A Novel Mutation in *IRF6* Underlies Hearing Loss, Pulp Stones, Large Craniofacial Sinuses, and Limb Anomalies in Van der Woude Syndrome Patients

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Summary: Van der Woude (VWS) and popliteal pterygium syndromes are caused by mutations in the interferon regulatory factor (*IRF6*) gene. Two Thai VWS families demonstrating newly recognized findings of VWS are reported. The phenotype in the first family includes sensorineural hearing loss, cleft lip and palate, lower lip anomalies, ankyloglossia, hypodontia, dental pulp stones, large craniofacial sinuses, and limb anomalies. Molecular analysis of *IRF6* revealed an 11 bp deletion in exon 4. This frameshift mutation truncates IRF6 just after the DNA binding domain. The mutation implies that IRF6 can affect dental pulp calcification, pneumatization of craniofacial sinuses, and ear and limb development. The second family consists of an affected brother and sister. Both have lower lip anomalies and the sister has cleft lip and palate. Interestingly, both have abnormal shape of the mandibular deciduous and permanent molars. Mutation analysis of *IRF6* was negative, suggesting that the mutations may be located outside of the coding exons or in another locus.

Key words: dental pulp stone, IRF6 gene, limb anomaly, hearing loss, Van der Woude syndrome

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INTRODUCTION

Van der Woude syndrome (VWS; OMIM 119300) is characterized by congenital lip pits, cleft lip with or without cleft palate, hypodontia, ankyloglossia, and cutaneous syndactyly of toes. The disorder is caused by mutations in interferon regulatory factor (*IRF6*) and mutations in the same gene also cause popliteal pterygium syndrome (PPS; OMIM 119500) (Kondo et al, 2002). In addition to the VWS phenotype, patients with PPS exhibit webbing of the limbs, toe nail dysplasia, and genital anomalies (Gorlin et al, 2001). From their analysis of 49 VWS mutations and 13 PPS mutations, Kondo et al (2002) suggested that VWS is caused by haploinsufficiency of *IRF6*, whereas PPS is caused by mutations that have a dominant negative effect on IRF6 function.

Recently, we reported a four-generation Thai family with a unique VWS-like phenotype. In addition to lip anomalies, hypodontia, and cleft lip and cleft palate, affected members of this family exhibited sensorineural hearing loss, large craniofacial sinuses, dental pulp stones, and minor limbs anomalies (Kantaputra et al, 2002). Given the VWS-like orofacial features in this

family, we hypothesized that affected members of the family would have a mutation in *IRF6*. With the unique anomalies in this family, we further hypothesized that the mutation would be novel and may provide clues to the structure and function of *IRF6*. Here we report a novel *IRF6* mutation found in this family and suggest that this mutation affects ear and limb development, calcification in the dental pulps, and pneumatization of craniofacial sinuses.

In addition, a new VWS family affected with abnormal shape of mandibular deciduous and permanent molars is reported. Abnormal shape of teeth has never

