

INVESTIGATIVE REPORT

Connexin 26 (*GJB2*) Mutations in Two Swedish Patients with Atypical Vohwinkel (Mutilating Keratoderma plus Deafness) and KID Syndrome Both Extensively Treated with Acitretin

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Neuroectodermal syndromes involving the skin and inner ear may be associated with mutations in connexin proteins, which form gap junctions important for intercellular communication. Vohwinkel syndrome (keratoderma mutilans with hearing loss) and keratitis-ichthyosis-deafness (KID) syndrome are rare ectodermal dysplasias associated with dominant mutations in the *GJB2* gene encoding connexin 26. We report here two patients, one with KID and one with Vohwinkel syndrome. Both displayed unusual clinical features and responded well to long-term treatment with oral retinoid. Mutation analysis revealed a novel *GJB2* mutation p.Gly59Ser in the patient with Vohwinkel syndrome, whereas a recurrent mutation (p.Asp50Asn) was found in the patient with KID syndrome. The clinical features, particularly a proneness to skin cancer in the patient with Vohwinkel syndrome, are discussed in relation to the identified genotypes. **Key words:** genodermatosis; deafness; cancer; acitretin; Cx26, mutation.

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Dominant negative mutations in the human connexin (Cx) gene *GJB2* have recently been shown to cause two rare diseases: keratitis-ichthyosis-deafness (KID) syndrome (OMIM 148210) and keratoderma hereditarium mutilans with sensorineural deafness (Vohwinkel syndrome; VS) (OMIM124500). *GJB2* codes for Cx26, a protein component of the gap junction which is essential for cellular communication in many epithelial cells (1).

Depending on the location of the gene mutation, different types of Cx26 mutations can cause either isolated hearing loss (DFNA3 or DFNB1) or combinations of hearing and skin problems with variable phenotypes,

such as KID and VS (<http://davinci.crg.es/deafness>). The skin problem is characterized by disturbed epidermal differentiation manifested by hyperkeratosis especially on the palms and soles (keratoderma), which, in the case of VS, often becomes mutilating with starfish-shaped proximal extensions and hyperkeratotic bands around the fingers, so-called pseudoainhum, sometimes leading to auto-amputation (2, 3). KID syndrome is usually associated with less severe keratoderma and a milder hearing problem, but the eye involvement (keratitis) may eventually lead to impaired vision (4).

The treatment of VS and KID syndrome involves oral retinoids, which reduce the hyperkeratosis but do not affect the underlying disease mechanism. The first successful use of etretinate in VS was reported in 1981 (5), and since then several equally positive reports have appeared using both acitretin and isotretinoin (6–8). Much less has been written about retinoid therapy in KID syndrome, although this disease has a proneness to skin cancer (4) which *per se* is an indication for retinoids. In this report, we describe a man with VS associated with a novel Cx26 mutation, p.Gly59Ser, and a woman with KID syndrome caused by the recurrent mutation p.Asp50Asn, but with an unusual clinical presentation. Both patients have been treated extensively with retinoids with variable results.

MATERIALS AND METHODS

All clinical investigation and genetic analyses were conducted in accordance with the guidelines in the Declaration of Helsinki. The study was approved by the local ethics committee. Informed consent was obtained from all the individuals included in the study.

Case 1 (Vohwinkel syndrome)

A 75-year-old man with congenital ichthyosis, massive keratoderma and deaf-mutism was first seen by us at the age of 60 years. His parents (deceased many years previously) had both been healthy and there was no family history of a similar disease. He has no siblings or children. His psychomotoric development was normal apart from the congenital sensorineural deafness and associated mutism. He was diagnosed as having VS in the late 1970s, due to a typical combination of neuroectodermal symptoms.

At first examination in 1988 (by AV), he presented with mild, generalized ichthyosis and a verrucous type of palmar keratoderma extending beyond the wrists and with pseudoainhum

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Fig. 1. Case 1 (Vohwinkel syndrome) showing honeycomb keratoderma of the hand with constriction band on one finger (top, left and right) and mutilating keratoderma of the sole before and after oral retinoid therapy (bottom, left and right, respectively).

