



SHORT REPORT

# Connexin mutations associated with palmoplantar keratoderma and profound deafness in a single family

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Recently, mutations in two gap junction genes, *GJB2* and *GJB3* (encoding Connexin 26 and Connexin 31, respectively), have been shown to underlie either inherited hearing loss and skin disease or both disorders. In this study, we have extended our analysis of a small family in which palmoplantar keratoderma and various forms of deafness is segregating. In addition to the previously described sequence variant M34T in *GJB2*, two other sequence variants were identified: D66H also in *GJB2* and R32W in *GJB3*. As D66H segregated with the skin disease, it is likely to underlie the palmoplantar keratoderma. The other two gap junction variants identified may contribute to the type of hearing impairment and the variable severity of the skin disease in the family. *European Journal of Human Genetics* (2000) 8, 141–144.

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## Introduction

Germline mutations in the gene encoding the gap junction protein Connexin 26 (*GJB2*) cause autosomal recessive deafness<sup>1</sup> and, in some populations, underlie between 30–50% of genetic deafness.<sup>2,3</sup> Although two reports have demonstrated that *GJB2* mutations can also cause autosomal dominant deafness,<sup>4,5</sup> it remains controversial as to whether the first described autosomal dominant deafness-associated variant, termed M34T, is actually disease-causing<sup>1</sup> or just a low frequency non-consequential polymorphism.<sup>6</sup> An *in vitro* assay for connexin channel activity suggests that M34T is not a recessive or a neutral allele of *GJB2* but acts as a dominant inhibitor of channel activity.<sup>7</sup> However, the increasing reports of normal hearing M34T carriers from distinct ethnic populations suggests that the inheritance of this sequence variant is not sufficient to cause hearing loss. To date, no M34T homozygotes have been reported. Here we provide a

possible molecular explanation for the association of M34T with profound deafness.

The profound deafness-associated M34T<sup>1</sup> was identified in a small family in which palmoplantar keratoderma (PPK) and deafness was segregating. The PPK is diffuse and its distribution resembles that of a mild form of Vohwinkel's syndrome.<sup>8</sup> The skin disease in individual II:2 differed from that seen in other members of the family in that there was a more extensive involvement of the palmoplantar skin. The non-volar epidermis of this individual was also affected (data not shown). M34T was found in the DNA of two sisters (individuals II:2 and II:5; Figure 1) who presented with the skin disease and profound hearing loss from birth. DNA was available from only one other family member; the daughter of one of the sisters (individual III:1) who was shown not to carry the M34T variant. As III:1 had the skin disease but not the profound form of hearing loss, this suggested that the M34T variant was associated with the profound deafness occurring in the family.

One explanation for M34T as being more significant than a neutral polymorphism is that its expressivity is dependent on its genetic background. However, no second-site mutations in *GJB2* which may nullify the putative dominant effect

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of M34T in normal hearing carriers have been reported. Conversely, it is possible that M34T only causes genetic deafness when associated with a second mutation in either *GJB2* or in another gene associated with hearing loss, for example, the gene encoding another gap junction protein, Connexin 31 (*GJB3*).<sup>9</sup> Autosomal dominant mutations in *GJB3* have also been associated with the skin disease, *Erythrokeratoderma variabilis*.<sup>10</sup> In addition, the mitochondrial A7445G mutation has also been associated with both inherited deafness and a palmoplantar keratoderma.<sup>11</sup>

To investigate the latter possibility, DNA from three members of the PPK/deafness family in which M34T is segregating were reassessed for a second-site mutation in *GJB2* and also for mutations in *GJB3*. Previously, none of the three family members were shown to carry the mitochondrial A7445G mutation (data not shown).

## Materials and methods

### Family material

The clinical features of the palmoplantar keratoderma and the different types of hearing impairments (including audiograms) segregating in the family have been described previously.<sup>1,8</sup> The structure of the family is shown in Figure 1c. DNA was available from only three members of the family (individuals: II:2, II:5 and III:1). Genomic DNA extracted from 122 unrelated individuals was used as a control population.

