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A missense mutation in connexin26, D66H, causes mutilating keratoderma with sensorineural deafness (Vohwinkel's syndrome) in three unrelated families

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The multiplicity of functions served by intercellular gap junctions is reflected by the variety of phenotypes caused by mutations in the connexins of which they are composed. Mutations in the connexin26 (Cx26) gene (*GJB2*) at 13q11–q13 are a major cause of autosomal recessive hearing loss (DFNB1), but have also been reported in autosomal dominant deafness (DFNA3). We now report a Cx26 mutation in three families with mutilating keratoderma and deafness [Vohwinkel's syndrome (VS; MIM 124500), as originally described]. VS is characterized by papular and honeycomb keratoderma associated with constrictions of digits leading to autoamputation, distinctive starfish-like acral keratoses and moderate degrees of deafness. In a large British pedigree, we have mapped the defect to the Cx26 locus. All 10 affected members were heterozygous for a non-conservative mutation, D66H, in Cx26. The same mutation was found subsequently in affected individuals from two unrelated Spanish and Italian pedigrees segregating VS, suggesting that D66H in Cx26 is a common mutation in classical VS. This mutation occurs at a highly conserved residue in the first extracellular domain of the Cx26 molecule, and may exert its effects by interfering with assembly into connexons, docking with adjacent cells or gating properties of the gap junction. Our results provide evidence that a specific mutation in Cx26 can impair epidermal differentiation, as well as inner ear function.

INTRODUCTION

The inherited palmoplantar keratodermas (PPKs) are a clinically and genetically diverse group of conditions characterized by punctate, focal or diffuse hyperkeratosis of palms and soles, sometimes accompanied by other cutaneous or systemic features. In 'mutilating' PPKs, circumferential hyperkeratosis of digits can result in constricting bands (pseudo-ainhum) sufficient to cause autoamputation. The constriction is perhaps due to creases in stiff epidermis at points of flexion, causing local ischaemic damage. In 1929, Vohwinkel (1) and Wigley (2) independently reported mutilating PPK associated with honeycomb-like keratoderma and starfish-like keratoses on the knuckles. In Vohwinkel's report, a mother and daughter were affected. Moderate sensorineural deafness was also a feature in this family (3), as in most other clear cases of Vohwinkel's syndrome (VS) (4–7). However, classification of PPKs often is confounded by clinical overlap between genetically distinct entities, and pseudo-ainhum is found in several other transgradient PPKs. Recently, insertional mutations in the gene encoding loricrin (8–10), a component of the cornified cell envelope, have been described in families manifesting a clinical variant of VS associated with generalized ichthyosis without deafness (11–12). Korge *et al.* (9) studied a family with deafness and starfish keratoses but not ichthyosis (i.e. VS as originally described), demonstrating that it was clinically and ultrastructurally distinct from the variant with ichthyosis and that it did not map to the loricrin locus. Therefore, insertional mutations in loricrin are most likely unique to variant VS, mutilating keratoderma with ichthyosis. Here we report that a mutation in the *GJB2* gene, encoding the gap junction protein connexin26 (Cx26), is associated with classical VS.

Connexins are the building blocks of gap junctions, which are plasma membrane complexes facilitating and regulating the passage of small (<1 kDa) molecules between cells (13–15). Connexons, hexamers of connexin subunits, dock with those of adjacent cells to produce a direct inter-cytoplasmic channel. These gated channels are applied to different functions in different tissues, for example non-synaptic transmission of neural impulses, chemical signalling such as in growth regulation, or nutrient exchange. There are thought to be >14 genes in the vertebrate connexin family (15), which are

