Exome sequencing reveals mutation in GJA1 as a cause of keratoderma-hypotrichosis-leukonychia totalis syndrome

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Keratoderma-hypotrichosis-leukonychia totalis syndrome (KHLS) is an extremely rare, autosomal-dominant disorder characterized by severe skin hyperkeratosis, congenital alopecia and leukonychia totalis. The genetic defect underlying KHLS remained undetermined. By performing whole-exome sequencing in a family with KHLS, we identified a heterozygous mutation (c.23G>T [p.Gly8Val]) in GJA1, which cosegregated with the phenotype in the family. In an additional affected individual, we also found the identical de novo mutation which was absent in his unaffected family members. GJA1 encodes a gap junction protein connexin 43 (Cx43) which is ubiquitously expressed in various organs, including the epidermis and hair follicles. In vitro studies on HEK293 cells expressing Cx43Gly8Val found that the protein formed gap junction plaques between adjacent transfected cells, as observed in the wild-type. Dye-transfer experiments by microinjection of Lucifer yellow displayed functional gap junction of the Cx43Gly8Val mutant. Using patch clamp and Ca2+ imaging methods, we observed that the Cx43Gly8Val hemichannel had significantly more openings than Cx43WT, facilitating Ca2+ influx at resting potential. Such gain-of-function effect might result in cytoplasmic Ca2+ overload, accelerated apoptosis of keratinocytes and subsequent skin hyperkeratosis. Taken together, our results demonstrated that, with probably enhanced hemichannel activities, a mutation in GJA1 is linked to KHLS without extracutaneous involvement.

INTRODUCTION

Connexins consist of 21 different members which are widely expressed in the majority of human tissues (1, 2). Six connexins oligomerize to form a connexon, which, when trafficked to the cell surface membrane, functions as a hemichannel allowing the transfer of small molecules and ions, including Ca2+, between the cell and extracellular environment. Two connexons from adjacent cells dock with each other and bridge the extracellular space to form a gap junction channel, which mediates intercellular communication by transferring ions and small molecules. Connexin isoforms may selectively combine to form heteromeric/homomeric connexons or hemichannels, as well as heterotypic or homotypic gap junctions (2). Certain connexin members may be able to interact with other connexin members, and the trans-dominant effect, where one mutant connexin affects the co-expressed wild-type connexin, has been verified, e.g. mutant connexin 26 inhibiting connexin 43...
(Cx43) trans-dominantly (3). Defects in connexin genes have been associated with a broad scope of Mendelian disorders, and the pathophysiology of mutant connexins contributing to these disorders generally arises from three mechanisms: retention of connexin proteins in the cytoplasm [predominantly in endoplasmic reticulum (ER)], aberrant hemichannel function and loss of gap junction function (4,5).

Keratodera-hypotrichosis-leukonychia totalis syndrome (KHLS) is an extremely rare disorder characterized by severe skin hyperkeratosis, congenital alopecia and leukonychia totalis. It was first described in a Turkish family with proposed autosomal-dominant inheritance (6). The affected individuals were born with sparse scalp hair, eyebrows and eyelashes. Secondary sexual hair was also affected. Widespread hyperkeratosis, especially keratosis pilaris, could coalesce into plaques on the elbows, knees and perianal region. Palmoplantar keratodermwas well-defined, focal or linear, non-mutilating and transgredient. Leukonychia affected 20 nails, without nail dystrophy or subungual hyperkeratosis. Affected individuals were otherwise healthy. Sweating, dentition and hearing were normal. KHLS was classified as a subtype of palmoplantar keratoderma-congenital alopecia syndrome (MIM 104100), of which the genetic basis remained undetermined (7). We herein demonstrated a missense mutation in the GJA1 gene (MIM 121014), which exerted a gain-of-function effect on the connexin 43 (Cx43) hemichannel, as a probable underlying genetic basis of KHLS.

