A Connexin40 Mutation Associated With a Malignant Variant of Progressive Familial Heart Block Type I

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Background—Progressive familial heart block type I (PFHBI) is a hereditary arrhythmia characterized by progressive conduction disturbances in the His-Purkinje system. PFHBI has been linked to genes such as SCN5A that influence cardiac excitability but not to genes that influence cell-to-cell communication. Our goal was to explore whether nucleotide substitutions in genes coding for connexin proteins would associate with clinical cases of PFHBI and if so, to establish a genotype-cell phenotype correlation for that mutation.

Methods and Results—We screened 156 probands with PFHBI. In addition to 12 sodium channel mutations, we found a germ line mutation in a connexin gene that associates with inherited ventricular arrhythmias and emphasizes the importance of Cx40 in normal propagation in the specialized conduction system. (Circ Arrhythm Electrophysiol. 2012; 5:163-172.)

Key Words: heart block ■ genes ■ ion channels ■ death sudden ■ gap junctions

Clinical Perspective on p 172

Cardiac myocyte excitability in atria, His-Purkinje system, and ventricles is largely determined by the properties of voltage-gated sodium channels. Once activated, excitatory currents rapidly propagate to neighboring cells through low-resistance intercellular channels called gap junctions, which facilitate the synchronous contraction of the heart. Loss of expression and function of cardiac gap junctions and sodium currents can severely impair action potential propagation, which sets the stage for life-threatening arrhythmias. Although multiple mutations in genes coding for components of the voltage-gated sodium channel complex have been previ...
association between germ line mutations in gap junction proteins and inherited ventricular arrhythmias in humans.

In this study, we investigated a group of patients with progressive familial heart block type I (PFHBI) (Online Mendelian Inheritance in Man 113900), also known as progressive cardiac conduction defect or Lenègre-Lev disease,\(^6\,^7\) is a dominant inherited disorder of the His-Purkinje system. Affected individuals show electrocardiographic evidence of bundle branch disease (ie, right bundle branch block, left anterior or posterior hemiblock, complete heart block) with broad QRS complexes. The disease can progress from a normal ECG to right bundle branch block and from the latter to complete heart block. Affected individuals often present with family history of syncpe, pacemaker implantation, and sudden death.\(^8\) Although structural abnormalities have been invoked as a cause of the disease,\(^6\,^7\) a number of patients present with normal cardiac structure and contractile function. Linkage analysis in a large South African PFHBI kindred\(^9\) and a Lebanese kindred\(^10\) mapped a causal locus on chromosome 19q13.3, and further work identified mutations in genes encoding for the transient receptor potential nonselective cation channel, subfamily M, member 4 (TRPM4) gene\(^11\) at this locus. Haploinsufficiency of SCN5A and aging have been implicated in PFHBI,\(^8\) and age-dependent manifestations of the disease have been recapitulated in mice.\(^12\)

Here, we sought to expand on the association between PFHBI and mutations in genes relevant to action potential propagation; in particular, we assessed the possible association between nucleotide substitutions in connexin-coding genes and PFHBI. We evaluated 156 probands of diverse ethnic backgrounds from Asia, Europe, and North America given a clinical diagnosis of PFHBI. In addition to the sodium channel mutations previously reported,\(^13\,^15\) we identified a germ line missense mutation in GJA5 in a family with severe, early onset disease. This gene codes for the gap junction protein connexin40 (Cx40), which predominantly expresses in the atria and His-Purkinje system.\(^16\) Heterologous expression experiments revealed that this novel mutation (Cx40-Q58L) significantly impaired the ability of Cx40 to form gap junction channels. Confocal microscopy showed that the Cx40-Q58L mutant but not the wild type (WT) failed to form plaques at sites of cell-cell apposition. Coexpression experiments indicated that the Cx40-WT protein provided only partial rescue of the Cx40-Q58L cellular phenotype. To our knowledge, this is the first description of a germ line mutation in a connexin gene associated with inherited ventricular arrhythmias. The results open the possibility of GJA5 as a candidate gene for screening in patients with PFHBI, yet in the absence of further evidence, screening may be limited to the research environment rather than included as a part of the routine diagnostic examination.\(^17\) The data also emphasize the importance of Cx40 in the maintenance of normal propagation in the specialized conduction system of the human heart.

