Molecular Pathogenesis of Genetic and Inherited Diseases

A Novel Missense Mutation in the Second Extracellular Domain of GJB2, p.Ser183Phe, Causes a Syndrome of Focal Palmoplantar Keratoderma with Deafness

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Gap junctions, which consist of connexins, are intercellular channels that mediate rapid intercellular communication. In the skin, connexins are involved in the regulation of epidermal growth and differentiation. GJB2 encodes connexin26, which is an important skin-expressed gap junction protein. Mutations in GJB2 cause a wide variety of unique disorders, but despite extensive research, their mechanisms of action are poorly understood. The identification of novel diseases caused by mutations in GJB2 may help to illuminate the genotype-phenotype correlation and elucidate the function of different regions of the protein. Here, we report the first account of a family with a GJB2 missense mutation in the second extracellular domain (p.Ser183Phe) that causes skin abnormalities in addition to sensorineural hearing loss. Using fluorescent connexin26-EGFP fusion proteins, we showed that the mutation induces a partial protein transport defect that cannot be rescued by wild-type protein. Dye-transfer experiments using a parachute assay revealed channel functionality. Although p.Ser183Phe affects the second extracellular domain, mutations in the first extracellular domain also lead to focal palmoplantar keratoderma and likewise perturb protein transport in a dominant-negative manner. Therefore, we hypothesize that focal palmoplantar keratoderma in gap junction skin disease may be specifically associated with connexin trafficking de-

fects as well as with mutations affecting its extracellular domains, thus broadening the spectrum of GJB2-associated diseases. (Am J Pathol 2008, 173:1113–1119; DOI: 10.2353/ajpath.2008.080049)

Connexins are small transmembrane proteins that belong to an extensive protein family found in most metazoans.¹ Six connexins can oligomerize to form a hemichannel (connexon) in the cell membrane. Two opposite channels connect, or dock to form an aqueous channel that is called a gap junction and facilitates intercellular communication. In the skin, gap junction intercellular communication is essential for regulation of its growth and differentiation.² Many different skin diseases of widely varying severity are associated with a large number of gap-junction gene mutations. In the case of the GJB2 gene, which codes for connexin26, the list of mutations is still growing. Sensorineural deafness is common to all GJB2-associated phenotypes, but the skin symptoms are quite distinct and specific to the mutation.

Why the different mutations cause such widely varying cutaneous manifestations is poorly understood. Some mutations can form heteromeric connexins with wild-type counterparts causing a gain of function of the mutant connexin, whereas other mutations give rise to transport defects, which may affect gap junction stoichiometry and lead to a loss of function.^{1–3} How these functional consequences relate to the skin and hair abnormalities is

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unclear, although mutations associated with skin symptoms, in particular palmoplantar keratoderma, so far seem to cluster in the first cytoplasmic and extracellular loop of connexin26 (for an overview see de Zwart-Storm et al⁴). Thus, one might infer that these domains are of particular importance for gap junction function in the skin.

In the present study we report on the first missense mutation in the second extracellular domain (E2) of *GJB2* that causes cutaneous symptoms in addition to hearing impairment. Functional analysis of the mutation suggests that it leads to a partial protein transport defect that is dominant-negative with respect to the wild-type protein. Dye transfer assays show permeability of mutant gap junction channels to the fluorescent dye calcein. With these new data, the spectrum of *GJB2*-associated disease continues to broaden.

Case Reports

The probanda, a 43-year-old woman of Dutch descent, consulted the Department of Dermatology of the Laurentius Hospital (Roermond, The Netherlands) for a long-standing complaint of palmoplantar keratoderma. In addition, she had sensory hearing loss with preferential high-tone loss. The patient's youngest daughter had sensory hearing loss that had been diagnosed at 5 years of age and was said to have similar skin changes (Figure 1A). As far as our patient knew, no other members of the family were deaf or had skin abnormalities. The probanda and her daughter were available for physical examination. In the probanda, flat, hyperkeratotic translucent plaques consisting of confluent hyperkeratotic papules each with a diameter of a few millimeters were present on the palms, in particular on the border of the thenar and wrist (Figure 2, A–C). On the feet we likewise saw hyperkeratotic plaques (Figure 2, D–F). Most were seen in areas exposed to mechanical stress, such as heels, balls of feet, knuckles, and wrists. At the proximal interphalangeal joints of the hands, we saw a more pronounced hyperkeratosis, sometimes in the form of knuckle pads. Knees and elbows were not involved, and other skin abnormalities were not found. In particular, there were no hypotrichosis, mucositis, nail abnormalities, or pseudo-ainhum. Hair and teeth were normal. Audiological examination showed preferential sensory high-tone hearing loss (Figure 3). Similar but less pronounced skin alterations and high-tone sensory hearing loss were found in the affected daughter. The coincidence of hearing impairment and palmoplantar hyperkeratosis prompted us to look for mutations in the *GJB2* gene.

