO) in 10 families, cleft lip and palate (CLP) in 52 families (at least one member affected with CLP), Pierre Robin sequence in nine families and mixed clefting (CL/P and CPO) in 14 families. No mutation was found in 92/95 tested individuals. Mutations were found in the three remaining individuals from families A, B and C. In family A, in which four members were affected with CPO in three consecutive generations (fig. 1A), a c.16C>T change located in exon 3 and resulting in a p.Arg6Cys substitution was identified. The mutation co-segregated with the disease in the family. When the family was seen for genetic counseling, an additional clinical examination regarding minor VWS signs was performed, but no evidence of lower lip pits was found in any of the affected members or in the family history. However, clinical examination of the proband's brother revealed the presence of paramedian small bulges/nodules below the lower lip on its external side (patient III.1) (fig. 1B). This patient was also missing his right lower lateral incisive. The father and the proband did not show any tooth agenesis or lip anomaly. The affected grandfather was not available for examination.

In family B, with three affected members in three consecutive generations (fig. 2A), a c.749G>A mutation was found. The same change was identified in a VWS family (VWS-63, table 1). This change occurs in exon 7 substituting the arginine 250 to a glutamine. In family B, the change was found in the proband, affected with CPO, as well as in his father and paternal grandfather, both affected with bilateral cleft lip and palate (BCLP). A posteriori counseling regarding VWS signs revealed that patient III.1 had lip pits at birth (as per communication with his mother) which were surgically removed during infancy. Patient II.1 had no lip pits, but stated the presence of two lip pits in his mother and a small cleft lip in a maternal cousin. The index case had no lip pits, nor fistulae, but two small soft extended nodules below the vermillion

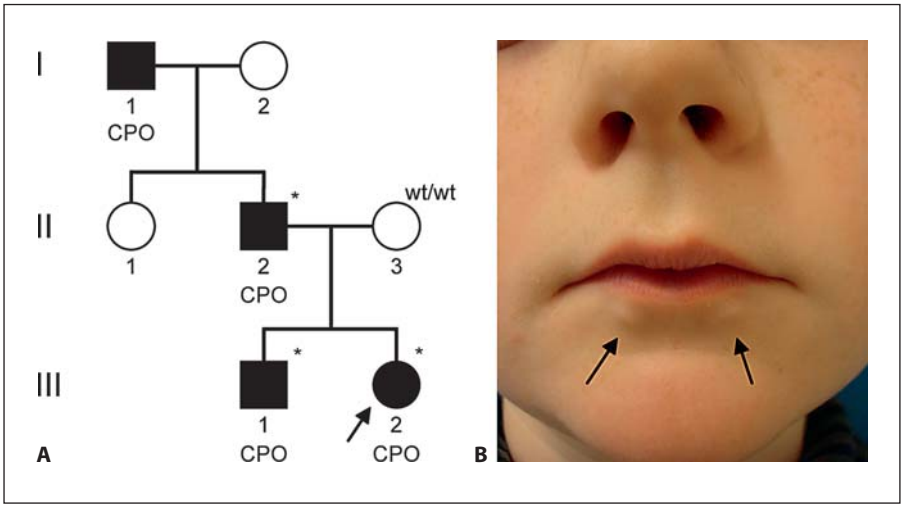

Fig. 1. Family A. **A** Pedigree. Proband indicated by arrow. *, mutation carrier; wt, wild type; CPO, cleft palate only. **B** Proband's brother. Two lower lip bulges indicated by arrows.