**Methods**

**Genetic Screening of PFHBI**

Genomic screening by polymerase chain reaction and DNA sequencing was performed for GJA5 (Cx40), GJA1 (Cx43), GJC1 (Cx45), KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, KCNJ2, SCN1B, SCN4B, HCN4. Primer information is provided in the online-only Data Supplement. All participating probands and family members gave written informed consent in accordance with standards (Declaration of Helsinki) and local ethics committees.

**Plasmid Construction**

A 1.1-kb Cx40-DNA fragment was subcloned into bichistronic plasmids pRES2-EGFP and pRES2-DsRED2. An EGFP or FLAG epitope was added at Cx40 C terminal to generate EGFP- or FLAG-tagged Cx40. Site-directed mutagenesis (Q58L) was performed with QuikChange. Primer information and additional details are provided in the online-only Data Supplement.

**Cell Culture and Transfection**

Constructs were introduced into connexin-deficient HeLa cells or mouse neuroblastoma (N2A) cells using Lipofectamine as per manufacturer’s protocol.

**Electrophysiology**

Gap junction currents were recorded from transiently transfected N2A cell pairs using whole-cell double-patch clamp techniques as previously described.\(^18\,^19\) Further details are provided in the online-only Data Supplement.

**Statistical Analysis**

Results are presented as mean±SEM. Mann-Whitney rank sum tests with Bonferroni post hoc correction were used in comparisons for which normality or equal variance assumptions were invalid. In other instances, differences between groups were assessed by 1-way ANOVA followed by Bonferroni post hoc correction. Statistical significance was assumed for \(P<0.05\).

**Results**

**Genetic Screening of PFHBI Probands**

We genetically screened 156 probands given a clinical diagnosis of PFHBI. We identified 4 novel and 5 previously reported mutations in SCN5A,\(^13\,^15\) 3 mutations in SCN1B,\(^14\) and a novel germ line heterozygous missense mutation in exon 2 of the Cx40 gene GJA5 (online-only Data Supplement Table I). Mutations were not found in connexin genes GJA1 (Cx43) or GJC1 (Cx45) or in the other genes screened (KCNQ1, KCNH2, KCNE1, KCNE2, KCNJ2, HCN4, or SCN4B). Of the novel SCN5A mutations, 1 caused a modification of the amplitude and voltage gating kinetics of the sodium current in heterologously expressing cells (online-only Data Supplement Figure I); 3 other mutant constructs failed to express functional channels, suggesting that patients carrying the mutation were functionally haploinsufficient for Nav1.5 (online-only Data Supplement Figure I). The GJA5 mutation (c.173A>T) caused an amino acid substitution (glutamine [Q] replaced by leucine [L]) at position 58 in Cx40 (Cx40-Q58L) (Figure 1A and 1B). The mutation was absent in 400 alleles from unaffected control subjects and in the other 155 PFHBI probands. Screening of the entire gene...
panel (including SCN5A and SCN1B) revealed no other sequence modification in the DNA of this proband. Topological analysis placed amino acid 58 of Cx40 within the first extracellular loop (Figure 1C). The presence of glutamine in this position is highly conserved among GJA5 orthologs, and 2 other cardiac connexins, Cx43 and Cx45 (Figure 1D). The clinical and genotypic characteristics of proband and tested family members are described next.

**Clinical Phenotypes and Genotype of the PFHBI Pedigree With the GJA5 Mutation**

The proband, an 11-year-old boy at time of death, was first referred for evaluation when he was age 6 years because of ECG abnormalities. Although asymptomatic at that time, his ECG showed advanced atrioventricular block, complete left bundle branch block, and left axis deviation (Figure 2A). Echocardiography and cardiac scintigraphy did not reveal signs of structural heart disease. He experienced an episode of syncope at age 9; implantation of a permanent pacemaker was recommended by the physician but not authorized by the legal guardian. The proband died suddenly 2 years later during exercise (running), and the family declined postmortem examination. The proband’s younger sister shares the Cx40-Q58L mutation. She is asymptomatic, with a QRS duration at the upper limit of normal, left axis deviation that has been progressive (online-only Data Supplement Table II), and QRS notch. These findings are consistent with impaired intraventricular conduction (Figure 2B). The mother died suddenly at age 30 after delivering the second child. An ECG on record, obtained when she was age 16, was similar to that of the proband (compare Figure 2C with 2A). In addition, a ventricular tachycardia was recorded during the recovery phase of an exercise stress test (online-only Data Supplement Figure II). DNA from the mother was not available for