RESULTS
Clinical findings of the patients with KHLS

One familial and one sporadic cases of KHLS in China were included in this study. The proband of the familial case, also designated as patient 1, was a 25-year-old male who was born to non-consanguineous parents at full term following an uneventful pregnancy (Fig. 1A). At birth, he was noted to have leukonychia totalis involving 20 nails and alopecia affecting his scalp hair, eyebrows and eyelashes. He never had his hair trimmed or his beard shaved. At the age of 2 years, skin hyperkeratosis developed on his perianal areas, auricles, knuckles, and gradually spread to his trunk, extremities, hands and feet. He had normal sweating, tear production, dentition, hearing and vision. Physical examination revealed diffuse hyperkeratosis, especially keratosis pilaris distributing on his trunk and extremities, which coalesced into dark plaques with sharp demarcation on friction areas (Fig. 1B). Hyperkeratosis was particularly prominent on his elbows, axillae and popliteal fossae, with multiple spiky, horn-like lesions reminiscent of ichthyosis hystrix (Fig. 1C). Focal palmoplantar keratoderm was found preferentially on weight-bearing areas and was transgredient over the dorsal aspects of his hands and feet (Fig. 1D). All 20 nails were chalky white, without nail dystrophy or subungual hyperkeratosis (Fig. 1D). Sparse, short, brittle hair was present on his scalp, axillae and other hair-bearing areas (Fig. 1E). His body hair was scanty. Anomalies of skeletal, neural, cardiovascular systems or symptoms reminiscent of oculodentodigital dysplasia (ODDD; OMIM 164200) were excluded by corresponding specialists. Skin biopsy taken from his trunk showed orthohyperkeratosis with follicular plugging and perivascular lymphocytic infiltration in the papillary dermis. Scanning electron microscopy of his hair shaft revealed multiple pits with cuticular weathering (Fig. 1G). His 4-year-old daughter had a nearly identical clinical presentation despite milder hyperkeratosis (Supplementary Material, Fig. S1), while his parents and three siblings were unaffected. Patient 2 was a 27-month-old boy born to non-consanguineous healthy parents. His clinical features closely resembled those of patient 1 (Fig. 1F).

Identification of a novel heterozygous GJA1 mutation in KHLS

Three family members (patient 1’s daughter, wife and one of his unaffected sisters) were chosen for whole-exome sequencing. We filtered all the detected variants against BGI inner database, 1000 Genomes Project and Hapmap 8 databases. According to the presumed autosomal-dominant pattern, we focused on heterozygous variants that were present in patient 1’s daughter but absent in his wife and sister. Filtered variants were predicted by the SIFT software (8). Among the remnant 215 variants which fulfilled the above criteria and were predicted to be ‘damaging’, 15 variants occurred in the genes that were predicted to be expressed in skin according to the UniGene expression database. Sanger sequencing was applied to verify these 15 candidates (Supplementary Material, Table S1). Co-segregation with the phenotype in the pedigree was confirmed in only one variant, c.23G>A (p.Gly8Val) in GJA1 (Fig. 1H). This mutation was absent in the parents of patient 1 and 212 ethnically matched normal controls, indicating a de novo mutation that is unlikely to represent a polymorphic variant. The Gly8 residue is highly conserved in evolution (Fig. 1I). Direct sequencing of GJA1 exons was subsequently performed in patient 2 (primers are listed in Supplementary Material, Table S2). Intriguingly, the identical mutation c.23G>A in GJA1 was identified, whereas his parents and two unaffected siblings were free of this mutation. Paternity and maternity were confirmed in both patients, indicating the mutation c.23G>A was an independent de novo event.

Cx43^{Gly8Val} forms functional gap junction

To determine whether Cx43^{Gly8Val} impairs its localization to the plasma membrane, HEK293 cells were co-transfected with plasmids carrying ER-mCherry and EGFP tagged wild-type (Cx43 WT) or Cx43 Gly8Val. After 24 h, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, sigma) and imaged on an Olympus FV1000 confocal microscope. Microscopy revealed that, rather than being retained in the ER, Cx43 Gly8Val could traffic to the cell membrane and form bright gap junction-like aggregates of similar abundance and size to Cx43 WT at the interface between adjacent cells (Fig. 2A and B).

