Revertant Mutation Releases Confined Lethal Mutation, Opening Pandora’s Box: A Novel Genetic Pathogenesis

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Abstract

When two mutations, one dominant pathogenic and the other “confining” nonsense, coexist in the same allele, theoretically, reversion of the latter may elicit a disease, like the opening of Pandora’s box. However, cases of this hypothetical pathogenic mechanism have never been reported. We describe a lethal form of keratitis-ichthyosis-deafness (KID) syndrome caused by the reversion of the GJB2 nonsense mutation p.Tyr136X that would otherwise have confined the effect of another dominant lethal mutation, p.Gly45Glu, in the same allele. The patient’s mother had the identical missense mutation which was confined by the nonsense mutation. The biological relationship between the parents and the child was confirmed by genotyping of 15 short tandem repeat loci. Haplotype analysis using 40 SNPs spanning the >39 kbp region surrounding the GJB2 gene and an extended SNP microarray analysis spanning 83,483 SNPs throughout chromosome 13 in the family showed that an allelic recombination event involving the maternal allele carrying the mutations generated the pathogenic allele unique to the patient, although the possibility of coincidental accumulation of spontaneous point mutations cannot be completely excluded. Previous reports and our mutation screening support that p.Gly45Glu is in complete linkage disequilibrium with p.Tyr136X in the Japanese population. Estimated from statistics in the literature, there may be approximately 11,000 p.Gly45Glu carriers in the Japanese population who have this second-site confining mutation, which acts as natural genetic protection from the lethal disease. The reversion-triggered onset of the disease shown in this study is a previously unreported genetic pathogenesis based on Mendelian inheritance.

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Introduction

A nonsense mutation may, in theory, disrupt and thus “confine” the effects of another dominant pathogenic mutation when the two mutations coexist in the same allele of a single gene. Furthermore, in such cases, reversion of the confining nonsense mutation may paradoxically elicit a congenital disease, although proven cases of this hypothetical pathogenesis have not been reported.

Keratitis-ichthyosis-deafness (KID) syndrome (OMIM 148210) is a rare congenital ectodermal disorder characterized by vascularizing keratitis, ichthyosiform erythroderma and sensorineural hearing loss [1]. KID syndrome is mainly caused by a heterozygous germ line missense mutation in GJB2 (Entrez Gene ID: 2706) encoding connexin 26 (Cx26) (RefSeq: NM_004004.5) [2–4].

Here we report a case of KID syndrome where the reversion of a missense mutation induced a lethal disease. We encountered a girl with KID syndrome from obviously healthy parents, and sequence analysis of GJB2 revealed a heterozygous missense mutation, p.Gly45Glu, in the patient. Unexpectedly, her healthy mother also had the heterozygous missense mutation p.Gly45Glu, as well as another heterozygous nonsense mutation: p.Tyr136X. From these findings, we hypothesized that the p.Tyr136X mutation confines the pathogenic effect of p.Gly45Glu in the mother and that the reversion of p.Tyr136X triggered the onset of KID syndrome in the patient. In the present study, TA cloning and haplotype analysis of the family confirmed that an allelic recombination event involving the maternal allele carrying the two mutations generated the pathogenic allele unique to the patient. Furthermore, cotransfection experiments and a neurobiotin uptake assay clearly demonstrated that the p.Tyr136X mutation confines the pathogenic effects of the p.Gly45Glu mutation. Thus, to our knowledge, the present findings provide the first evidence of reversion-triggered onset of a congenital disease.

Results

The Patient’s Mother Had Both GJB2 Lethal Missense Mutation and Confining Nonsense Mutation, Although the Patient Had Only the Lethal One