Materials and Methods

Informed consent was obtained from the patient and her family. DNA was isolated from peripheral blood leukocytes using a simple salt precipitation method.⁵

GJB2 Mutation Analysis

The *GJB2* gene was amplified by standard polymerase chain reaction (PCR) using primers Cx26F 5'-GCATGCT-

Figure 1. Presence of the mutation within the index family. **A:** Pedigree of the family, with the filled symbols representing the affected members and the proband indicated by an arrow. **B:** Restriction analysis with *NciI*; mutation present in the mother and daughter (lanes 1 and 3, respectively; 535, 440, 318, and 122 bp), not in the father or a son (lanes 2 and 4, respectively; 535, 318, and 122 bp).

TGCTTACCCAGACTC-3' and Cx26R 5'-AGGGGAGCAGAGCTCCATTG-3'. The gene was analyzed using the PCR primers and the sequencing primers: Cx26FS 5'-CAGAAGGTCCGCATCGAAGG-3', Cx26RS 5'-GCTTCGAAGATGACCCGGAAG-3', using the BigDye Deoxy terminator system and an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). The mutation eliminates an *NciI* (New England Biolabs, Frankfurt am Main, Germany) restriction site (the wild type contains two *NciI* sites). With this enzyme, we checked the patient's family and 100 unaffected controls by restriction analysis.

GJB2 Cloning Strategy

The wild-type *GJB2*/EGFP-fusion construct coding for ~54 kDa was produced as previously described (*GJB2*, 26.2 kDa).⁴ The *GJB2* c.548C>T (p.Ser183Phe) mutation was introduced into the wild-type *GJB2*/EGFP construct with the Quick-Change site-directed mutagenesis (SDM) kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Clones were sequenced to confirm that they contained the correct insert and muta-

Figure 2. Clinical phenotype of the index patient. **A:** Diffuse, flat, hyperkeratotic translucent plaques were present on the palms, in particular on the border of the thenar and wrist. **B:** Close-up view of the mild palmar hyperkeratosis. **C:** Hyperkeratotic plaques were seen in areas exposed to mechanical stress, such as heels, balls of feet, knuckles, and wrists, sometimes in the form of knuckle pads. **D:** Hyperkeratotic plaques (knuckle pads) on the toes. **E:** Mild diffuse plantar hyperkeratosis. **F:** Close-up of the plantar surface, where areas with hyperkeratosis are clearly visible. Note that the plaques seem to actually be formed by confluence of smaller hyperkeratotic papules.

tion. The complete insert was sequenced to verify if no additional mutations had occurred. Plasmid DNA for transfection was isolated using Nucleobond PC 100 (Machery-Nagel GmbH & Co. KG, Düren, Germany).

Cell Culture

HeLa Ohio wild-type (wt) cells, that do not endogenously express Cx26, Cx30, or Cx43 and are communication-deficient, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum, 100 μ g/ml penicillin/streptomycin, and 2 mmol/L L-glutamine (cDMEM; Lonza Biologics, Cambridge, UK). HeLa cells stably expressing Cx26 were maintained in cDMEM supplemented with 0.5 μ g/ml puromycin (Sigma-Aldrich, Gillingham, UK) selective antibiotic.⁶

Figure 3. Pronounced sensory hearing loss, in particular for higher frequencies (vertical: decibels; horizontal: the frequency in KHz). Air conduction: cross/round marks; bone conduction: triangles, arrows pointing downwards indicate the loudest tone was not heard). The curves of a normal-hearing person are almost horizontal at ~10 to 15 decibels.

Immunofluorescence

Wild-type or Cx26-expressing HeLa Ohio cells (1×10^5 cells) were plated onto 16-mm² glass coverslips in a 12-well plate. After 24 hours, at 70% confluency, cells were transfected with 0.5 μ g of mutant p.Ser183Phe-GJB2/EGFP fusion construct or GJB2/EGFP using Lipofectamine 2000 (Invitrogen Ltd., Paisley, UK) according to the manufacturer's protocol. After 5 hours the medium was replaced and 24 hours later the cells were fixed with ice-cold methanol (-20°C).⁷ Briefly, cells were permeabilized with phosphate-buffered saline (PBS) + 0.1% Triton X-100 (v/v), blocked with 5% (w/v) powdered milk-PBS solution followed by staining with appropriate primary antibody for 1 hour at 37°C . Connexin 26wt localization was assessed using a mouse anti-Cx26 (1:50 dilution, Invitrogen). Cells were then washed in PBS and incubated with anti-mouse secondary antibody conjugated to Alexa 594 as previously described.⁸ Finally, cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (10 ng/ml, w/v, in PBS). Cells with intermediate expression levels of the mutant protein were selected for study.