### Mutation analysis

The entire protein encoding region of both genes was amplified by PCR. *GJB2* was performed as described previously.<sup>1</sup> For *GJB3*, the coding sequence of human connexin 31 (*GJB3*) was amplified by PCR using the primer pair 5' GTCAGAACTCAGAACACTGCC 3" corresponding to bases 681–701 and 5' CCTATACCCGGCTAGACAGC 3" corresponding to bases 1787–1768 (Genbank AJ004856) under the following PCR conditions: 35 cycles of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C. Each PCR fragment was screened for sequence variants by heteroduplex analysis and Denaturing High Performance Liquid Chromatography (DHPLC). All PCR reactions were heated to 95°C for 5 min followed by cooling to a final temperature of 25°C. The samples were then transferred to the autosampler block, maintained at 7°C, for analysis. DHPLC was performed using a WAVE™ DNA Fragment analysis system (Transgenomic, Crew, Cheshire, UK). Following DHPLC, each variant PCR product was directly sequenced by PCR-cycle sequencing (Big-Dye terminator system, PE Applied Biosystems, Warrington, Cheshire, UK) and analysed on an ABI377 automated sequencer (PE Applied Biosystems, Warrington, Cheshire, UK). The two further mutations identified, D66H in *GJB2* and R32W in *GJB3*, were confirmed and used to screen their presence in the control population by restriction digestions using the restriction enzymes DpnII and HpaII, respectively. Cloning of the

*GJB2* PCR products were performed using the pGEM-T Easy Vector system (Promega, Southampton, UK) according to the manufacturer's instructions.

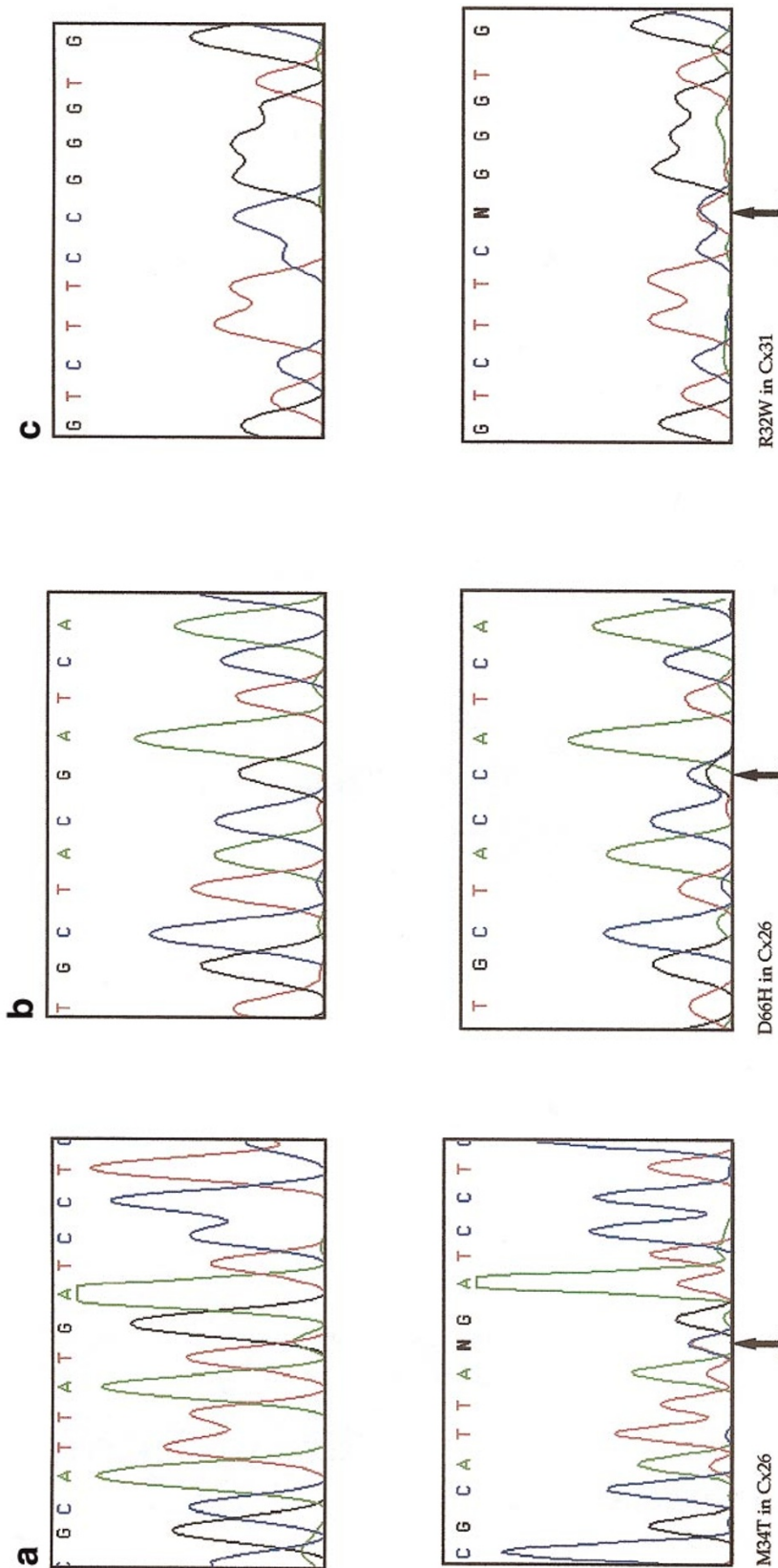
## Results

Heteroduplex and DHPLC analysis revealed three variants in *GJB2* and *GJB3* in the family (Figure 1a). Sequencing identified the two new coding sequence variants, D66H in *GJB2* and R32W in *GJB3* (Figure 1b). Restriction enzyme digests also confirmed the presence of these sequence variants (data not shown).