The KID syndrome patient is a girl born from apparently healthy Japanese parents. She showed ichthyosiform erythroderma
Loss of gene functions due to nonsense mutations is a typical pathogenic mechanism of hereditary diseases. They may, however, in certain genetic contexts, confine the effects of other dominant pathogenic mutations and suppress disease manifestations. We report the first instance in the literature where the reversion of a “confining” nonsense mutation in \textit{GJB2} gene released the dominant pathogenic effect of a coexisting gain-of-function mutation, eliciting the lethal form of keratitis-ichthyosis-deafness syndrome (KID). We describe this form of KID syndrome caused by the reversion of the \textit{GJB2} nonsense mutation p.Tyr136X that would otherwise have confined the effect of another dominant lethal mutation, p.Gly45Glu, in the same allele. The patient’s mother had the identical missense mutation which was confined by the nonsense mutation. An epidemiologic estimation demonstrates that approximately 11,000 individuals in the Japanese population may have the same lethal \textit{GJB2} mutation, nonetheless protected from the manifestation of the syndrome because they also inherit the common “confining” nonsense mutation. The reversion-triggered onset of the disease shown in this study is a previously unreported genetic pathogenesis based on Mendelian inheritance.

Revertant Mutation of Confining Nonsense Mutation Occurred in the Patient’s Pathogenic Allele of \textit{GJB2}

To elucidate the origin of the c.134G>A mutation in the patient, haplotype analysis was performed. Forty SNPs annotated by the International HapMap Project [6] spanning the >39 kbp region surrounding the \textit{GJB2} gene were sequenced. Fourteen SNPs were found to be heterozygous in one or more of the family members (Figure 1C and S2). TA cloning analysis mapped the heterozygous SNPs into three separate genetic regions (Figure 1C). All family members had at least one common haplotype in each genetic region, suggesting that they share a haplotype in the >39 kb genetic region we studied. Unexpectedly, the patient harbored a unique haplotype that was not seen in either of her parents (Figure 1C). No evidence of spontaneous mutations was found besides these SNP sites through the direct sequencing of the entire coding region of \textit{GJB2}.

We performed an extended SNP microarray analysis spanning 83,483 SNPs throughout chromosome 13. No apparent chromosomal aberration was detected besides a 1,430 kbp copy-number neutral loss-of-heterozygosity region on 13q31.1 which was unique to the patient’s genome.

From these findings, we reasoned that an allelic recombination event involving the shared allele (Figure 1C, shown in blue) and the maternally unique allele (Figure 1C, shown in orange) generated the haplotype unique to the patient (see also the Discussion section below), since it differs by three or more base pairs from the counterparts carried by either parent, giving only a remote possibility of coincidental accumulation of spontaneous point mutations at these specific SNP sites. The latter possibility, however, cannot be completely excluded.

The blood cells of the patient did not show mosaicism, and the patient’s skin symptoms were fairly evenly distributed over the entire body surface. These findings suggest that the patient was not mosaic for the \textit{GJB2} mutation. Thus, we consider the reversion leading to the pathogenic allele in the patient to be a pre-zygotic event.

Gap Junctions Containing p.Gly45Glu-Mutant Connexin 26 (CX26) Showed Aberrant Gating Activity, Whereas p.Gly45Glu/p.Tyr136X-Mutant CX26 Were Excluded from Functional Gap Junction Formation

As described above, the patient who harbored the p.Gly45-Glu mutation manifested the disease, while the mother who harbored the mutations p.Gly45Glu and p.Tyr136X was apparently unaffected (Figure 2A). A heterozygous \textit{de novo} p.Gly45Glu mutation is known to cause the lethal form of KID syndrome [3], and its molecular pathogenic mechanism has been well described [7–9]. Cx26, the product of \textit{GJB2}, is a gap junction protein with four transmembrane domains and two extracellular domains (Figure 2B). The Cx26 molecule is a protomer of a hexameric connexon, and two connexons expressed on the membranes of neighboring cells connect to form a gap junction channel [10]. Gly45 locates at a domain that lines the channel pore and probably mediates voltage sensing [10]. Connexons containing p.Gly45Glu mutants function as hemichannels with aberrantly increased activity [8,9] that leads to the disease manifestations [3,9]. It is also known that, besides KID syndrome, biallelic loss of function of \textit{GJB2} causes autosomal recessive non-syndromic hearing loss (NSHL) [11]. The fact that the p.Gly45Glu/p.Tyr136X mutation homozygously or compound heterozygously causes NSHL suggests that this mutation leads to total loss of function for the \textit{GJB2} product [5].