formations around some of the fingers (Fig. 1). His plantar keratoderma in particular was severely mutilating and occasionally erosive. He was prescribed oral etretinate 25 mg bid for the first time in 1980; this was later changed to acitretin 25 mg 5 days per week, which has been continued until the present. Although this reduced his ichthyosis and palmoplantar keratoderma quite substantially (Fig. 1), the mutilating foot lesions became more erosive and painful despite intermittent antibiotic therapy. In 1986, his right big toe was amputated due to an acral malignant melanoma. One year later, a chronic ulcer developed on the anterior of the left sole, which did not heal on conservative therapy. An incisional biopsy eventually showed invasive squamous cell carcinoma (SCC), which led to amputation below the knee in 1992. In 1999 he developed a verrucous type of basal cell carcinoma on his right lower limb, which was removed surgically. A concurrent punch biopsy specimen of the keratotic sole showed pseudoepitheliomatous hyperplasia and massive hyperkeratosis, but no atypia (results not shown). Apart from retinoid-induced hyperlipidaemia (controlled by diet and gemfibrozil 600 mg bid) and short episodes of liver transaminase elevations, etretinate/acitretin was well tolerated for over 20 years.

Case 2 (KID syndrome)

A 26-year-old woman was seen by us (A.V.) for the first time in 1986 at the age of 10 years (Fig. 2 top). She was born following an uncomplicated pregnancy, but showed widespread scaling of the skin at birth. There was no history of ichthyosis in the family; she has two healthy siblings and no children. Her psychomotor development has been normal, except for a mild sensorineural deafness of the left ear detected at the age of 4 years, and short heel cords corrected by orthopaedic surgery on three occasions between 1979 and 1992. Her hearing problem has remained constant and she only occasionally needs to use a hearing aid. Since early childhood she has had a mild, segmental keratitis with residual small epithelial defects and scarring, but no visual problems. If anything, the eye problems have diminished over the years. She occasionally uses corticosteroid eye drops due to allergic seasonal conjunctivitis. She has had no dental dysplasia, caries or any other mouth problems. Her skin problems have been fairly constant over the years, except for transient hypohidrosis during childhood. She now presents with diffuse, non-scaly palmo-plantar keratoderma and well-circumscribed hyperkeratotic plaques with follicular accentuation on her knees, elbows and back of her hands (Fig. 2 top), as well as in her face. On the inside of her thighs, the skin is erythematous with peculiar punched-out, non-inflamed areas of normal-looking skin (Fig. 2, bottom). In addition, she has slightly keratotic and pigmented (possibly post-inflammatory) lesions on the trunk, which were also noted in childhood (Fig. 2). Her hair growth is normal. Except for chronic furuncles in the axillae, there is no history of increased susceptibility to infections. Histopathology of a skin biopsy specimen taken in 1986 and 2001 showed a verrucous orthohyperkeratosis and a mild dermal inflammatory infiltrate (results not shown).

She was diagnosed as having KID syndrome at the age of 10 years, based on the triad of characteristic skin lesions, hearing problems and keratitis. She first received topical retinoids with some improvement of the keratotic lesions in the face, although not sufficient to motivate continued therapy. In March 2001, she was put on oral acitretin 25 mg/day, which greatly reduced the hyperkeratosis but caused more erythema especially on her thighs. The dose was tapered to 25 mg twice per week after one month, but since skin irritation remained a problem she stopped retinoid therapy altogether after 5 months. She is presently using only emollients.

Mutation analysis

Genomic DNA was extracted from peripheral blood by standard procedures. The entire protein encoding and flanking region of *GJB2* (GenBank accession number: M86849) was amplified by polymerase chain reaction (PCR) in two overlapping segments using the following two primer pairs with added M13 sequences: Cx26:1 fwd; 5'-TGT AAA ACG ACG GCC AGT CAT TCG TCT TTT CCA GAG CA-3' and Cx26:2 rev; 5'-CAG GAA ACA GCT ATG ACC AGC CTT CGA TGC GGA CCT T-3' and Cx26:3 fwd; 5'-TGT AAA ACG ACG GCC AGT ACC GGA GAC ATG AGA AGA AG-3', and Cx26:4 rev; 5'-CAG GAA ACA GCT ATG ACC GTT GCA TTT AAG GTC AGA ATC-3'. PCR conditions were as follows: 500 ng of genomic DNA was amplified in a 50 µl reaction volume of 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 100 µM dNTPs, 100 µM of each primer and 0.2 U AmpliTaqGold polymerase (Applied Biosystems, Roche, USA). An initial denaturation step of 10 min at 94°C was followed by 35 cycles of 94°C for 30 s 55°C for 30 s and 72°C for one min. and one final cycle of 72°C for 7 min. PCR products were purified (QIAquick PCR purification kit, QIAGEN, Germany) prior to bidirectional sequencing using M13 universal forward (5'-TGT AAA ACG ACG GCC AGT-3') and reverse primers (5'-CAG



Fig. 2. Case 2 (KID syndrome) showing eythrokeratotic, follicular plugging in the face at the age of 6 (top, left) and thickened skin on the back of the hand (top, right), and areas of atypical erythema and pigmentation on the thigh and trunk at the age of 26 (bottom, left and right, respectively)

GAAACA GCT ATG ACC-3'). For sequencing the ABI PRISM Big Dye Primer v3.0 Cycle Sequencing Ready Reaction kit was used (Applied Biosystems, USA) and the reaction products were analysed on an ABI 377 automated sequencer. For restriction enzyme digestions of PCR products the endonucleases *Tth1111* and *BstNI* (Amersham, Biosciences, Sweden) were used according to the manufactures recommendations. The products were analysed by electrophoresis in a 4% NuSieve gel.