Parachute Assay

A co-culture system was used to examine the ability of p.Ser183Phe-GJB2/EGFP to form functional channels. Briefly, 24 hours after transfection of wild-type HeLa cells with p.Ser183Phe-GJB2/EGFP or GJB2/EGFP, these donor cells were loaded with the fluorescent tracer calcein AM (2.5 μ mol/L, Mw 623 Da, charge -4) and the cell membrane marker CM-Dil (Mw 1053 Da, 1 μ g/ μ l) as previously described.⁹ HeLa cells stably expressing wild-type Cx26 (acceptor cells, 1×10^5) were seeded onto 24-mm² cover chambers. Donor cells were subsequently washed in PBS, trypsinized, resuspended, and centri-

fused at 1500 rpm for 5 minutes before final resuspension in cDMEM and seeding onto the acceptor cells at a ratio of 1 donor to 50 acceptor cells. Co-cultured cells were then incubated for a minimum of 4 hours at 37°C to allow the donor cells to settle onto the acceptor cells and gap junctions to form. After 4 hours cells were examined on a confocal microscope (Zeiss, Oberkochen, Germany) using dual wavelengths to examine transfer of calcein from the CM-Dil loaded donor cells to the acceptor cells.

Microscopy

Fluorescent samples were viewed using a Zeiss Axiovert 200 confocal microscope linked to a LSM510 META laser-scanning system. Images were acquired under similar conditions and magnifications using either an argon (488-nm excitation, to detect calcein and GFP) or a HeNe laser (543-nm excitation, to detect CM-Dil and Alexa 594) as appropriate. Optical sections were recorded in the Kalman filtering mode using four scans for each picture. Images were further processed using Adobe Photoshop software (Adobe, San Jose, CA).

Results

In the patient and her daughter (Figure 1), we found a heterozygous c.548C>T transition at codon 183 of the *GJB2* gene, resulting in a substitution of a serine by a phenylalanine (p.Ser183Phe, Figure 4). The mutation was not found in unaffected family members or in 100 unrelated controls from the Dutch population, using restriction analysis (Figure 2B), nor was the mutation found in the SNP database (NCBI dbSNP Build 128).

When HeLa Ohio cells were transiently transfected with *GJB2*/EGFP, intercellular gap junction plaques were seen at points of cell-to-cell contact with limited intracellular staining (Figure 5A). By contrast, in HeLa Ohio cells transiently transfected with p.Ser183Phe-*GJB2*/EGFP, the mutant fusion protein had a predominantly cytoplasmic localization, where it accumulated diffusely (Figure 5B). Brighter fluorescence was seen in the endoplasmic reticulum. Some punctate fluorescence, suggestive of

membrane localization was also present, as were small intercellular gap junction plaques (Figure 5B). The cellular localization of p.Ser183Phe-*GJB2*/EGFP was not significantly altered after transfection into HeLa Ohio cells stably expressing Cx26, suggesting a dominant-negative effect of the mutation, although almost no protein was retained in the endoplasmic reticulum (Figure 5D). A control experiment showed that *GJB2*/EGFP and wild-type Cx26 detected with a Cx26-specific monoclonal antibody (red) co-localized at points of cell-to-cell contact in gap junction plaques in HeLa Ohio cells stably transfected with Cx26wt (Figure 5C).

To determine whether this novel Cx26 mutation formed functional gap junction channels, we performed a parachute assay and showed that HeLa Ohio cells, transiently transfected with *GJB2*/EGFP or p.Ser183Phe-*GJB2*/EGFP, could transfer the tracer dye calcein (Mw 623 Da, charge -4) to a monolayer of HeLa Ohio cells stably expressing Cx26wt, suggesting that channels containing mutant protein are at least partly functional (Figure 6, A and B). Control, nontransfected wild-type HeLa Ohio cells were unable to communicate with the stably Cx26wt-expressing HeLa cells (Figure 6C).

Discussion

We report a novel missense mutation, p.Ser183Phe, in the second extracellular loop (E2) of connexin26. To the best of our knowledge, this is the first report of an E2 mutation causing skin symptoms. The pathogenesis of the keratoderma is not clear, but our results may offer a clue. We recently reported that a dominant E1 mutation, p.His73Arg, associated with a quite similar palmoplantar keratoderma, causes defective protein transport.⁴ We therefore looked for a similar effect, which we confirmed in this study. We also demonstrated that the mutant protein cannot be completely rescued by its wild-type counterpart, although when co-expressed there is less accumulation of mutant protein in the endoplasmic reticulum. The parachute assay demonstrates residual channel activity, which might explain the mildness of the phenotype. Mutations causing more severe phenotypes such as p.Asp66His (Vohwinkel's syndrome) are reported to show less dye transfer than p.Ser183Phe.¹⁰ However, other pathogenic mechanisms must also be considered. The p.Ser183Phe mutation occurs in the highly conserved second extracellular loop of Cx26. The serine at position 183 of connexin26 is highly conserved between species and even between connexins, suggesting that this residue is critical for protein function (Figure 7). Because the p.Ser183Phe change replaces a polar with an aromatic amino acid, this substitution can be expected to modify local conformation. The two extracellular domains play a major role in connexon assembly and docking.¹¹ Serine 183 is the first amino acid of the Gap27 mimetic peptide, which can inhibit intercellular coupling, lowers channel conductance, and may prevent gap junction assembly.¹² Thus, p.Ser183Phe may influence assembly and the interconnexon docking process, which might lead to the skin phenotype. It is of interest to