These considerations lead us to hypothesize that the p.Tyr136X mutation confines and rescues the dominant pathogenic effect of the p.Gly45Glu mutation. Since inter-protomer interactions of Cx26 require the fourth transmembrane domain [10] that is lost in the p.Gly45Glu/p.Tyr136X mutation, it is clear that the functional gap junction is unlikely to be formed. This phenomenon, in which a second-site mutation cancels an existing pathogenic mutation, was previously reported; it is called “partial reversion”, because the wild-type allele itself is not
attained, although the second-site mutation rescues the disease [12].

To test this hypothesis, we observed the colocalization of fluorescent-tagged Cx26 variants in HeLa cells. The father had wild-type and p.Val27Ile/p.Glu114Gly variant alleles (Figure 2A). When these Cx26s were cotransfected, they together formed gap junctions, suggesting that both proteins retain their native functions (Figure 3A). The Cx26 p.Gly45Glu/p.Tyr136X mutant failed to enter the gap junction generated by Cx26 p.Val27Ile/p.Glu114Gly, demonstrating that only the latter form comprises the functional gap junctions in the mother (Figure 3A). Cx26 p.Gly45Glu colocalized with the p.Val27Ile/p.Glu114Gly variant but failed to form gap junctions (Figure 3A). In a neurobiotin uptake assay, which monitors channel activity as cellular uptake of a neurobiotin tracer [9], only the p.Gly45Glu mutant and not the p.Gly45Glu/p.Tyr136X mutant induced the aberrant uptake (Figure 3B).

Discussion

Many cases of revertant mosaicism have been reported as “natural gene therapy” where the mitotic recombination results in revertant mutations that mitigate the disease symptoms [13–16]. However, the present study is the first report to demonstrate a mutant reversion triggering a genetic disease.

The present data of genomic DNA sequencing and haplotype analysis demonstrate that the patient and her father share an identical haplotype (Figure 1C, shown in blue). We hypothesized that the entire blue allele in the patient’s genome was derived from the father, while the other allele (Figure 1C, shown in yellow) was basically derived from the mother. It seemed, however, that this allele underwent pre-zygotic reversion during meiosis of the maternal gamete. The fact that the patient’s unique allele (Figure 1C, shown in yellow) differs by three non-continuous SNPs from the unique maternal allele (Figure 1C, shown in orange) while the neighboring SNPs are conserved might be explained by multiple events of gene conversion involving both of the maternal alleles (Figure 1C, shown in blue and orange) that may have occurred in this genetic region.

Double cross-over also might account for the recombination, but it is less likely, considering that the non-conserved and conserved SNPs in the patient’s allele reside within close proximity (Figure 1C); the average length of the gene conversion tract is estimated to be in the range of 55–290 bp, whereas the cross-over tracts are typically longer [17].
Mitotic gene conversion has been found in some cases of revertant mosaicism in cutaneous disease, including generalized atrophic benign epidermolysis bullosa [12,13]. We are unaware of any previous report of multiple gene conversions within a relatively short genetic segment as in the present case. However, the present data compel us to consider that it occurred. Since the patient’s unique allele differs by three or more base pairs from the counterparts carried by either parent, we judge the possibility of coincidental accumulation of spontaneous point mutations at these specific SNP sites to be highly unlikely. This possibility, however, cannot be completely excluded.

As evidence supporting our hypothesis, consistent with a previous report [9], we clearly demonstrated that Cx26 p.Gly45Glu colocalized with the p.Val27Ile/p.Glu114Gly variant but failed to form gap junctions (Figure 3A). Previous studies have shown that Cx26 p.Gly45Glu forms hemichannels that are aberrantly activated at low extracellular Ca2+ levels [9]. The present study used a neurobiotin uptake assay [9] to show that only the p.Gly45Glu mutant and not the p.Gly45Glu/p.Tyr136X mutant induces the aberrant uptake (Figure 3B). These results taken together support the model in which the p.Tyr136X mutation confines the dominant gain-of-function effect of the p.Gly45Glu mutation to prevent the onset of the disease (Figure 4). Such secondary effects of revertants may pose a challenge in genetic analyses of extended genes or more than one gene with functional interactions.