We next examined whether the Cx43 Gly8Val mutant would disable cell coupling by assaying the intercellular transfer of the low molecular mass tracers, Lucifer yellow (LY, charge = −2, molecular weight = 457; Sigma). Here, mCherry was added to the C-terminus of all Cx43 constructs to allow identification of the LY-injected cell. Cell pairs that had clear gap junction plaques at the cell–cell interfaces were selected for dye transfer experiments. One cell of each pair was impaled with a patch pipette filled with 1% LY. Eight out of 9 cells expressing Cx43 WT permitted LY to spread beyond the Cx43 Gly8Val cell, indicating a functional gap junction.
expressing Cx43Gly8Val mutant coupled to each microinjected cell and only two of injected cells showing no dye passage, suggesting that intercellular coupling was unimpaired between cells expressing Cx43Gly8Val (Fig. 2C–F).

**Cx43Gly8Val showed gain-of-function hemichannel activity and increased basal Ca2+ level**

We assayed the conducting properties of the Cx43Gly8Val hemichannel in HEK293 cells by patch clamp technique (9,10). We first characterized the hemichannel behavior of the Cx43WT channels to establish a baseline. During applications of slow voltage steps (6 s) from −100 to +100 mV, pronounced increases in currents were recorded in Cx43WT and Cx43Gly8Val cells at large positive potential, indicating that both can form conductive hemichannels. Hemichannel currents were completely blocked by the application of La3+ (100 μM) and the Cx43-specific blocker, 43Gap26 (11), further confirming that the currents indeed are generated by functional Cx43 hemichannels (Fig. 3A–C). However, at resting potential, in most cells, if not all, Cx43WT did not show any hemichannel openings, whereas much more openings were observed in cells expressing the Cx43Gly8Val mutant. The current density observed in the Cx43Gly8Val hemichannels was significantly larger than that of Cx43WT, implying a gain-of-function hemichannel activity (Fig. 3D).

As openings of Cx43 hemichannels may trigger Ca2+ influx (12), we deduced that the gain-of-function hemichannel activity would increase intracellular basal Ca2+ concentration. We then assessed the Ca2+ permeability, one of the major Cx43 hemichannel functionality (13), by GCamP6s-based Ca2+-imaging. The GCamP6s protein is an ultrasensitive protein Ca2+ sensor (14). The HEK293 cells were co-transfected with the GCamP6s plasmid and Cx43WT or Cx43Gly8Val. Ca2+ influx through open channels could be evaluated by observing the intracellular fluorescence. In cells expressing Cx43Gly8Val, the fluorescence was much brighter than in cells expressing Cx43WT, suggesting that the gain of function of the Cx43Gly8Val hemichannel resulted in significantly increased Ca2+ influx (Fig. 3E and F).

**Cx43Gly8Val increases transfected cell death in vitro and keratinocyte apoptosis in vivo**

As overactivity of hemichannels may increase metabolic stress leading to excessive cell death, we next evaluated whether
Cx43\textsuperscript{Gly8Val} could trigger enhanced cell death in transfected HEK293 cells. Comparison of Cx43\textsuperscript{Gly8Val} with the Cx43\textsuperscript{WT} group was performed by t-test. At 24 h post-transfection, cells expressing Cx43\textsuperscript{Gly8Val} showed a significantly higher death rate than those expressing Cx43\textsuperscript{WT} (Fig. 4A).

As increased extracellular Ca\textsuperscript{2+} could rescue cell death by suppressing hemichannel currents (9,15), transfected cells were cultured in DMEM media supplemented with CaCl\textsubscript{2} at a concentration ranging from 0 to 4 mM. We found that, in Cx43\textsuperscript{Gly8Val} transfected HEK293 cells, increasing the concentration of extracellular Ca\textsuperscript{2+} rescued the cells in a dose-dependent manner (Fig. 4B), suggesting that massive cell death was likely to result from increased activity of the mutant hemichannels. These data further corroborated our electrophysiological results.

To determine the apoptosis of keratinocytes in vivo, in situ apoptosis on keratinocytes from skin lesions of patient 1 was evaluated by the TUNEL assay. Skin sections from the normal edge of a surgically excised nevus in unrelated healthy subjects were used as controls. Significantly larger amounts of apoptotic keratinocytes were observed in the epidermis of patient 1 than those of normal controls (Fig. 4C).