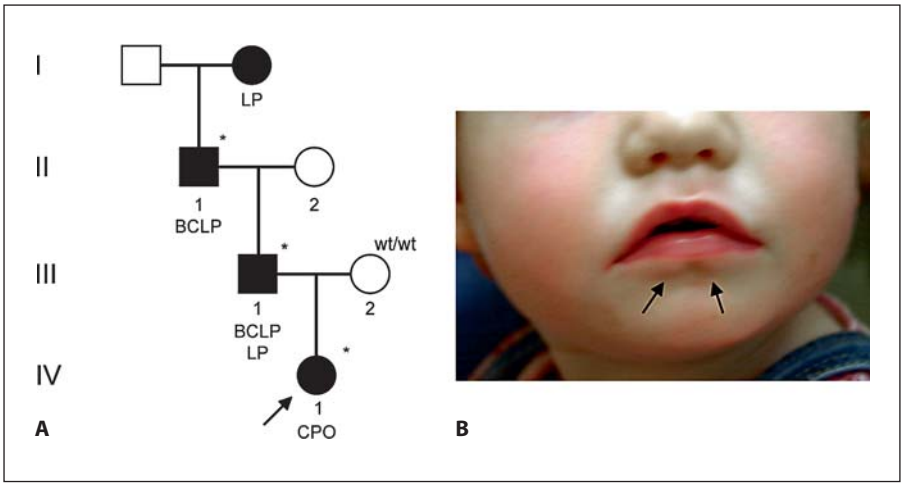

Fig. 2. Family B. **A** Pedigree. Proband indicated by arrow. *, mutation carrier; wt, wild type; CPO, cleft palate only; BCLP, bilateral cleft lip and palate; LP, lip pit. **B** Proband. Two bulges indicated by arrows.

of the lower lip on both sides of the median line (fig. 2B). These were similar to the bulges observed on the proband's brother in family A. Hypodontia was difficult to appreciate because of the young age or absence of any record of dental extractions in the adults. The a posteriori clinical features of family B are consistent with a diagnosis of VWS.

A c.1199G>A change in exon 9, resulting in p.Arg400Gln substitution, was identified in the 3rd family (family C); the male proband had a unilateral CL. Apart from him, only a distant maternal cousin presented a bilateral CLP (fig. 3A). The mutation occurred de novo in the index patient, as neither the unaffected parents nor the two unaffected siblings carried the change. When the family was seen for genetic counseling, the clinical geneticist observed a tiny pit-looking lesion on the inner-side of the lower lip, rejecting the a priori non-syndromic phenotype in this patient (fig. 3B). All three mutations identified in families A, B and C have been reported in other VWS families [Kondo et al., 2002; Wang et al., 2003, 2005; Ghassibe et al., 2004; Peyrard-Janvid et al., 2005; de Lima et al., 2009].

Discussion

We screened a total of 170 patients with CL/P; 75 syndromic and 95 a priori multiplex non-syndromic families with at least two affected members. We detected mutations in 66.7% of our VWS patients in accordance with

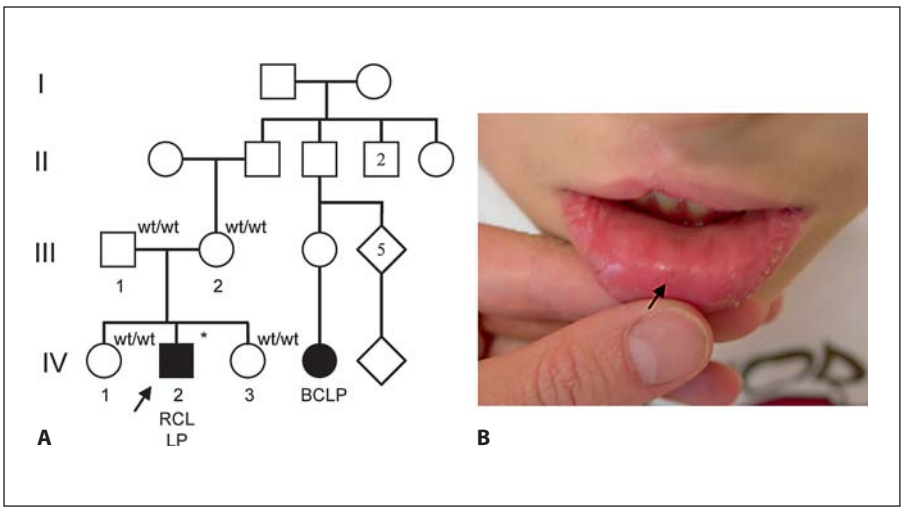

Fig. 3. Family C. **A** Pedigree. Proband indicated by arrow. *, mutation carrier; wt, wild type; RCL, right cleft lip; BCLP, bilateral cleft lip and palate; LP, lip pit. **B** Proband. A posteriori identified tiny lip pit.