Interestingly, in the group of Japanese patients with bilateral sensorineural hearing loss, it is not uncommon to find GJB2 p.Gly45Glu carriers, but none of them are affected by KID syndrome [5]. They uniformly have a tandem p.Tyr136X mutation, as in the mother of the present case [5]. Thus, we hypothesized that, in the Japanese population, carriers of p.Gly45Glu are protected from the lethal form of KID syndrome by the tandem, confining mutation p.Tyr136X.

To clarify the frequency of the p.Gly45Glu mutation in the entire Japanese population, we performed screening analysis for the two mutations p.Gly45Glu and p.Tyr136X in a normal control group consisting of 920 overall healthy Japanese individuals (1,840 alleles) and these results support the idea that p.Gly45Glu and p.Tyr136X mutations are infrequent in the general Japanese population. However, in the epidemiological statistics of Tsukada et al. [5], we found screening data for GJB2 mutations in Japanese patients with sensorineural hearing loss. The report revealed that, among 1,343 Japanese patients with hearing loss, 33 patients had one or two p.Gly45Glu alleles (34 p.Gly45Glu alleles in 2666 alleles for an allele frequency of 1.27%; 33 carriers in 1,343 patients for a carrier rate of 2.46%). This means 2.46% of Japanese patients with bilateral sensorineural hearing loss have one or two p.Gly45Glu alleles. As for the prevalence of sensorineural hearing loss, it was reported that 3.5 per 1,000 individuals in the entire population have bilateral sensorineural hearing loss [18]. Thus, calculating from these epidemiological statistics, we estimate that 8.6 per 100,000 individuals, or approximately 11,000 individuals in the entire Japanese population, have one or two p.Gly45Glu alleles. However, no patient with the lethal form of KID syndrome due to p.Gly45Glu has been reported in the Japanese population as far as we know, although the mutation p.Gly45Glu has been reported as a cause of the lethal form of KID syndrome in several European patients [3,19–22].

Tsukada et al. [5] reported that all 34 alleles with p.Gly45Glu found in the Japanese patients with sensorineural hearing loss also had p.Tyr136X, suggesting that p.Gly45Glu is in complete linkage disequilibrium with p.Tyr136X in the Japanese population. In our mutation screening, no allele carrying either or both mutations, p.Gly45Glu and p.Tyr136X, was found in 920 Japanese individuals (1,840 alleles) and these results support the idea that p.Gly45Glu is in complete LD with p.Tyr136X in the Japanese population.

In light of this, we conclude that, even though individuals may have the dominant lethal mutation p.Gly45Glu, the confining mutation p.Tyr136X in cis configuration protects against the disease, KID syndrome, in the approximately 11,000 Japanese individuals in the entire Japanese population who harbor p.Gly45Glu. The allele with the tandem mutations p.Gly45Glu and p.Tyr136X causes hearing loss in an autosomal recessive manner. Most carriers of the tandem mutations in the Japanese population are heterozygous for the allele, such as the patient’s mother in the present study, and are not affected with hearing loss.

In summary, our findings demonstrate that the second-site confining mutation is an important genetic protection mechanism, and its loss, like the opening of Pandora’s box, is a novel genetic pathogenesis that releases the hidden genetic disease.
Figure 3. The p.Tyr136X mutation confines the effect of the p.Gly45Glu mutation. (A) Gap junction formation by the transfected Cx26 variants. Each panel contains two co-transfected cells connected to each other. Wild-type, p.Gly45Glu/p.Tyr136X and p.Gly45Glu mutants of Cx26 were tagged with monomeric Red Fluorescent Protein (mRFP) and co-transfected with Green Fluorescent Protein (EGFP)-tagged Cx26 p.Val27Ile/p.Glu114Gly into HeLa cells as indicated. Gap junction formation sites are indicated by arrowheads. The combination of WT Cx26 and Cx26 p.Val27Ile/p.Glu114Gly (top row) results in gap junctions that consist of both Cx26 proteins (yellow signal). The combination of Cx26 p.Gly45Glu/p.Tyr136X and Cx26 p.Val27Ile/p.Glu114Gly (middle row) results in gap junctions with only Cx26 p.Val27Ile/p.Glu114Gly (green signal). No apparent gap junction formation is seen when Cx26 p.Gly45Glu and Cx26 p.Val27Ile/p.Glu114Gly are cotransfected (bottom row). (B) Aberrant gate opening detected with neurobiotin uptake assay. Fluorescent-tagged Cx26s were cotransfected into HeLa cells as indicated, and treated with neurobiotin in a calcium-free condition. Uptake was detected with AlexaFluor350 streptavidin dye (blue). Aberrant uptake of neurobiotin is observed only in cells cotransfected with Cx26 p.Gly45Glu and Cx26 p.Val27Ile/p.Glu114Gly (bottom row). doi:10.1371/journal.pgen.1004276.g003
Genotyping of GJB2 Mutations in 920 Japanese Control Individuals

Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Maxi Kit (Qiagen). Real-time PCR-based genotyping of the GJB2 mutations was performed with TaqMan MGB probe genotyping assay according to the manufacturer’s instructions provided by Roche Diagnostics. To detect an allele of each mutation, a set of two TaqMan MGB probes labeled with a fluorescent dye (FAM or VIC) and a quencher dye (non-fluorescent dye; NFD) followed by minor groove binder (MGB), and sequence-specific forward and reverse primers were synthesized by Life Technologies Corporation. The sequences of assay probes/primers are shown in Table S3 in this article’s supplementary material. Real-time PCR was performed with LightCycler 480 system II 384 plate (Roche Diagnostics) in a final volume of 5 μl containing 2× LightCycler 480 Probes Master (Roche Diagnostics), 200 nM probes for wild type and mutant each and 900 nM forward and reverse primers each, with 5 ng genomic DNA as the template. The thermal conditions were the following: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 1 s, with a final cooling at 40°C for 30 s. Endpoint fluorescence was measured for each sample well. Afterward, genotyping was performed using endpoint genotyping analysis in LightCycler 480 software.

Eight hundred and twenty controls were analyzed with the real-time PCR-based genotyping of GJB2 mutations, and another 100 controls were analyzed with the direct automated sequencing for the entire coding region of GJB2.

GJB2 Expression Study

Total RNA from the formaldehyde-fixed paraffin-embedded skin sample of the patient was extracted using the RNeasy FFPE kit (Qiagen) and Deparaffinization Solution (Qiagen) according to the manufacturer’s instructions. The total RNA was reverse-transcribed with a GJB2 specific primer, 5’-GGATGTGGGAGATGGG-GGAAGTAGTG-3’, using PrimeScriptII 1st strand cDNA synthesis kit (Takara, Japan). The PCR fragment harboring c.134G>A mutation was amplified with primer sets, 5’-GGAAGATCTCGGCTCAGCGCTCCTC-3’ and 5’-CGTAGCACACGTCTTTCTTCAGCTC-3’, and directly sequenced with the same primers.

SNP Array

Chromosome-wide genotyping was performed using HumanOmni2.5–8 BeadChip (Illumina), which covers a total of 2,379,855 SNPs throughout the genome, including 83,482 SNPs on chromosome 13. Genomic DNA was hybridized according to the manufacturer’s instructions and data analysis was carried out using GenomeStudio software (Illumina).

Cell Culture

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. For transfection of plasmids, cells were seeded onto 8-well LabTek chamber slides (Thermo Scientific) and transfected with FuGene HD Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions.

Plasmid Construction

The coding sequences of Cx26 variants were amplified from the genome of the patient or the parents, fused to cDNAs coding enhanced green fluorescent protein (EGFP) (Clontech) or monomeric red fluorescent protein (Clontech), and subcloned into

Materials and Methods

Ethics Statement

This study was approved by the Bioethics Committee of the Nagoya University Graduate School of Medicine and was conducted according to The Declaration of Helsinki Principles. Written informed consent was obtained from the parents.

The Patient and Her Parents

The patient was referred and seen at the Outpatient Clinic of Dermatology, Nagoya University Hospital.