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**Figure 1.** GJA5 mutation identified in a family given the clinical diagnosis of progressive familial heart block type I. A, Family pedigree. Genetically affected and unaffected individuals are shown with closed and open symbols, respectively. The hatched circle indicates the proband’s mother not genotyped; clinical data suggest that she was a de novo mutation carrier. Number below each symbol indicates the age at registration or age of SCD (parenthesis). B, Sequence electropherogram of exon 2 GJA5 of proband. Arrow indicates heterozygous missense mutation of leucine (CTG) for glutamine-58 (CAG). C, Cx40 predicted membrane topology indicating position Q58 in first extracellular loop. D, Sequence alignment of human Cx40 and its homologues (residues 45–70). Notice the conservation in human Cx43 and Cx45. Dashes indicate residues identical with the top sequence. Cx indicates connexin.
examination. Other family members, including the proband’s father, showed normal ECGs. DNA analysis of proband’s father and maternal grandparents revealed absence of the Cx40-Q58L mutation. On the basis of clinical data and genotypic features of the proband and sister, it is most likely that the Cx40-Q58L mutation appeared de novo in the proband’s mother. The data also indicate an early onset of PFHBI in this family compared with the natural history of the disease in most other cases. As an initial step to assess the functional implications of the Cx40-Q58L mutation, modified constructs were transiently expressed in an exogenous system and evaluated for localization and function.

Electrophysiological Properties of Mutant Cx40-Q58L Channels

Connexin-deficient N2A cells were transiently transfected with cDNA for Cx40-WT or Cx40-Q58L; electrophysiological properties of homologous Cx40 channels were analyzed by conventional dual whole-cell patch clamp. Figure 3A shows representative junctional current traces elicited by a transjunctival voltage gradient of −60 mV. Average junctional conductance (Gj) decreased from 22.2±1.7 nS in cells expressing Cx40-WT (n=14) to 0.56±0.34 nS in cells expressing the Cx40-Q58L mutant (n=14; P<0.001). The probability of functional coupling, calculated by dividing the number of electrically coupled pairs by the number of pairs tested, was 100% and 57.1% for Cx40-WT and Cx40-Q58L, respectively.

Figure 3B depicts representative single-channel recordings elicited by a transjunctional voltage of −60 mV in cell pairs expressing Cx40-WT or Cx40-Q58L. Unitary events for WT channels displayed current transitions corresponding to 2 conducting states (O1 and O2) of 43.3 and 119.5 pS, respectively. Figure 3C shows the event histograms for both cell
types (Cx40-WT, 3 cell pairs and 303 events; Cx40-Q58L, 3 cell pairs and 416 events). The histogram for the Cx40-WT channels was best described by 2 Gaussian distributions centered at 136.2 ± 2.3 and 53.1 ± 5.3 pS. In contrast, the histogram for Cx40-Q58L channels was best described by a single Gaussian function centered at 40.2 ± 0.3 pS. Moreover, the length of time that a channel dwelled in the open state (dwell open time) was substantially longer for the Cx40-Q58L channels (92.0 ± 7.8 ms, 3 cell pairs, 163 events) than for Cx40-WT channels (27.9 ± 0.5 ms, 4 cell pairs, and 186 events) (Figure 3D). Of note, the Q58L mutation had a strong dominant effect on formation of heterotypic functional gap junctions. Cells were transfected with either pIRES2-EGFP-Cx40-WT or pIRES2-DsRED2-Cx40-Q58L, and heterotypic pairs were identified by fluorescence microscopy (an EGFP-expressing cell paired with a DsRED-expressing cell). We recorded from 8 cell pairs and detected unitary current events in only 2 pairs. A total of 57 events were recorded, and average macroscopic junctional conductance was 0.04 ± 0.03 nS. Collectively, the data demonstrated that the Q58L mutation significantly affects the biophysical properties of Cx40 channels and the overall ability of Cx40 gap junctions to form a low-resistance pathway between cells.