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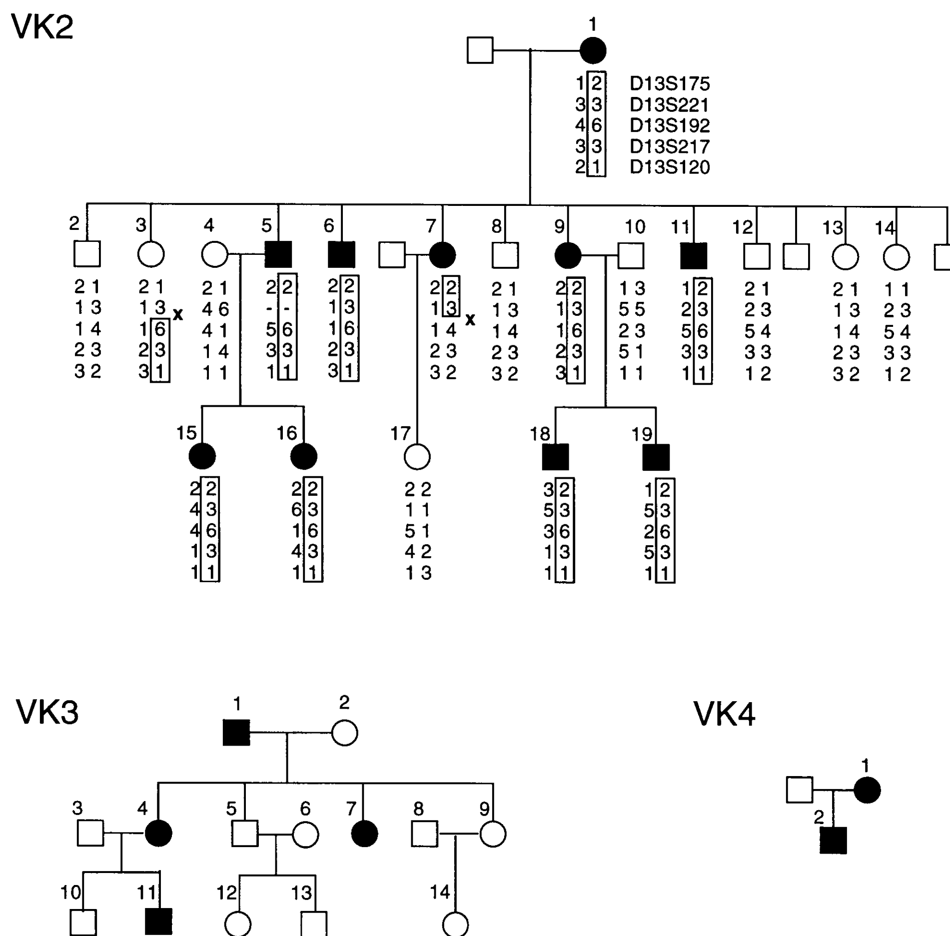

Figure 1. Pedigrees studied. In VK-2, haplotypes for microsatellite markers in the region of the connexin 26 locus at 13q11–q13 (*D13S175*, *D13S221*, *D13S192*, *D13S217* and *D13S120*) are shown. Recombination events defining the locus of VS are indicated by crosses.

expressed in a tissue- and differentiation-specific manner. The connexin sequence is predicted to produce four transmembrane domains, with intracellular N- and C-termini, and two extracellular loops (16). Mutations in connexin genes are now known to cause several inherited human disorders, whose phenotype at least in part reflects the distribution and inferred function of the affected genes. These include a progressive degenerative neuropathy, X-linked Charcot–Marie–Tooth disease [CMTX; connexin32 (Cx32)] (17), non-syndromic deafness (Cx26 and connexin31) (18–24), zonular pulverent cataract (connexin50) (25) and erythrokeratodermia variabilis (connexin31) (26).

RESULTS

Patients and clinical features

We studied a family with mutilating PPK and deafness (9), in which 10 of 22 individuals, aged 10–76 years, were affected (family VK2; Fig. 1). In the milder or younger cases, the keratoderma consisted of translucent horny papules, in places becoming confluent (Fig. 2a). Confluent lesions on the palms in older cases were responsible for the 'honeycomb' pattern of keratoderma, although some cases had only callosities at pressure points, or even striate lesions. Linear lesions, including some at sites of injury, suggested the isomorphic (Köbner) phenomenon (Fig. 2c). Warty papules coalesced into typical starfish keratoses over the knuckles, and sometimes other prominences. The edges of the palmar keratoderma demonstrated similar spiky extensions into normal skin. In two individuals, keratoderma extending around small digits had resulted in pseudo-ainhum (Fig. 2b), and one woman had lost a little toe. Adult members of the family suffered from moderate to severe sensorineural deafness (Fig. 2d), although the children (aged 8–15 years) were only mildly affected at the time of assessment.