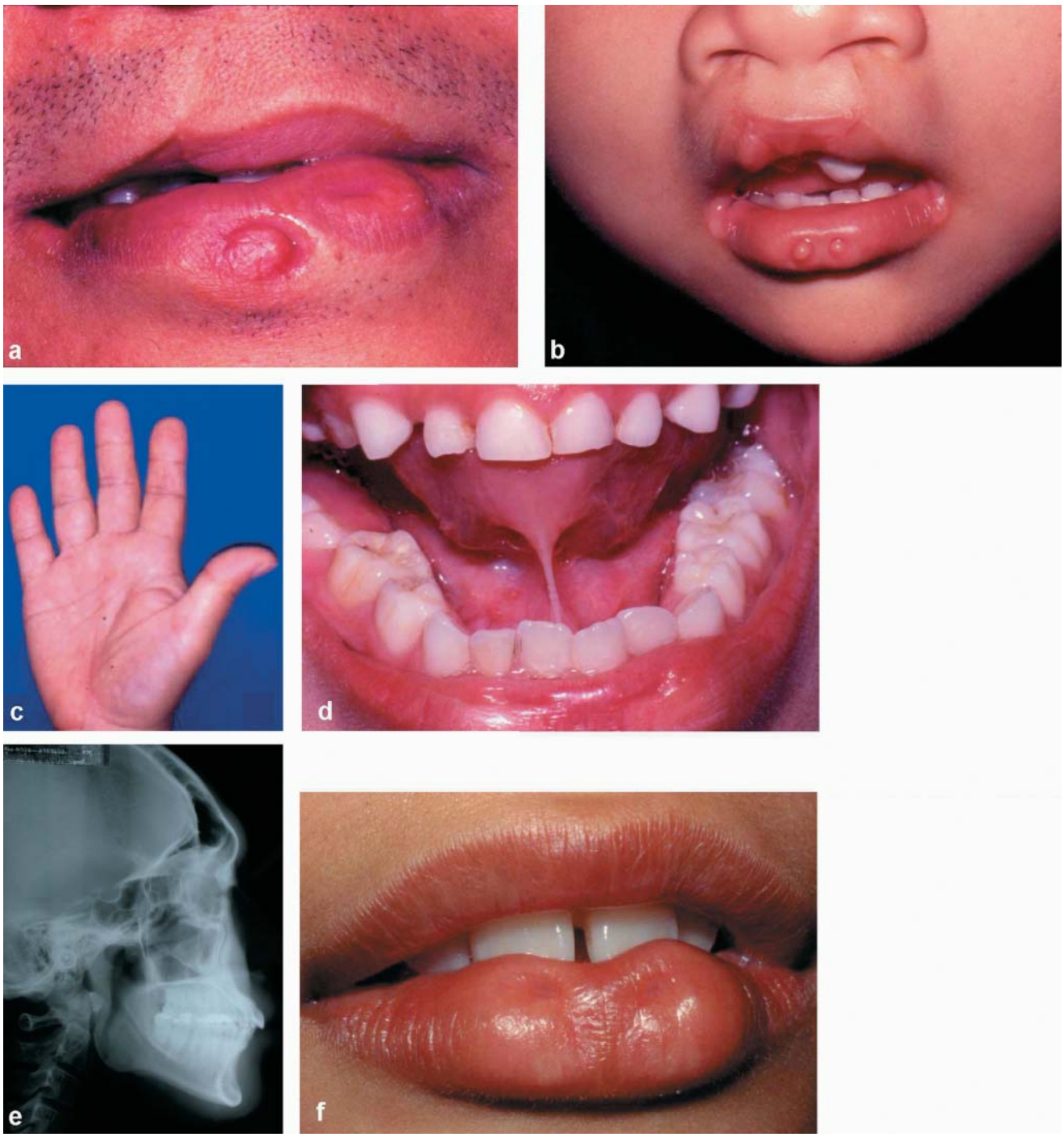

Fig. 1 Family 1: a) Repaired unilateral cleft lip and palate. Lip pit and sulcus; b) Repaired bilateral cleft lip and palate. Lip nipples; c) Single flexion crease of the right fifth finger; d) Ankyloglossia; e) Large frontal sinus; f) Lip sulcuses.

been described to be associated with VWS. The molecular analysis of *IRF6* was negative, supporting the possibility of VWS-causing mutations outside the coding region, or deletions or mutations in another locus (Kondo et al, 2002).

MATERIALS AND METHODS

Clinical Details

Family 1: This four-generation Thai family consists of 23 individuals, of which seven are affected with VWS. The clinical findings include cleft lip with cleft palate, lower lip anomalies, congenital absence of the mandibular second premolars, ankyloglossia, sensorineural hearing loss, large craniofacial sinuses, long tooth roots, and dental pulp stones. The limb anomalies include a single crease of the fifth finger (Figs. 1a-f), short middle phalanges of the fifth fingers, short middle phalanges of toe 4, short distal phalanges of toes 2 and 3, brachydactyly of fingers, cutaneous syndactyly of toes 2 and 3, and hyperphalangy of toes (Kantaputra et al, 2002).

Family 2: This family consists of an unaffected mother and father, and two affected children (Fig. 1a). The father died of an unrelated disease a few years ago. Clinical manifestations found in the affected daughter (Patient 2.1) were repaired unilateral cleft lip with cleft palate, supernumerary right maxillary lateral incisor, lower lip anomaly, and congenital absence of the mandibular second premolars (Figs. 2a,b). She had cosmetic surgery for her lower lip at age 12 years. The mandibular second deciduous molars had numerous secondary grooves, with prominent lingual cusps. The right mandibular first permanent molar had prominent distolingual cusps. The left mandibular first permanent molar had a very unusual shape. The occlusal table was round, with three prominent lingual cusps and many secondary grooves. The left mandibular second permanent molar was very small with an unusual occlusal configuration (Figs. 2d,e). She had crowding of the maxillary anterior teeth and anterior cross-bite as a result of the hypoplastic maxilla.