**Electrophysiological Properties and Gap Junction Plaque Formation in Cells Coexpressing WT and Q58L Proteins**

In the clinical cases identified, the Q58L mutation was detected in only 1 carrier allele. Therefore, we assessed the function of gap junctions in cells coexpressing WT and mutant proteins. N2A cells were cotransfected with cDNA for both GFP-tagged Cx40-WT and Cx40-Q58L (0.5 μg of pEGFPN1-Cx40-WT combined with 0.5 μg of pEGFPN1-Cx40-Q58L). Results were compared with those obtained when only 1 of the constructs (1 μg) was transfected. Cells expressing both constructs (WT/Q58L) showed intermediate conductance (15.4 ± 3.7 nS, n = 16) between WT (28.8 ± 3.6 nS, n = 16, P < 0.001) and Q58L (0.28 ± 0.11 nS, n = 14, P < 0.001) (Figure 4A). These values were comparable to those obtained using the bicistronic pIRE2-EGFP constructs (WT, 22.2 ± 1.7 nS, n = 14; WT/Q58L, 13.0 ± 2.4 nS, n = 17; Q58L, 0.56 ± 0.34 nS, n = 14). The coexpression results were consistent with those obtained using pIRE plasmids that tagged the cells both green and red, if cotransfected (online-only Data Supplement Figure I). The probability of finding functional coupling in cotransfected cells was 76.5%, which was intermediately between WT (100%) and Q58L (57.1%).

The characteristics of gap junction plaques observed in cells coexpressing WT and Q58L varied significantly between pairs (Figure 4B). Nearly one half of transfected (fluorescence-positive) cells exhibited clear and discrete gap junction plaques (arrow a), whereas the rest of fluorescent-positive cells showed a diffuse expression pattern and absence of well-defined plaques (arrow b). Fluorescence-positive and gap junction plaque-positive cells were counted in 10 different views for each group, and efficacy of gap junction plaque formation was statistically analyzed (Figure 4C) by calculating the ratio of cells with gap junction plaques...
to the number of fluorescence-positive cells. In the Cx40-WT group, almost all fluorescent-positive cells exhibited clear gap junction plaques (94.9±1.9%, n=940), whereas there was a more-diffuse and homogenous pattern with only occasional plaque formation in the Cx40-Q58L group (6.6±0.7%, n=1318, *P*<0.001 compared with WT). In contrast, results varied widely in cells cotransfected with WT/Q58L; nearly one half of fluorescence-positive cells exhibited gap junction plaques similar to those observed in cells transfected with the WT construct (48.2±2.4%, n=855; Q58L, n=1318). Representative images of phase contrast (left), EGFP fluorescence (middle), and junctional conductance (right) from neuroblastoma cells cotransfected with pEGFPN1-Cx40-WT (0.25 μg) and pEGFPN1-Cx40-Q58L (0.25 μg). Three different examples illustrate the relation between plaque morphology and recorded junctional conductance. WT indicates wild type. ***P*<0.001 compared with WT.

Figure 4. Macroscopic conductance and gap junction plaque morphology in cells coexpressing connexin40 (Cx40)-WT and Cx40-Q58L. A, Junctional conductance of cells transfected with plasmid pEGFPN1-Cx40-WT (1 μg), pEGFPN1-Cx40-Q58L (1 μg), or cotransfected with WT and Q58L (WT/Q58L, pEGFPN1-Cx40-WT 0.5 μg + pEGFPN1-Cx40-Q58L 0.5 μg). B, Phase contrast/fluorescence overlay image of neuroblastoma cells transfected with WT/Q58L constructs. Arrow a points to gap junction plaque; arrow b points to an example of cells transfected but devoid of gap junction plaque. C, Efficacy of gap junction plaque formation was measured as the ratio between the number of gap junction plaque-positive cells and the number of fluorescent-positive cells (WT, n=940; WT/Q58L, n=855; Q58L, n=1318). D, Representative images of phase contrast (left), EGFP fluorescence (middle), and junctional conductance (right) from neuroblastoma cells cotransfected with pEGFPN1-Cx40-WT (0.25 μg) and pEGFPN1-Cx40-Q58L (0.25 μg). Three different examples illustrate the relation between plaque morphology and recorded junctional conductance. WT indicates wild type. ***P*<0.001 compared with WT.