DNA was also obtained from seven members of a Spanish VS family, VK3 (Fig. 1), in whom identical clinical features have been reported (5). We also studied two affected members of an Italian family, VK4 (Fig. 1). The mother (VK4-1), reported by Sensi *et al.* (27), had mutilating PPK with bilateral symmetric hearing loss of cochlear origin, but also congenital anomalies including cleft lip and palate, microcephaly and

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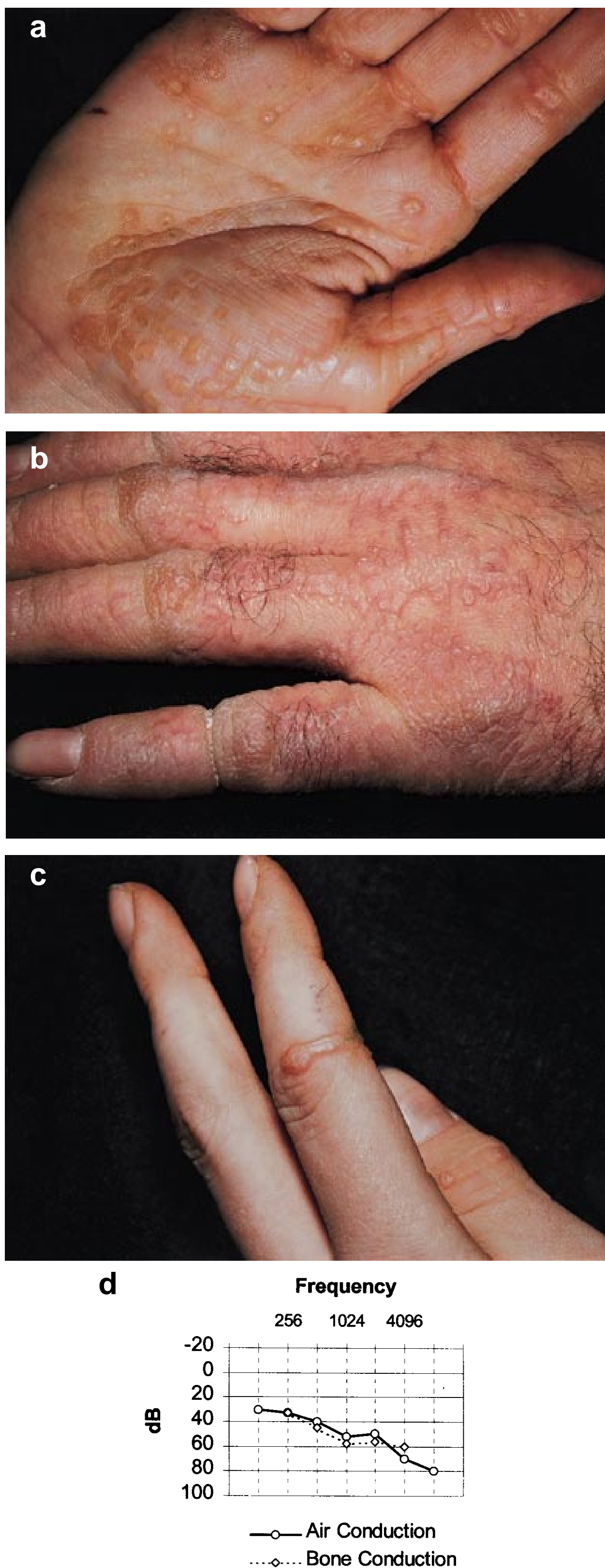

Figure 2. Clinical features of VS in VK-2. (**a**) Papular and confluent keratoderma in a child, VK2-16. (**b**) Pseudo-ainhum and starfish keratoses in an adult, VK2-6. (**c**) Linear lesions arising at the site of a cut in VK2-19. (**d**) A representative audiogram of the right ear in an affected adult, VK2-9; the other ear, and audiograms from other adult cases, exhibited a similar degree of sensorineural hearing loss (data not shown).

facial asymmetry. Her son (VK4-2), who is 2 years old at the time of writing, began to develop hyperkeratotic papular lesions on both palms and soles at the age of 7 months which have increased progressively in number and begun to coalesce. He has no craniofacial anomalies.

Linkage and mutation analysis

Linkage analysis was performed on 19 individuals (10 affected and nine unaffected) from family VK2 (Fig. 1). Having excluded a number of other loci of genes involved with keratinization (data not shown), a genome search was started. Potential evidence for linkage was detected with the microsatellite marker *D13S221*, mapping to chromosome 13q11–q12 (pairwise lod score = 1.51, $\theta = 0$). Other markers in the region (*D13S175*, *D13S192*, *D13S217* and *D13S120*) also provided positive pairwise lod scores. By multipoint analysis, a maximum lod score of 3.73 was obtained at *D13S175*. Inspection of the haplotypes revealed a recombination event between *D13S221* and *D13S192* in the affected individual VK2-7, which mapped the disease locus proximal to *D13S192*. Another recombination event in the unaffected individual VK2-3 confirmed this localization (Fig. 1).