The affected son (Patient 2.2) had congenital lip pits and congenital absence of mandibular left second premolar. The shape of his right first permanent molar was unusual. The centrolingual cusp was very prominent, connecting with the mesiobuccal cusp with an oblique ridge (Fig. 2f). The left mandibular second deciduous molar was large mesiodistally, with very a prominent centrolingual cusp (Fig. 2g). The permanent teeth of both patients were affected with dental fluorosis, as a result of high fluoride consumption.

Mutation Analysis

Blood was obtained from all affected individuals and the unaffected mother of Family 2 after the written informed consents were given. DNA was extracted using a conventional technique. Exons 1-8 and part of exons 9 and 10 were amplified by polymerase chain reaction (PCR), using primer sequences as reported elsewhere (Schutte et al, personal communication). The amplified products were purified (Qiagen) and directly sequenced using BigDye labelling in an ABI 377 automated DNA sequencer (Applied Biosystems).

RESULTS

Mutation analysis of all coding exons of *IRF6* for Family 1 revealed an 11 bp deletion within exon 4, 358del(CAG GGC TCG AT), in all affected individuals (Fig. 3). This frameshift mutation truncates IRF6 beyond amino acid P119 and adds a single histidine residue. The DNA binding domain (amino acids 7 to 113) does not appear to be affected, but the remainder of IRF6, including the SMIR/IAD protein binding domain (Eroshkin and Mushegian, 1999) is absent. This mutation has not been reported previously and may explain the unique phenotypic features in this family.

Mutation analysis of *IRF6* for Family 2 detected no disease-causing mutations. The lack of a defined mutation in the coding region of *IRF6* in this family is consistent with the results of Kondo et al (2002); mutations were found in only 50% of the VWS families. As suggested in that study, such families may have a mutation elsewhere in *IRF6* or have a deletion or mutation in a second locus.

DISCUSSION

We discovered a novel 11 bp deletion mutation in exon 4 of *IRF6* in a four-generation Thai family with a unique form of VWS that included sensorineural hearing loss, large craniofacial sinuses, and limb anomalies. These anomalies also distinguish this family from families with PPS. Although individuals with PPS exhibit limb anomalies, they are generally restricted to syndactyly of the second and third toes and webbing of the lower limbs which probably reflects a defect in skin development rather than skeletal development. Also, 40% of