Subcellular Distribution of WT and Q58L Cx40 in Transiently Transfected Cells

To further analyze the subcellular distribution of Cx40-WT and Cx40-Q58L proteins, the C terminal of Cx40-WT was

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A very small Gj (<5 nS) and very few or negligible gap junction plaques (a). In the other half of cell pairs, small, dot-like junctional plaques correlated with intermediate Gj values (b), and there were clear, extensive gap junction plaques associated with Gj values >25 nS (c). Overall, we found significant heterogeneity in the extent of electric coupling, although the measurements of Gj correlated with the localization of proteins in transfected cells. These results indicate that the Q58L mutation significantly impairs the ability of cells to form gap junction plaques, although the effect is not purely dominant when both WT and mutant proteins are coexpressed.
tagged with GFP, whereas the C terminal of Cx40-Q58L was FLAG tagged. After transfection of N2A cells with the tagged constructs, the distribution of each protein was examined by confocal microscopy. As shown in Figure 5, green color indicates the position of GFP-tagged molecules, whereas red indicates the position of FLAG-tagged molecules. In cells transfected only with GFP-tagged Cx40-WT, fluorescence was consistently detected at sites of cell-cell apposition, following the pattern previously described for GFP-labeled gap junction plaques (Figure 5A). A similar distribution was found when cells were transfected with FLAG-tagged Cx40-WT (not shown). In contrast, most FLAG-tagged Cx40-Q58L signals were evenly distributed around the cell in the vicinity of the plasma membrane (Figure 5B). Biotinylation experiments showed that the Q58L mutation did not prevent the Cx40 protein from inserting into the membrane and presenting a domain-reachable form in the extracellular space (online-only Data Supplement Figure II). Microscopy experiments in cells coexpressing GFP-tagged Cx40-WT and FLAG-tagged Cx40-Q58L proteins yielded results intermediate to those obtained when only 1 construct was expressed. Nearly one half of cell pairs showed that both proteins distributed homogenously at or near the cell membrane, without the formation of well-defined gap junction plaques (Figure 5C). These images resembled those obtained when only Cx40-Q58L proteins were expressed (Figure 5B, FLAG). In contrast, other cell pairs showed clustering of fluorescent signals within closely confined areas that appeared to be gap junction plaques (Figure 5D).

The experiments described herein led us to speculate that the distribution and function of heteromeric connexons is determined by their mutant subunit content, whereby formation (or not) of plaques and channels are determined, at least in part, by the abundance of expression of one protein over the other. As an initial step to probe this hypothesis, we took advantage of the characteristics of the bicistronic plasmid pIRES, in which the expression rate of the upstream gene is several-fold greater than that of the downstream gene, and explored the functional properties of heteromeric connexons. Cx40-WT and GFP-tagged Cx40-Q58L were subcloned into the pIRES vector, either alone or in combination, in the specific orientations shown in Figure 6A. Protein expression levels of Cx40-WT and Cx40-Q58L were determined by immunochemistry. In contrast to the data obtained when Cx40-WT and GFP-tagged Cx40-Q58L plasmids were cotransfected at a 1:1 ratio (lane 6), expression of heteromeric pIRES plasmids WT-IRES-Q58L-EGFP (lane 3) and Q58L-EGFP-IRES-WT (lane 4) resulted in uneven protein expression levels of WT (40 kDa) and Q58L-EGFP (67 kDa), depending on their orientation in the pIRES vector. Based on

Figure 5. Subcellular distribution of connexin40 (Cx40)-WT and Cx40-Q58L in transiently transfected cells. HeLa cells were transiently transfected with pEGFPN1-Cx40-WT (3.0 μg) (A), pCMV-FLAG-Cx40-Q58L (3.0 μg) (B), or pEGFPN1-Cx40-WT (1.5 μg) plus pCMV-FLAG-Cx40-Q58L (1.5 μg) (C); immunostained for the respective tag protein; and visualized by confocal laser scanning microscopy. Notice that gap junction plaques (A) are absent in Q58L transfectants (B) and present in some (D) but not all (C) cotransfected cells. Bar=20 μm. WT indicates wild type.
these observations, we constructed a homomeric Cx40-WT plasmid (WT-IRES-WT) and heteromeric plasmids of Cx40-WT and Cx40-Q58L with different orientations (WT-IRES-Q58L and Q58L-IRES-WT) (Figure 6B). The junctional conductance of cell pairs expressing WT-IRES-WT (n=17) was comparable to heteromeric construct WT-IRES-Q58L (n=17). However, converse heteromeric construct Q58L-IRES-WT (n=15) showed significantly reduced conductance (P<0.001 versus WT-IRES-WT and WT-IRES-Q58L). **P<0.001. NS indicates not significant; WT, wild type.