DISCUSSION

Despite its rarity, KHLS is considered to be a distinct entity from all the other disorders, such as hidrotic ectodermal dysplasia (OMIM 129500), keratitis-ichthyosis-deafness syndrome (OMIM 148210), Lelis syndrome (OMIM 608290), Bart-Pumphrey syndrome (OMIM 149200), Olmsted syndrome (OMIM 614594), etc. (6,16). Both the patients 1 and 2 in this study harbored a de novo mutation in \textit{GJA1}, which cosegregated with the phenotype in the patient 1’s family, suggesting \textit{GJA1} mutations were genetically associated with KHLS. Since Cx43 is highly expressed throughout the epidermis and hair follicles (17,18), it is conceivable that mutations in \textit{GJA1} may cause a constellation of skin hyperkeratosis, alopecia and nail abnormality, as seen in KHLS. Genetic screening in additional cases of KHLS is needed to clarify whether KHLS is Cx43\textsuperscript{Gly8Val} specific or is associated with other mutations in \textit{GJA1}.

Heterozygous mutations in \textit{GJA1} have also been demonstrated to cause ODDD, a pleiotropic disorder characterized by craniofacial and limb dysmorphisms, as well as neurodegeneration (19). However, careful re-examination on our affected patients has excluded all the extracutaneous involvements reminiscent of ODDD, indicating that KHLS and ODDD are allelic disorders caused by \textit{GJA1} mutations. This perspective was further strengthened by the comparison of different functional properties between the ODDD-associated mutants and Cx43 \textsuperscript{Gly8Val}. For ODDD, most of the associated \textit{GJA1} mutations, such as p.Tyr230CysfsX7 and p.Gly138Arg, result in either retention of the mutant protein in ER, or decreased permeability of the channels (Supplementary Material, Fig. S2), leading to non-functional gap junctions (20–22). A homozygous nonsense mutation in the N-terminus of Cx43 (p.Arg33\textsuperscript{*}) has been reported in a Pakistani family with classic ODDD (23) but lacking skin hyperkeratosis, suggesting that loss of function of Cx43 is unlikely to result in severe skin hyperkeratosis. For KHLS, Cx43\textsuperscript{Gly8Val} forms competent gap junction channels comparable with the wild-type in terms of the properties of
trafficking, cell coupling and dye transfer between cells (Fig. 2). Moreover, our results revealed that Cx43 Gly8Val exerted a gain-of-function effect on the hemichannel activity. The result-ant overactivation of the Cx43 Gly8Val hemichannel facilitated transmembrane passage of small molecules, including Ca2+ (12). As a consequence, cytoplasmic Ca2+ overload may induce apoptosis of keratinocytes, leading to skin hyperkeratosis (16). Such distinct functional discrimination of these mutants may, in part, contribute to different organ predilections of ODDD and KHLS. Nevertheless, skin involvement was occasionally reported in ODDD, which included Ca2+ (12). As a consequence, cytoplasmic Ca2+ overload may induce apoptosis of keratinocytes, leading to skin hyperkeratosis (16). Such distinct functional discrimination of these mutants may, in part, contribute to different organ predilections of ODDD and KHLS. Nevertheless, skin involvement was occasionally reported in ODDD, which included palmoplantar keratoderma, hair and nail anomalies with varying severity, though milder than KHLS (24–27). Therefore, we speculate that GJA1 mutations can cause a wide spectrum of skin symptoms, with KHLS at its most severe end.

Phenotypic overlaps are very common in connexin-related skin disorders. KHLS also bears remarkable clinical resemblance to connexin 26 (Cx26, encoded by GJB2)-associated conditions, i.e. spiky skin hyperkeratosis with hystrix-like ichthyosis-deafness syndrome (OMIM 602540), follicular keratosis, alopecia and palmoplantar keratoderma with keratitis-ichthyosis-deafness syndrome, and leukonychia with Bart-Pumphrey syndrome (28–33). Therefore, another plausible explanation for the pathogenesis of KHLS is a trans-dominant effect interfering Cx26, which has been demonstrated to be co-localized with Cx43 in the keratinocytes in vivo (3). Selective Cx26 mutants with skin symptoms were previously shown to exert a trans-dominant effect on the intracellular conductance of co-expressed Cx43 in vitro (3). In Cx26-associated disorders, skin hyperkeratosis is always associated with certain mutations leading to aberrant hemi-channels, most of which are constitutively active (34–36). Thus, it is reasonable to speculate that the gain of function of the Cx43 Gly8Val mutant may also exert a trans-dominant positive effect on the activity of the Cx26 hemichannel, leading to the skin phenotypes. Further studies are required to clarify this postulation.