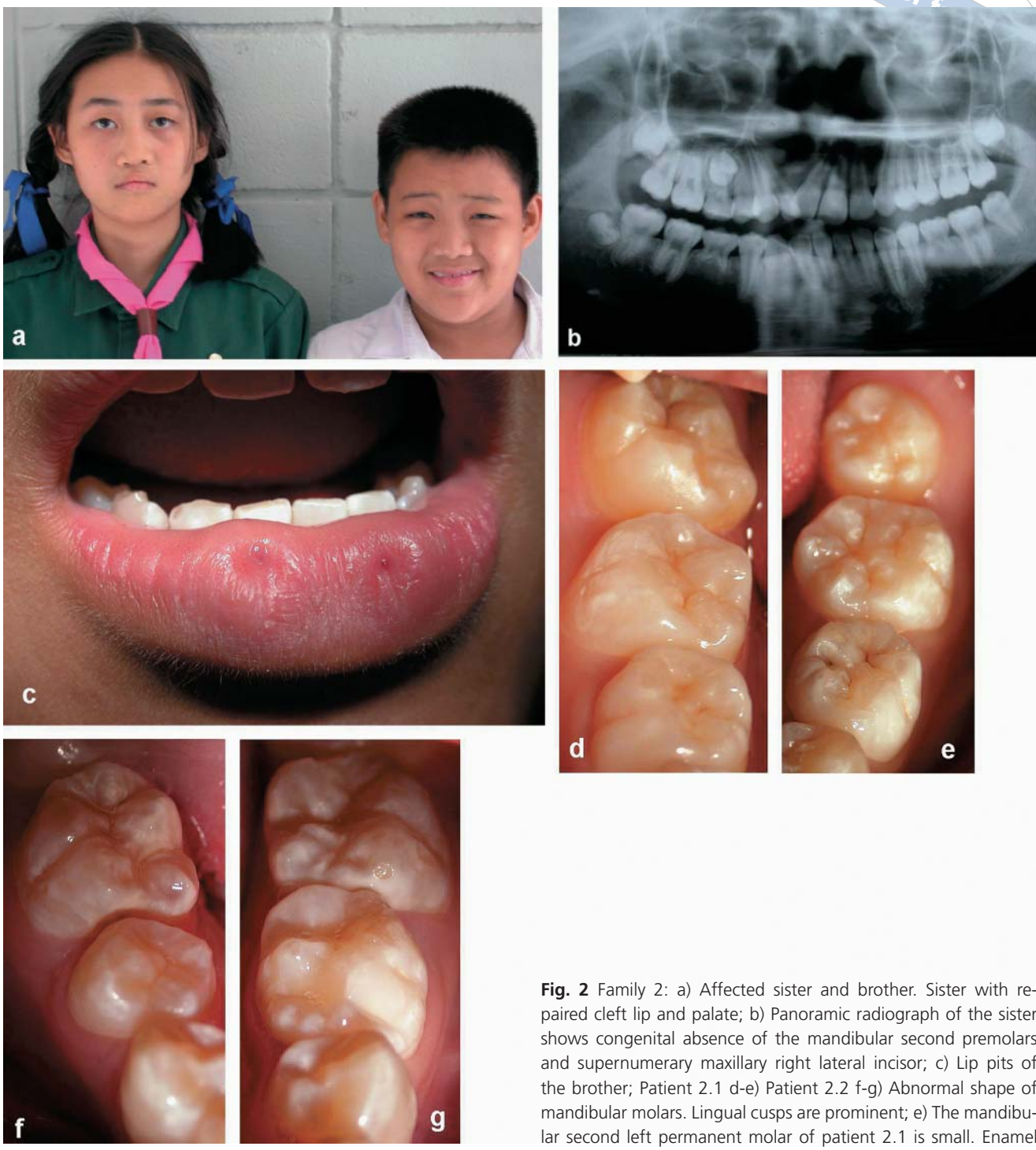

Fig. 2 Family 2: a) Affected sister and brother. Sister with repaired cleft lip and palate; b) Panoramic radiograph of the sister shows congenital absence of the mandibular second premolars and supernumerary maxillary right lateral incisor; c) Lip pits of the brother; Patient 2.1 d-e) Patient 2.2 f-g) Abnormal shape of mandibular molars. Lingual cusps are prominent; e) The mandibular second left permanent molar of patient 2.1 is small. Enamel fluorosis is noted.

PPS cases exhibit genital anomalies which were not observed in this family. We conclude that this family displays a unique form of VWS. Is the novel mutation found in this family responsible for the unique phenotype?

First, we can try to exclude other genetic and environment factors that might contribute to the novel phenotype. As seen in other VWS families (Burdick et al, 1985), the phenotype in this family is extremely variable and is consistent with the existence of modifying

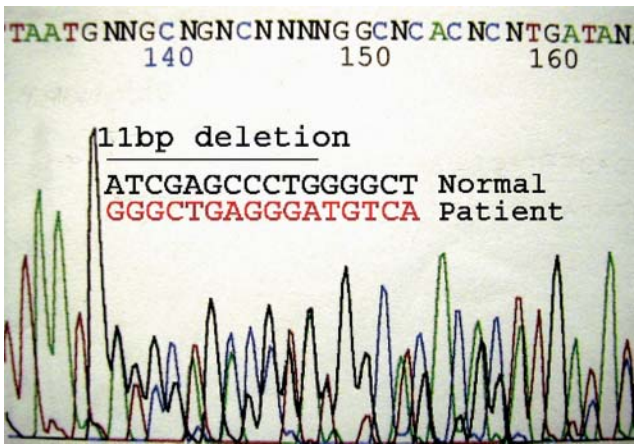

Fig. 3 Sequence from VWS patient with deletion in exon 4 of IRF6. The reverse sequence is shown for the wild type (wt) and deleted (del) alleles. The deleted allele is missing 11 nucleotides (lower case) and then picks up the wild type sequence (underlined).

factors. However, six of seven affected individuals in this family had a limb anomaly, four of six had dental anomalies and three of six exhibited deafness. The clustering of these additional anomalies in the affected members of this family suggest a single genetic factor with variable penetrance. The unique phenotype could be due to an intra-genic or linked modifier; however, no other mutations of interest were found in our mutation analysis of *IRF6* in this family. Also, the phenotype is probably not due to a common environment factor since the mothers in this family lived in different geographical regions, different socioeconomic conditions during their pregnancy, and the phenotype of most individuals with VWS in Thailand do not differ from other parts of the world (Shotelersuk et al, 2003).