RESULTS

Case 1 is the first patient with VS and case 2 the second KID patient reported from Scandinavia (9). Both patients had characteristic skin and inner ear involvement consistent with mutations in the *GJB2* gene.

DNA analysis

The coding and flanking sequences of the *GJB2* gene were sequenced by bidirectional sequencing of two overlapping PCR segments. The fragments were derived from genomic DNA using the primer pairs Cx26:1fwd and Cx26:2rev and Cx26:3fwd and Cx26:4rev. In the patient diagnosed with VS (case 1), DNA sequencing revealed a heterozygous G > A base substitution at position 175 in *GJB2* (Fig. 3A). The c.175G > A alteration results in an amino acid substitution p.Gly59Ser and abolishes a BstNI restriction site.

To confirm the base substitution a second PCR amplification was performed using primer pair Cx26:1fwd and Cx26:2rev followed by *BstNI* digestion of the 465 bp PCR product. The wild-type allele produces three fragments of 84 bp, 145 bp and 236 bp, whereas the mutated allele generates only two fragments with the sizes 84 bp and 381 bp (Fig. 3B).

The p.Gly59Ser (c.175G > A) variant has not been reported previously and therefore represents a novel base substitution in the *GJB2*. Since no additional family members were available for analysis, investigation of a control population was performed to exclude that c.175G > A constitutes a common polymorphism in the *GJB2* gene. PCR amplification of genomic DNA followed by restriction enzyme digestion with *BstNI* revealed that the p.Gly59Ser, c.175G > A variant was absent in all the 100 normal alleles analysed.

In the patient diagnosed with KID syndrome, DNA sequencing revealed heterozygosity for a recurrent mutation in the *GJB2* gene, p.Asp50Asn (c.148G > A) (Fig. 3C). The presence of the mutation was confirmed by PCR amplification using primers Cx26:1 fwd and Cx26:2 rev followed by restriction digestion with the enzyme *Tth1111*. Digestion of the 465 bp PCR product from the wild-type allele produces two fragments of 207 bp and 258 bp, whereas the mutant allele remains undigested (Fig. 3D).