Two connexin genes are known to map to the 13q11–q12 region: *GJB2* encoding Cx26 and *GJA3* encoding connexin46 (Cx46) (28). *GJB2* was considered a good candidate gene given its expression in the cochlea and keratinocytes, and its role in recessive non-syndromic sensorineural deafness (DFNB1) and in autosomal dominant deafness (DFNA3). Cx46 is not known to be involved in human diseases, although homozygous disruption of this gene in mice resulted in development of nuclear cataracts associated with proteolysis of crystallins (29). The coding region of the Cx26 gene was amplified by PCR and screened for mutations by direct sequencing in two affected members of family VK2. Sequence analysis revealed a heterozygous G→C transversion in codon 66 (Fig. 3a), which results in a non-conservative amino acid substitution from aspartic acid (GAT) to histidine (CAT), D66H. This mutation affects a residue highly conserved across connexins (Fig. 3c). No other sequence variants were found in the rest of the coding sequence of Cx26. As the G→C transversion causes the loss of a recognition site for *Mbo*I endonuclease, PCR products from all available family members were subjected to restriction analysis with *Mbo*I, demonstrating the presence of the heterozygous mutation in all affected individuals and its absence in all those unaffected (Fig. 3b). In addition, the mutation was not detected in 145 Caucasian controls.

Families VK3 and VK4 were also screened for Cx26 mutations. Sequence analysis of affected individuals VK3-1 and VK4-1 revealed that both were heterozygous for the same D66H mutation detected in family VK2. The presence of the mutation in all affected family members was again demonstrated by restriction analysis with *Mbo*I (Fig. 3b).

DISCUSSION

Cx26 is expressed in a wide variety of tissues, including the epithelial supporting cells surrounding the sensory hair cells of the cochlea and in the fibrocytes lining the cochlear duct (18,30). A possible role for Cx26 in the cochlea may be to mediate the recycling of potassium ions passing through the

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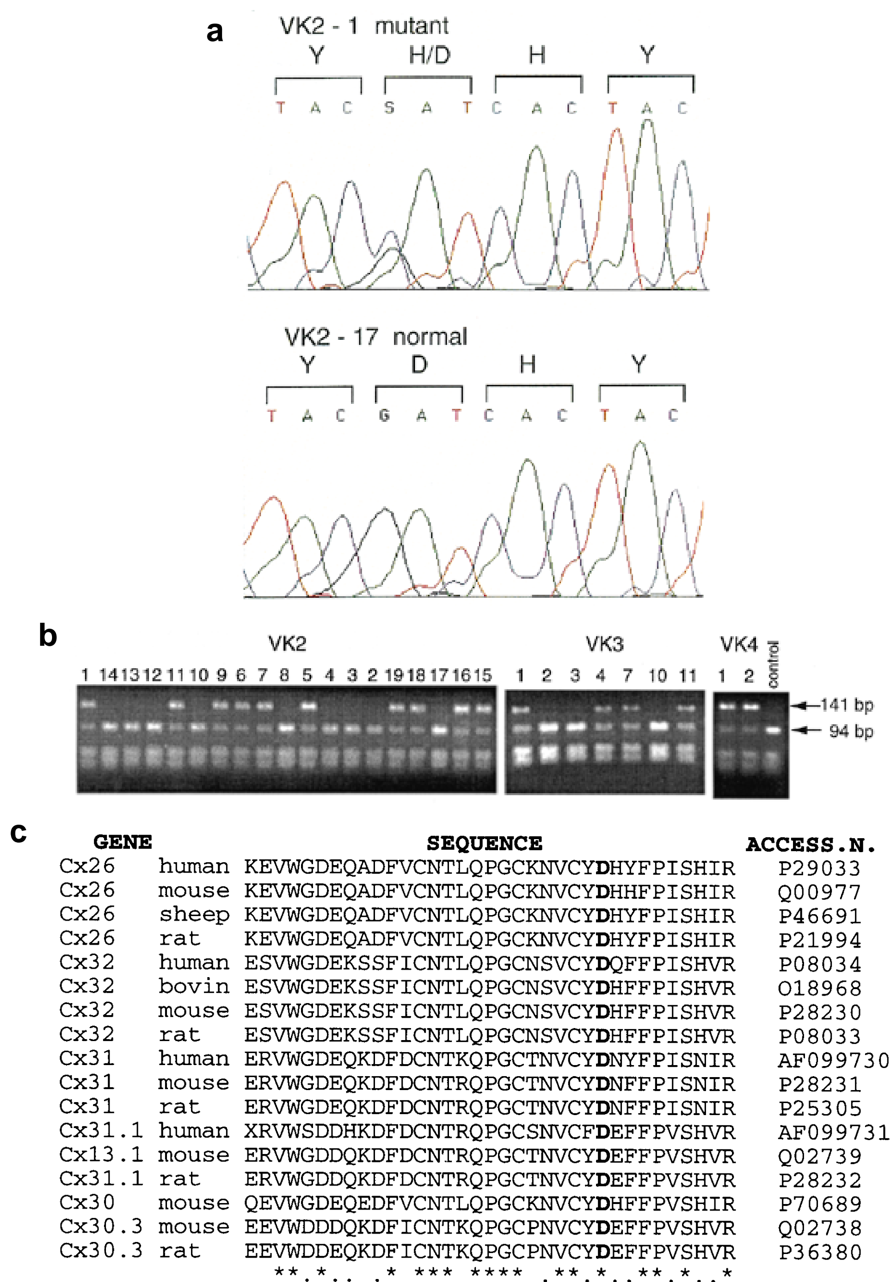