Sequence Analysis and TA Cloning

Genomic DNA extracted from peripheral blood was used as a template for PCR amplification, followed by direct automated sequencing. The entire coding regions of GJB2 including the exon/intron boundaries were sequenced as reported elsewhere [4]. TOPO-TA cloning kit (Life Technologies) was used for TA cloning analyses. PCR primers were designed to amplify the genetic regions containing the selected SNPs, and the acquired PCR products were analyzed by direct sequencing. For amplification of PCR fragments longer than 1,000 base pairs, KOD-Plus-Neo polymerase (Toyobo) or PrimeScriptGXL polymerase (Takara Bio) was used and the PCR products were cloned with the TOPO XL-TA Cloning Kit (Life Technologies) after the addition of a 3’-adenine overhang.

Verification of the Parent-Child Relationship

The parent-child relationship was validated using AmpFISTR Identifier plus PCR amplification kit (Applied Biosystems) according to the manufacturer’s instructions. The combined probability of exclusion and the combined probability of paternity (in the case of the odds ratio for prior probability = 1) for 13 STR loci were calculated to confirm the authenticity of the biological relationship between the parents and the child.
PBS containing 10 mM. Then the cells are stained with 3% BSA/0.1% Triton X-100/blocked with 3% BSA/0.1% Triton X-100/PBS for 1 hour.

Neurobiotin Uptake Assay
HeLa cells were cotransfected with EGFP-tagged and mRFP-tagged Cx26 variant expressing vectors, and neurobiotin uptake assay was performed as described elsewhere [9]. Briefly, cells were washed with calcium free Hank’s buffered salt solution for 20 minutes and incubated with phosphate-buffered saline (PBS) containing 0.1 mg/ml neurobiotin (Vector Laboratories) for another 20 minutes. Cells were washed three times with PBS supplemented with 2 mM CaCl2 for 10 minutes at 37°C. The cells were fixed with 4% formaldehyde and permeabilized and blocked with 3% BSA/0.1% Triton X-100/PBS for 1 hour. Then the cells are stained with 3% BSA/0.1% Triton X-100/PBS containing 10 μg/ml Alexa Fluor 350-streptoavidin (Life Technologies) for 1 hour, followed by three washes with 0.1% Triton X-100/PBS. Stained cells were fixed with ProLong Gold antifade reagent (Life Technologies) and fluorescent images were obtained.

Supporting Information
Figure S1 The GJB2 mRNA harboring the missense mutation is expressed in the patient’s skin. (A) RT-PCR from the total RNA extracted from a formaldehyde-fixed paraffin-embedded skin sample of the patient. A 136-bp PCR fragment was amplified from the GJB2 cDNA obtained from the skin sample of the patient. (B) Detection of GJB2 cDNA harboring the c.134G>A missense mutation. The PCR fragment was directly sequenced to confirm the expression of the mutant GJB2 mRNA.

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Author Contributions
Conceived and designed the experiments: YO KS MA. Performed the experiments: YO TT MK TY. Analyzed the data: YO KS MK TY MA. Contributed reagents/materials/analysis tools: NH. Wrote the paper: YO MK.

Supporting Information
Figure S1 The GJB2 mRNA harboring the missense mutation is expressed in the patient’s skin. (A) RT-PCR from the total RNA extracted from a formaldehyde-fixed paraffin-embedded skin sample of the patient. A 136-bp PCR fragment was amplified from the GJB2 cDNA obtained from the skin sample of the patient. (B) Detection of GJB2 cDNA harboring the c.134G>A missense mutation. The PCR fragment was directly sequenced to confirm the expression of the mutant GJB2 mRNA.

Figure S2 Summary of 40 SNPs spanning the >39 kbp region including GJB2. The SNPs inside the red box reside within the GJB2 gene. Note that the nucleotides are in the strand opposite those shown in Figure 1.

Table S1 Genotyping of the patient and her parents for 15 short tandem repeat (STR) loci.
Table S2 The combined probability of exclusion and the combined probability of paternity.
Table S3 The sequence of probes/primers for real-time PCR-based genotyping of GJB2 mutations.