previous studies (68%). However, the proportion of PPS patients with an identified mutation (76.5%) was lower than previously observed (97%) [Kondo et al., 2002; de Lima et al., 2009]. This may be explained by the fact that three patients with popliteal pterygium also had additional unusual features. One had multiple pterygia (neck, shoulder, hip and knees), spina bifida, clubfeet, facial dysmorphism, but no cleft. Post-mortem examination of the 2nd patient, a foetus, revealed facial dysmorphism without cleft, syndactyly, popliteal pterygium and clubfeet. No intrabuccal anomalies were observed. The 3rd patient had multiple congenital anomalies: a large unilateral cleft encompassing the nose, the lip and the palate, distal or complete amputation of fingers, clubfoot, left popliteal pterygium, pulmonary and genital anomalies (clitoral hypertrophy and small labia). Amniotic bands were observed in the placenta. If these three patients with PPS and multiple congenital anomalies are not taken into account, all but one of the remaining PPS patients carry a mutation in *IRF6*; more in-keeping with previous reported mutation frequency in *IRF6* in PPS [Kondo et al., 2002; de Lima et al., 2009]. The distribution of mutations was also non-random. While the majority of the VWS mutations was spread over exons 3, 4, 7, 8 and 9 (94.1% of the mutations), the majority of PPS mutations was concentrated in exons 3 and 4 (92.3%). This distribution supports previous reports and helps define diagnostic screening strategies.

Unraveling the etiology of NSCL/P is challenging. A gold standard for identifying causal genes is to study genes mutated in rare Mendelian disorders characterized by the presence of CL/P. *IRF6* is one such gene. VWS phenocopies non-syndromic cleft lip and palate, as lip pits, the main characteristic feature of VWS, are absent in 15% of the patients. Moreover, polymorphisms in the *IRF6* locus are associated with CLP in Caucasians and Asians [Zucchero et al., 2004; Srichomthong et al., 2005; Vieira et al., 2007], and so far, *IRF6* mutations were reported in 3/108 NSCL/P families [Jehee et al., 2009]. Interestingly, we identified exonic *IRF6* mutations in two a priori diagnosed non-syndromic multiplex CL/P families with an autosomal dominant inheritance and one sporadic case. Scrupulous anamnesis is crucial, including inspection of pictures at birth and/or before any surgery, as patients may not consider lip anomalies as signs of the disease or forget about them after surgery [Birnbaum et al., 2008].

Re-examination of the members of families A, B and C, in which an *IRF6* mutation was identified, to look for minor VWS signs, with careful scrutiny for the lower lip and family history proved to be very informative. Patient III.1 (family A) was found to have a missing tooth on the mandibular side, which to the best of our knowledge is not associated with VWS. Usually, the missing teeth in VWS are upper incisors and premolars, and lower premolars [Rizos and Spyropoulos, 2004]. This patient also had two nodules between the lower lip and the chin (fig. 1B). Such masses have not previously been reported in VWS or PPS. The two nodules have no opening inside the mouth and are situated in an atypical region for characteristic lower lip anomalies of VWS, which can adopt a wide range of phenotypes and even microforms have been described. The microforms consist of transverse mucosal ridges and conical elevations (nipple-like) with/without fistula on the lower lip. In addition, lip pits are

usually located between the vermillion part of the lower lip towards the mucosal side [Gorlin et al., 2001]. Patient III.1 does therefore not fulfill the diagnostic criteria for VWS and the other affected family members available for study were ascertained to have CP only. Thus, according to current diagnostic criteria, this family has NSCL/P. However, the identification of an *IRF6* mutation leads us to conclude that lower lip nodules are likely to be a novel sign of VWS and should be considered as one of the phenotypic features.

After specific clinical examination and familial history investigation, family B was diagnosed to have VWS. Interestingly, the index patient has two nodules below the external lower lip, similar to patient III.1 from family A, supporting our conclusion that these bilateral external lower lip nodules are a minor sign of VWS. Although their exact histological nature and embryologic origin are unknown, ultrasound examination could help describe them and identification of additional patients should help confirm their presence and prevalence among VWS and PPS patients.

In the third family, a priori classified as a multiplex NSCL/P, a de novo mutation was identified. Clinical examination a posteriori of the sporadic patient revealed a small pit on the internal lower lip. The diagnosis was therefore revised as VWS. This demonstrates how the identification of the genetic basis, i.e. an *IRF6* mutation, is of importance for the patient and his descendants, as the recurrence risk increases from 3% for NSCL/P to 50% for VWS/PPS. In our cohort of multiplex families, two affected patients are generally separated by several generations and assimilated to sporadic-like families. We conclude that without family history of orofacial clefting, *IRF6* screening is still relevant as de novo mutations occur.

In summary, we have identified an *IRF6* mutation in two families a priori classified as having NSCL/P with autosomal dominant inheritance and in one sporadic case. Clinical examination revealed a novel phenotypic sign: paramedian external lower lip nodules. This was the only other clinical sign in addition to the cleft in one of these families and thus they need to be looked for when considering VWS. Overall, these findings suggest that *IRF6* should be screened when (a) any doubt rises about the normality of the lip and (b) when there is familial history of NSCL/Ps with a dominant inheritance.

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