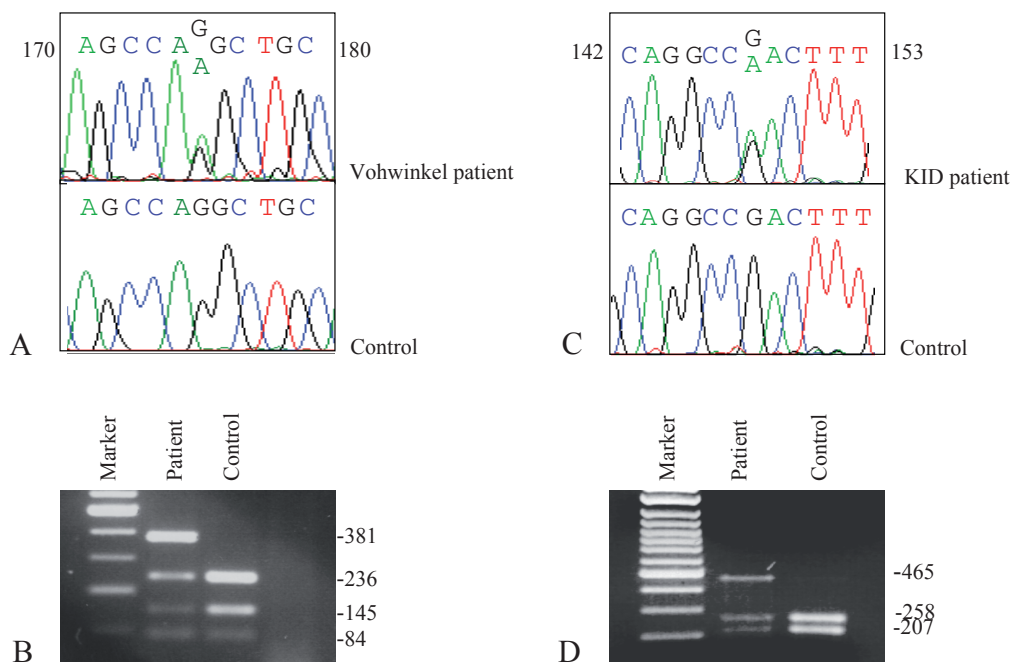


Fig. 3. *De novo* mutations in the *GJB2* gene in sporadic cases of Vohwinkel and KID syndromes. (A) Direct sequencing of *GJB2* in the Vohwinkel patient and a normal control. Positions in the nucleotide sequences are indicated. (B) Verification of the p.Gly59Ser(c.175G > A) mutation by PCR followed by *BstNI* restriction enzyme digestion. The PCR products were separated on a 4% NuSieve agarose gel using a 100-base pair (bp) ladder as a size marker. Fragment sizes in bp are shown to the right. *BstNI* digestion of the wild-type allele produces three fragments of 84, 145 and 236 bp, whereas the mutated allele generates two fragments of 84 bp and 381 bp. (C) Sequencing analysis of the *GJB2* gene in the patient with KID syndrome and a normal control. The p.Asp50Asn (c.148G > A) mutation was verified by PCR of genomic DNA followed by *Tth1111I* digestion as shown in (D). Digestion of the 465 bp PCR product produces two fragments of 207 and 258 bp, respectively, in the wild-type allele, whereas the mutant allele remains undigested.

DISCUSSION

Here, we report the identification of two dominant mutations in the *GJB2* gene, one of which is novel, that are associated with hearing impairment and cutaneous involvement. The symptoms and histopathological findings in the two investigated patients were more or less characteristic for VS and KID syndrome, respectively. However, whereas previously published cases of VS seem to have had severe symptoms from either the skin or the inner ear (7, 10–14), our case 1 has both a mutilating keratoderma (eventually leading to SCC) combined with generalized ichthyosis, and a complete congenital deafness. We excluded other forms of keratoderma, such as Mal de Meleda and Olmsted syndrome, based on the lack of hyperhidrotic, macerated and malodorous keratoderma and typical perioral lesions.

Case 2 also differs from previously published cases of KID syndrome, in that her keratitis and hearing problems were mild, and she had persistent erythematous plaques with punched-out holes of uninvolved skin on her thighs and several hyper-pigmented areas on the trunk. Both patients responded well to the anti-keratinizing effects of retinoids, but in case 2 the benefit of therapy was not enough to motivate long-term treatment.

To date, two missense mutations designated p.Asp66His and p.Gly130Val in the *GJB2* gene, have

been reported to be associated with classical VS (11–13). In addition, a p.Arg75Gln mutation has been disclosed in a Turkish family with VS-like symptoms (14).

By contrast, a variant form of Vohwinkel keratoderma without hearing loss and with more widespread skin involvement is associated with mutations in the *loricrin* gene on chromosome 1q21 (15, 16).

In the VS patient reported here, we identified a p.Gly59Ser (c.175G > A) mutation. This variant was not present in 100 normal alleles, suggesting that it does not represent a common polymorphism of the *GJB2* gene. Furthermore, c.175G > A has not been reported as a polymorphism neither in the Single Nucleotide Polymorphism (SNP) database (<http://www.ncbi.nlm.nih.gov/SNP/>) nor in the locus specific database provided by the Connexin deafness homepage (<http://davinci.org.es/deafness/>). The replacement of the amino acid glycine by serine at codon 59 represents a non-conservative alteration in the highly conserved first extracellular loop of Cx26 crucial for voltage gating and connexon-connexon interactions. The amino acid replacement p.Gly59Ser is therefore predicted to seriously compromise these functions. The VS patient reported here has no family history of the disease, suggesting that the p.Gly59Ser mutation represents a *de novo* mutation.

Interestingly, another mutation, designated p.Gly59Ala (c.176G > C), located at the same position in Cx26 has

previously been reported in a family with hearing loss and palmoplantar hyperkeratosis (OMIM 148350) (17). It remains to be elucidated whether a mutation at this position is associated with a higher risk of cutaneous malignancies (apparent in our patient) than in other causes of VS.

The mutation identified in the KID patient, p.Asp50Asn (c.148G > A), has been reported previously and our data support that this mutation frequently is associated with KID syndrome (9, 18–22). The mutation in this case most likely occurred *de novo*, which is consistent with the fact that most cases of KID syndrome are sporadic. It is noteworthy that the p.Asp50Asn mutation has also been found in a patient with the hystrix-like ichthyosis with deafness syndrome (HID), OMIM 602540 (19).