**Discussion**

Genetic screening confirmed the association of SCN5A and SCN1B with PFHBI11,13,14 and revealed novel mutations within these genes (online-only Data Supplement Table I). More importantly, we identified a particularly severe, early onset case of PFHBI associated with a germ line mutation in GJA5 in 2 blood relatives (proband and sister) given a clinical diagnosis of PFHBI. The data also indicate that the protein expressed (Cx40-Q58L) failed to form functional gap junctions in an exogenous expression system and decreased the probability of gap junction formation in cells coexpressing the WT protein.

So far, SCN5A, SCB1B, and TRPM4 are the only genes associated with PFHBI.11,13,14 The National Human Genome Research Institute database shows no association of GJA5 single-nucleotide polymorphisms with arrhythmias or conduction system diseases. PR interval and QRS have been associated with several loci, including SCN5A, SCN10A, NKX2.5, and TBX521,22 but not GJA5, which is located at chromosome 1q21.1. Overall, the present results suggest that GJA5 is a candidate gene associated with PFHBI, likely in a small fraction of the affected population. Yet, given the limited cosegregation observed in the reported family, we remain cautious in assigning a causative nature to the GJA5 mutation. It will be of great interest to expand the screening of GJA5 at the research level to identify other cases associated with amino acid changes in Cx40, although it may be premature to include GJA5 as a part of the routine diagnostic screen.17 The present results also emphasize the importance of Cx40 in the maintenance of normal cardiac rhythm.

To our knowledge, this is the first report of a germ line mutation in Cx40 associated with a high risk of ventricular arrhythmias (online-only Data Supplement Figure II). Other studies have shown somatic mutations of Cx40 or Cx43 in patients with idiopathic atrial fibrillation5,23; those mutations

**Figure 6.** Mutant subunit abundance correlated with gap junction function. A, Neuroblastoma cells were transiently transfected with 3 μg Cx40 constructs in IRES plasmids. Cell lysates were analyzed by western blot using anti-Cx40 (top) and anti-GAPDH antibodies (bottom). The number in each lane corresponds to the plasmid noted below the image. Samples from cells cotransfected with plasmids 1 and 2 (1.5 μg each) were loaded on lane 6. Double bands of Cx40-WT (40 kDa) and Q58L-EGFP (67 kDa) are shown in lanes 3, 4, 6, and 7. Results were repeated in 3 separate experiments. Overexposure (lane 7) confirmed expression of the high-molecular-weight protein in lane 3. B, Junctional conductance of homomeric and heteromeric constructs (WT-IRES-Q58L and Q58L-IRES-WT). Conductance of cell pairs expressing WT-IRES-WT (n=17) was comparable to heteromeric construct WT-IRES-Q58L (n=17). However, converse heteromeric construct Q58L-IRES-WT (n=15) showed significantly reduced conductance (P<0.001 versus WT-IRES-WT and WT-IRES-Q58L). **P<0.001. NS indicates not significant; WT, wild type.
were confined to the atria, and conduction abnormalities in the ventricles or His-Purkinje system were not observed. On the other hand, as in all cases involving identified genetic substrates for disease, the possibility of compound mutations in unexamined genes cannot be excluded. We do emphasize that the mutation led to a severe cellular phenotype in an exogenous expression system, supporting the argument that just the Q58L substitution can impair the formation of gap junctions necessary for propagation of action potentials between cells.