Figure 4. *GJB2* sequence chromatograms of an unaffected family member (Wt) and a patient (Mt) at the mutated location (arrow).

Figure 5. Results of *GJB2*/EGFP fusion protein (green: Cx26wt/mt, red: stably expressed Cx26wt) detection in HeLa Ohio cells. **A:** Gap junction plaque formation by wild-type *GJB2*/EGFP fusion protein (**arrows**). **B:** Some gap junction plaque formation is visible with p.Ser183Phe-*GJB2*/EGFP mutant fusion protein (**arrows**), but most of the protein accumulates in the cytoplasm and endoplasmic reticulum. **C:** Co-localization of stably expressed Cx26wt (red) and transfected Cx26wt (green) at the membrane and in intercellular gap junction plaques (merge = yellow, **arrow** gap junction plaque). **D:** There is no rescue of the mutant protein with Cx26wt; its primarily cytoplasmic distribution is suggestive for ER localization. Note that most of the wild-type protein co-localizes with the mutant, suggesting a dominant-negative effect on transport of the latter (**arrows**, gap junction plaques).

note in this regard, that connexon assembly takes place, at least partly, in the endoplasmic reticulum.¹¹ If the mutation interferes with assembly and correct protein folding, it might interfere with transport to the cell membrane.

Considering that we consistently find dominant-negative transport defects for mutations associated with keratoderma,^{4,10,13} we hypothesize that disturbed trafficking has an important role in the pathogenesis of gap junction diseases of the skin. One issue with this line of reasoning is that a transport defect per se is not typical for mutations

associated with PPK because it has also been observed for mutations causing deafness only.^{14,15} Furthermore, dominant mutations apparently without skin symptoms have been reported in E2; p.Met163Leu, p.Asp179Asn, and p.Arg184Gln.^{16–18} Besides P.Met163Leu is, however, not known whether these mutations affect protein transport. As a more parsimonious explanation, we suggest that skin symptoms have been overlooked in these patients. It would be of interest to re-examine them with particular attention to signs of keratoderma.

Figure 6. HeLa Ohio cells (marked with an asterisk) transfected with *GJB2*/EGFP, p.Ser183Phe-*GJB2*/EGFP, or untransfected, loaded with CM-Dil (red) and calcein (MW622 Da, charge -4) parachuted onto a monolayer of HeLa Ohio cells stably expressing Cx26wt. **A:** HeLa Ohio cells transfected with *GJB2*/EGFP show transfer of calcein to a monolayer of HeLa Ohio cells stably expressing Cx26wt. **B:** HeLa Ohio cells transfected with p.Ser183Phe-*GJB2*/EGFP show transfer of calcein to a monolayer of HeLa Ohio cells stably expressing Cx26wt. **C:** HeLa Ohio cells, untransfected, show no transfer of calcein to a monolayer of HeLa Ohio cells stably expressing Cx26wt.

In conclusion, we describe the first skin phenotype caused by a mutation in the second extracellular domain of connexin26, p.Ser183Phe. Using Cx26/EGFP fusion proteins, we show that the mutation leads to a partial transport defect with the mutant protein accumulating in the cytoplasm. Still, transport to the membrane does occur as evident from the ability to form gap junction plaques. These are (partially) functional with respect to transfer of calcein (Mw 623 Da, charge -4), which might explain the relatively mild phenotype compared to other mutations such as p.Asp66His that show very limited dye transfer. Our results suggest that, in light of the existing genotype-phenotype correlation for *GJB2*, mutations in E1 and E2 that cause dominant-negative transport defects with residual channel function lead to a focal palmoplantar keratoderma and sensory hearing impairment.

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Figure 7. Conservation throughout species and within connexins of E2 domain sequence. No Cx preface is Homo sapiens. Abbreviations: Mmul, *Macaca mulatta*; Bt, *Bos taurus*; Cf, *Canis familiaris*; Ec, *Equus caballus*; Mm, *Mus musculus*; Md, *Monodelphis domestica*; Oa, *Ornithorhynchus anatinus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*.

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