Second, is this frameshift mutation in this family structurally and functionally distinct from the known PPS-causing mutation in *IRF6*? Kondo et al (2002) observed that nearly all (11/13) PPS-causing-mutations were located in exon 4. However, all of these mutations were missense mutations at residues that contacted the DNA. Consequently, the PPS mutations are predicted to abrogate DNA binding, whereas the frameshift mutation in Family 1 is predicted to leave the DNA-binding domain intact. We conclude that the frameshift mutation in Family 1 is structurally distinct from the known mutations that cause PPS. Functionally, the PPS mutations are predicted to have a dominant negative effect on IRF6 by forming a dimer with the wild type allele, but the dimer is unable to bind to its DNA sites (Kondo et al, 2002). We predict that the frameshift mutation in Family 1 could also cause a dominant negative effect, but through a different mechanism. Since the frameshift mutation is located immediately after the DNA binding domain, the truncated IRF6 could compete for binding of DNA sites, but would not be capable of transactivation. This mode of dominant negative effect was observed previously in cells that express truncated isoforms of IRF7 that only encode the DNA binding domain (Au et al, 2001). Future studies will need to address how dominant negative effects on the same gene can alter the development of distinct sets of tissues.

Third, is the frameshift mutation in this family functionally and structurally distinct from the known VWS-causing mutations in *IRF6*? Functionally, previous studies discovered that distinct microdeletions that include the entire *IRF6* gene cause VWS, demonstrating that VWS is caused by haploinsufficiency of *IRF6* (Sander et al, 1994; Schutte et al, 1999). In support of this conclusion, Kondo et al (2002) observed that the 49 VWS-causing mutations are consistent with haploinsufficiency, including 22 of 23 protein truncation mutations. In fact, one of the protein truncation mutations from the earlier study, Q118stop, is predicted to be nearly identical structurally to the frameshift mutation in family 1. The Q118stop mutation is shorter by only three amino acids. Despite this structural similarity, the functional consequence of these two mutations must differ, as the 6 affected individuals in the family with the Q118stop mutation exhibit the classic VWS phenotype. One possible model is that despite their structural similarity, the Q118X mutation causes the mRNA or protein to be unstable; the functional consequence is haploinsufficiency. Whereas the frameshift mutation in Family 1 produces a stable mRNA and protein; the functional consequence is a dominant negative effect. Additional cellular and *in vivo* studies will be necessary to test whether the frameshift mutation in Family 1 produces a truncated protein that is stable and then able to cause a dominant negative effect through competitive binding at IRF6 target sites.

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REFERENCES

Au WC, Yeow WS, Pitha PM. Analysis of functional domains of interferon regulatory factor 7 and its association with IRF-3. Virology 2001;280:273-282.

Burdick AB, Bixler D, Puckett CL. Genetic analysis in families with Van der Woude syndrome. J Craniofacial Genet Dev Biol 1985;5: 181-208.

Eroshkin A, Mushegian A. Conserved transactivation domain shared by interferon regulatory factors and Smad morphogens. J Mol Med 1999;77:403-405.

Gorlin RJ, Cohen MM Jr, Hennekam R. Syndromes of the head and neck. New York: Oxford University Press 2001;775:905-907.

Kantaputra PN, Sumitsawan Y, Schutte BC, Tocharoentanaphol C. Van der Woude syndrome with sensorineural hearing loss, large craniofacial sinuses, dental pulp stones, and minor limb anomalies: report of a four-generation Thai family. Am J Med Genet 2002;108:275-280.

Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, et al. Mutations in *IRF6* cause Van der Woude and popliteal pterygium syndromes. Nat Genet 2002;32:285-289.

Sander A, Schmelzle R, Murray J. Evidence for a microdeletion in 1q32-41 involving the gene responsible for Van der Woude syndrome. Hum Mol Genet 1994;3:575-578.

Schutte, BC, Basart AM, Watanabe Y, Laffin JJ, Coppage K, Bjork BC, et al. Microdeletions at chromosome bands 1q32-q41 as a cause of Van der Woude syndrome. Am J Med Genet 1999;84: 145-150.

Shotelersuk V, Srichomthong C, Yoshiura K, Niikawa N. A novel mutation, 1234del(C), of the *IRF6* in a Thai family with Van der Woude syndrome. Int J Mol Med 2003;11:505-507.

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