Cx43 is abundantly expressed in most organs, and its mutations can cause a group of disorders with ocular, skeletal and dental involvement. Our preliminary data link, for the first time, the gain-of-function Cx43 Gly8Val mutation to the severe skin disorder KHLS, as opposed to ODDD which affects extracutaneous organs. Our study expanded the spectrum of both Cx43 disorders and skin channelopathies.
MATERIALS AND METHODS

Exome sequencing

This study was approved by the Clinical and Research Ethics Committee of Peking University First Hospital. Blood from participating family members (patient 1’s daughter, wife and one of his unaffected sisters) were collected after informed consent was obtained in accordance with principles of Declaration of Helsinki. Genomic DNA was extracted following standard procedures. A total amount of 3 μg genomic DNA per individual was used for exome capture with Nimblegen SeqCap EZ Library (Roche) for enrichment. Exome capture was carried out using the Nimblegen SeqCap EZ Library (Roche) for enrichment. Sequencing was performed on Hiseq 2000 (Illumina). Raw image files were processed by Illumina base calling Software 1.7 for base-calling with default parameters, and the sequences of each individual were generated as 90 bp paired-end reads. The high-quality reads were mapped to the human reference genome (hg1.9) using SOAP aligner (37). After filtering duplication reads, the consensus sequence in target regions was called by SOAP snp (38) using the unique mapped reads. The Genome Analysis Toolkit (GATK) (39) was used to identify indels with BWA (40)-aligned high-quality reads. Variant annotation, SNP filtering (1000 Genomes, HapMap, BGI inner databases) was attained using an in-house pipeline by BGI (BGI-Shenzhen).

Plasmids construction

The cDNA clone of the whole coding region of human GJA1 was purchased from Origene and GJA1 mutations were generated using a site-directed mutagenesis kit (Fast Mutagenesis System, Transgen Biotech, Beijing, China) (primers are listed in Supplementary Material, Table S3). For fluorescent microscopy, Cx43 was subcloned into either mCherry or EGFP, linking the fluorescent protein to the C-terminus of Cx43. ER-mCherry is constructed by adding an ER retention signal peptide (SEKDEL) to the N terminus of mCherry red fluorescence protein. The GCaMP6s plasmid is obtained from Addgene (plasmid 40753).

Cell culture and transfection

HEK293 cells were cultured in the DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C and 5% CO2. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.
Electrophysiology and microinjection

Currents were recorded using a HEKA EPC10 amplifier with Patch Master software (HEKA) coupled to a Nikon inverted fluorescence microscope. Patch pipettes were pulled from borosilicate glass (Sutter Instrument) and fire polished to a resistance of ~2 MΩ. Solution (containing 130 mM NaCl and 3 mM Hapes, pH 7.2) is free of divalent cations, since Cx43 hemichannels are highly sensitive to Ca2+. LaCl3 was purchased from Sigma and 43Gap26 was purchased from Genscript. HEK293 cells were microinjected with 1% LY (Molecular Probes) using patch pipettes. 1 min after microinjection, the incidence of dye transfer was determined. Images were collected by a Nikon inverted fluorescence microscope. Student’s t-test was performed for relevant comparisons.

Cell death assay and TUNEL assay

Cell death of transfected cells was detected by propidium iodide (PI)/Hoechst33424 staining. In brief, transfected HEK293 cells were cultured for 24 h and then stained by Hoechst 33342 and PI for 15 min before morphological cell death assessment. The stained cells were then analyzed with UV/488 nm dual excitation using inverted fluorescence microscope (IX71, Olympus). Dead cells, shown as PI positive, were counted in five randomly selected high-power fields with ImageJ (NIH software). The experiments were repeated independently for three times. TUNEL assays were performed using an In Situ Cell Death Detection Kit (Roche) principally according to the supplier’s instruction. Images of the whole epidermis were taken randomly with the same photograph parameters. The apoptotic cells were counted in the same way as above.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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REFERENCES