## Discussion

In addition to identifying the previously described M34T variant in II:2 and II:5, a second, previously undetected, sequence variant in *GJB2*, was present in all three individuals (Figure 1). This heterozygous G–C substitution in codon 66 results in a non-conservative amino acid change from an aspartic acid (GAT) to a histidine (CAT) (D66H) in the translated sequence. This sequence variant was not observed in 244 unrelated chromosomes and has not been reported in the literature as a polymorphism. The D66H carrier status was confirmed by restriction enzyme digestion of the PCR products as it results in the disruption of a DpnII site (data not shown). This aspartic acid residue is present in the first extracellular loop of *GJB2* and is conserved in both beta and alpha types of connexins across different species, suggesting it is important for connexin activity. As all three individuals had the skin disease but not the profound deafness, it is likely that D66H underlies the skin disease in the family (Figure 1c). From heteroduplex and DHPLC analysis plus sequencing of clones derived from the PCR products, M34T and D66H were shown to represent different alleles of *GJB2*.

A sequence variant in *GJB3* was also identified in this family (Figure 1). A single nucleotide substitution resulted in another non-conservative amino acid change from an arginine (CGG) to a tryptophan (TGG) in codon 32 of the translated sequence (R32W). The substitution disrupted a HpaII restriction site to allow independent confirmation of carrier status (data not shown). The arginine residue is present in the first transmembrane domain of the protein and is conserved in all connexin species. The R32W was present in individuals II:2 and III:1. Due to the association of *GJB3* mutations and bilateral high-frequency hearing impairment,<sup>9</sup> it is possible that R32W may contribute to the high frequency hearing impairment found in individual III:1. The panel of 244 unrelated chromosomes previously screened for the presence of D66H were also assessed for R32W. One individual with no obvious cutaneous disorder or hearing impairment was heterozygous for R32W. The high frequency deafness may result from defective C × 31/C × 26 channel formation and regulation when R32W in *GJB3* is associated with D66H in *GJB2*. However, it is equally plausible that D66H in *GJB2* underlies both the cutaneous disorder and the



**Figure 1** a. After heteroduplex formation of the PCR fragments, Denaturing High Performance Liquid Chromatography (DHPLC) was performed using a Transgenomic WAVE™ DNA Fragment analysis system. The Y axis represents the intensity of the DNA peak measured in mV. Time in minutes is on the X axis. Traces show differences in DNA heteroduplex formation in variants of Cx26 with that of the homoduplex wildtype trace consisting of a single peak (pink). Green and black traces show M34T/WT, generated by mixing cloned mutant DNA with wildtype prior to PCR amplification. The blue trace shows D66H/WT (individual III:1) and the red trace shows M34T/D66H (individual II:2). b. DNA sequence traces showing the three connexin mutations segregating in the family. Wildtype sequence trace is shown above that of the heterozygous substitution. c. Structure of the autosomal dominant palmoplantar keratoderma pedigree in which two distinct forms of hearing loss are segregating. Individuals II:6, III:4 and III:5 are additional to those reported previously.<sup>1</sup> The carrier status for the GJB2 and GJB3 mutations are shown under each family member for which a DNA sample was available. WT, wild-type allele. Squares indicate males and circles indicate females.

high frequency hearing loss in individual III:1. It is also possible that another mutation at a different locus, for example, that inherited from her deaf father (II:1), may contribute to the deafness in III:1.

As all tested individuals with PKK have only the D66H mutation in *GJB2* in common, this suggests that this mutation primarily underlies the cutaneous phenotype in the family. In strong support of this is the recent identification of D66H segregating with Vohwinkel's syndrome in three families, one of which was large.<sup>12</sup> In these families, the individuals affected with the mutilating keratoderma presented with mild progressive hearing loss. None of the affected individuals from the larger of the families were carriers of M34T or R32W (data not shown). These findings would suggest that M34T does underlie the profound deafness in the family (Figure 1c) but only on the genetic background of the second mutation in *GJB2*, D66H. Whether it acts as a recessive or modifying allele in combination with D66H to cause profound deafness remains to be elucidated. In addition, M34T in *GJB2* and R32W in *GJB3* may also contribute to the more extensive manifestation of the skin disease which occurs in individual II:2.

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