The Cx protein consists of four transmembrane domains, one cytoplasmic and two extracellular loops (Fig. 4). Both the N-terminal and C-terminal parts of the proteins are located in the cytoplasm. In the two patients reported here, both amino acid replacements have occurred in the first extracellular loop of Cx26, which is highly conserved among the connexins. Interestingly, most dominant negative *GJB2* mutations associated with hearing impairment and cutaneous involvement reported so far are located in the cytoplasmic N-terminal or in the first extracellular loop of Cx26 (Fig. 4), which are involved in the control of voltage gating and in the interaction between connexons. However, mutations located outside these domains have also been reported. The mutation p.Phe142Lys located in the third transmembrane domain of Cx26, causes muco-cutaneous findings different from those in HID, KID and VS (23). The p.Gly130Val mutation recently reported in a family with classical VS (13) is located in the intracellular domain of Cx26.

Interestingly, mutations in several other connexin proteins have also been associated with skin diseases such as erythrokeratoderma variabilis (Cx30.3 and Cx31) and Clouston ectodermal syndrome (Cx30) (for review see 24). Recently, Jan et al. (25) also described a patient with alopecia and KID syndrome who had an unusual Cx30 mutation.

The exact role of Cx26 mutations in skin disease and deafness remains unclear. The underlying molecular mechanism may involve either direct disruption of the gap-junction intracellular communication or include novel interactions of mutant proteins leading to cell death. The effect of mutant Cx26 upon other genes is supported by recent studies, where it has been demonstrated that mutant forms of Cx26 associated with skin disorders have an impact on the function of Cx43 and Cx30 (26, 27). Furthermore, mutations affecting both Cx26 and Cx30 are associated with pre-lingual deafness. A 342 kb deletion involving Cx30 (*GJB6*) is the second most frequent mutation causing pre-lingual deafness in the Spanish population (28). This deletion is associated with deafness both in the homozygous state and in the heterozygous state in combination with a *GJB2* mutation (<http://davinci.crg.es/deafness/>).

Due to the unusual clinical features of the VS patient reported here, presenting with both mutilating keratoderma, ichthyosis and complete congenital deafness, we also analysed his DNA for the presence of the large deletion involving *GJB6*. However, no such deletion was identified (data not shown). Further studies are needed to understand mechanistically how the p.Gly59Ser mutation in *GJB2* may contribute to the unusual clinical features of the VS patient described here, including his proneness to skin cancer.

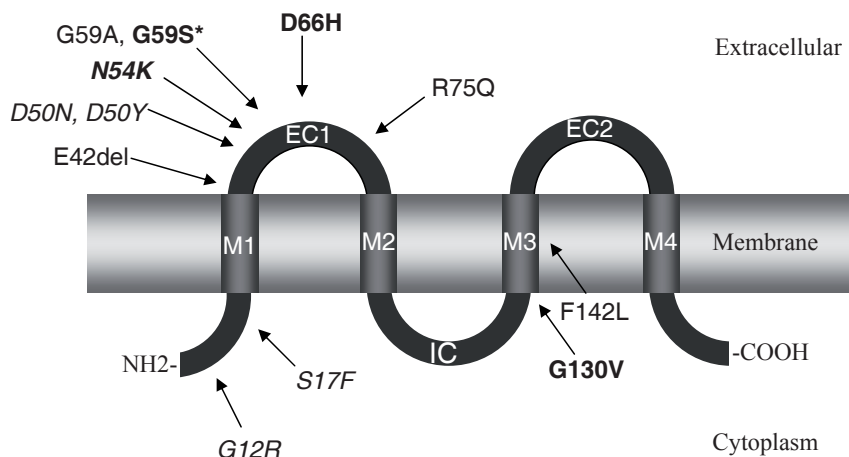


Fig. 4. The Cx26 polypeptide with four transmembrane domains (M1–M4), two extracellular domains (EC1, EC2) and cytoplasmic (IC), N- and C-terminal domains. The amino (-NH₂) and carboxy terminals (-COOH) are indicated. The arrows indicate the mutations identified in patients with palmoplantar keratoderma with hearing impairment, KID and Vohwinkel syndromes and are written in Roman, italic and bold text, respectively. A mutation identified in a family with Bart-Pumphrey syndrome (29) is indicated in bold italic text. The mutation p.Asp50Asn associated with KID syndrome has also been identified in a patient diagnosed with HID syndrome (19). The novel *GJB2* mutation reported here in the patient with Vohwinkel syndrome is indicated by an asterisk.

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