Figure 3. (**a**) Sequence data from an affected and an unaffected member of VK-2 showing the heterozygous G→C mutation at codon 66, encoding histidine (H, CAT) instead of aspartic acid (D, GAT). (**b**) *Mbo*I digestion of DNA amplified from all available members of the three pedigrees, showing an abnormal band of 141 bp in all affected individuals. (**c**) Multiple alignment of the first extracellular domain of the human Cx26 protein with other β-class gap junction proteins; the site of mutation is shown in bold; *, residues identical in all proteins; ·, similar residues; the Swissprot or GenBank accession numbers are shown on the right.

hair cells back to the endolymph. Mutations in its gene cause many cases of autosomal recessive and sporadic non-syndromic deafness (DFNB1) (18–22). One mutation, 30delG, is particularly common, accounting for two-thirds of all Cx26 mutations in DFNB1 patients, and is estimated to be responsible for 20% of all hereditary childhood hearing loss (22). Cx26 mutations also have been identified in autosomal dominant non-syndromic deafness (DFNA3). Specifically, three heterozygous missense mutations associated with deafness have been reported: W44C (23), M34T (18) and R75W (31). The W44C mutation is likely to have a dominant-negative effect and its significance is supported by linkage data in two large pedigrees (23). No cutaneous phenotype was associated with this mutation (23). The role of the other two variants is more problematic. The M34T variant is controversial, as it has been found in normal hearing individuals (22,32), suggesting that it is a polymorphism rather than a pathogenic mutation. However, this variant acted as a dominant inhibitor of wild-type Cx26 channel activity in the paired *Xenopus* oocyte expression system, a model of gap junction function (33). Interestingly, members of the family with the M34T variant reported by Kelsell *et al.* (18) also had a papular and confluent PPK with pseudo-ainhum (34);

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however, the M34T mutation segregated with the profound deafness phenotype but not the skin disorder in this family. The R75W mutation was identified in two affected members of an Egyptian family with pre-lingual deafness and a diffuse, fissured keratoderma (31). This mutation interfered with gap junction function in the *Xenopus* oocyte model, but was also found in an Egyptian control individual, with no skin disease but of unknown hearing function. Therefore, only the W44C mutation has been associated unambiguously with dominant deafness, while the relevance of dominant mutations in Cx26 to skin disease has remained enigmatic.

We have detected a dominant Cx26 mutation which co-segregated with mutilating keratoderma and deafness in three pedigrees, strongly suggesting that this mutation causes both the cutaneous and the hearing abnormalities observed in these families. The absence of a cutaneous phenotype associated with the W44C mutation suggests that the D66H and the W44C mutations affect different aspects of channel function, giving rise to different phenotypes. Similarly, mutations in the *GJB3* gene, encoding Cx31, have been reported recently both in patients with autosomal dominant deafness without apparent cutaneous phenotype (24) and in individuals affected by the skin disorder erythrokeratodermia variabilis (EKV) (26), without deafness. The main features of EKV are figurate hyperkeratotic plaques and transitory erythemas, but in some cases there is palmoplantar scaling or keratoderma clinically distinct from Vohwinkel's keratoderma. Overall, these findings imply that different mutations in connexin genes can produce cutaneous features and/or hearing loss, but that the precise phenotype depends on the nature and location of the mutation, and on the connexin gene involved.

The D66H mutation we describe has not been reported in other connexins, but it is a non-conservative change in a highly conserved sequence (Fig. 3b), and pathogenic mutations in adjacent residues have been reported in Cx32 in CMTX (36–38), suggesting that this region has functional importance. The D66H mutation affects a residue in the first extracellular domain, which is important in multimer assembly and docking with other connexons (16); thus it is likely that this mutation exerts a dominant-negative effect by impairing these abilities. The mutation could also selectively impair the ability of Cx26 to form heteromeric as well as homomeric connexons (39), which exhibit differential permeability to second messengers (40). Alternatively, it is possible that a change in charge or conformation of Cx26 could disrupt the gating functions of the connexon for certain molecules or ions only, by analogy with Cx32 mutations, which have distinct effects on gap junction function and gating properties (41). In HeLa cells, normal Cx26 regulates growth. Certain Cx26 mutants inhibit this control, while others do not (42). Hence, one possible consequence of gap junction dysfunction is impaired transfer of growth regulators between cells (43). Cx26 expression is up-regulated in hyperproliferative skin following tape-stripping and in psoriasis (35,44), and some of the lesions in our patients arose at sites of injury or stress. Defects in other genes expressed in proliferating epidermis, such as keratin 6 or 16, similarly produce keratoderma at pressure points (45). Keratoderma in VS may thus represent an abnormal healing response due to defects in growth regulation following induction of mutant Cx26 expression.

The D66H mutation is likely to have arisen independently in the three families reported herein, since they originated from different countries (the UK, Spain and Italy). Thus, it is possible that this mutation is common to the majority (if not all) of cases of classical VS. It is also possible that other mutations in Cx26, or in another connexin expressed in the epidermis, may lead to the same or a related disease phenotype. However, the combination of PPK and deafness (MIM 148350) (46–49) may also have other causes: in two families, mutations in mitochondrial DNA have been identified (50). The common ectodermal origin of the skin and of the non-sensory epithelial cells of the organ of Corti, which express Cx26, may result in several shared pathways in which different defects give rise to the same association of phenotypes.

MATERIALS AND METHODS

Linkage analysis

Microsatellite markers used for the genome search were part of the UK MRC Human Genome Mapping (HGMP) set (51). PCRs were performed using primers labelled with either 6-FAM, HEX or TET phosphoramidite and were analysed on a 373A Sequencer (Applied Biosystem). Two-point lod scores between the disease locus and genetic markers were calculated using MLINK from the LINKAGE package (52). The disease was considered fully penetrant with autosomal dominant inheritance. Multipoint linkage analysis was performed using the VITESSE algorithm (53). The marker map was cen–*D13S175*–7.2 cM–*D13S221*–2.2 cM–*D13S192*–2.3 cM–*D13S217*–2 cM–*D13S120*–tel.

Mutation detection

The coding sequence of the human Cx26 gene (GenBank accession no. M86849) was PCR amplified from genomic DNA using primers pairs producing two overlapping amplimers: pair A, tct ttt cca gag caa acc gcc (forward), gac acg aag atc agc tgc agg (reverse); pair B, gcc gac ttt gtc tgc aac acc (forward), cct cat ccc tct cat gct gtc (reverse). The PCR products were gel purified using the QIAquick Gel Extraction kit (Qiagen, Crawley, UK) and directly sequenced with the same PCR primers using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, UK). To check for the presence or absence of the mutation in all family members and in the controls, PCR products obtained using primer pair A were digested with *Mbo*I and the fragments were resolved on a 3% agarose gel. Normal individuals displayed fragments of 37, 42, 47, 55 (not resolved on gel) and 94 bp. Carriers of the mutant allele showed a 94 bp fragment of half the intensity and an additional fragment of 141 bp.

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