The results show that Cx40-Q58L was abundantly expressed in an exogenous system. The protein reached the vicinity of the cell membrane but failed to form gap junction plaques (Figure 5B). This result may be due to impaired docking of mutant hemichannels within the intercellular space because of the mutation in the extracellular loop (Figure 1C). During trafficking, connexin subunits oligomerize to form a hemichannel (or connexon). Once at the site of cell contact, connexons from apposing cells dock, sealing the hydrophilic path (the channel pore) from the extracellular space. The locking of 2 connexons into 1 gap junction channel is believed to stabilize connexin subunits in place, facilitating aggregation of other oligomers into their vicinity and eventually forming a plaque. Amino acid substitutions within the extracellular loop, as in Q58L, can prevent hemichannel docking and, thus, plaque formation.24 The present biotinylation experiments indicate that the Q58L protein integrates into the cell membrane, supporting the notion that the inability of the Q58L mutation to form functional gap junctions is related to events that occur after the oligomer is delivered to the cell membrane and before a functional dodecamer converts into a functional channel in a gap junction plaque.

Results obtained in cells coexpressing both mutant and WT proteins clearly show that one subunit can significantly influence the fate of the other (Figure 5). This suggests that Cx40-Q58L subunits retain their ability to oligomerize not only with other mutant subunits, but also with the WT protein. The results also present an interesting paradigm in that neither the WT nor the mutant construct exerted a dominant effect over the other. After transfection with equal amounts of cDNA, we found cells where both WT and mutant proteins displayed the phenotype of the mutant construct, whereas in other cases, junctional plaques could be easily discerned (although an outline of the cell, likely resulting from the presence of the FLAG-tagged mutant protein, could still be observed [see red signal in Figure 5D]). These results can be explained if we assume that the probability of proper targeting and integration of a connexon into a plaque decreases as a function of the number of mutant subunits contained. For cotransfection, we used equal amounts of cDNA; however, it is very likely that each cell was transfected with variable amounts of each construct and, thus, expressed variable amounts of each protein. We speculate that a majority (though of unknown stoichiometry) of WT connexin subunits are required in a connexon for proper formation of functional gap junctions. Thus, if a cell captures an abundance of Q58L cDNA, most oligomers will contain an excess of mutant subunits, and gap junction formation will fail. If, on the other hand, that cell captures and expresses more of the WT cDNA, the distribution of the subunits within the oligomer will contain a majority of WT connexins, and the connexon will be properly integrated into a channel. This hypothesis will require further testing, although data presented in Figure 6 support the concept that success or failure of functional channel formation may relate to relative abundance of each protein (WT or mutant). If our hypothesis is correct, it suggests that the distribution of functional gap junctions in the His-Purkinje network of affected individuals could vary significantly among cells, depending on the extent of expression of each allele in each cell. The resulting phenotype may be that of a Purkinje network where gap junction-mediated coupling could be heterogeneous, setting the stage for local conduction block, microreentry, and ventricular arrhythmias at the Purkinje network or at the Purkinje-muscle junction.1,2

Overall, we show that both proband and sister have a genotype that (1) is absent in hundreds of control subjects and in the unaffected parent (the father), (2) disrupts an important functional domain of the protein, and (3) disrupts the formation of gap junction channels. The data therefore support the notion of an association between the Cx40 mutation and the clinical phenotype and emphasize the importance of future studies to assess the possible involvement of Cx40 mutations as causative of the disease.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Progressive familial heart block type I, also known as progressive cardiac conduction defect, is an inherited form of cardiac conduction system dysfunction that can lead to severe heart rhythm disturbances, including sudden cardiac death. The genetic causes of this disease are poorly understood. Here, we genetically screened 156 patients with progressive familial heart block type I. In addition to mutations in genes of the voltage-gated cardiac sodium channel complex (SCN5A and SCN1B), we found a novel germ line mutation in GJA5, the gene encoding the gap junction protein connexin40. The disease had an early onset and was associated with otherwise unexplained sudden cardiac death in the proband and his mother. The proband’s sister is also affected. Cellular phenotype analysis revealed impaired gap junction formation at cell-cell interfaces and marked reduction of junctional conductance in cells expressing the mutated connexin40 protein. The results emphasize the importance of connexin40 in normal electrical propagation in the cardiac conduction system and open the possibility of including GJA5 as a target gene for study in patients with progressive familial heart block type I.
A Connexin40 Mutation Associated With a Malignant Variant of Progressive Familial Heart Block Type I

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

1. Genetic screening of PFHB1

The exon 2 of GJA5 and exon 3 of GJC1 that cover the entire coding region of the Cx40 and Cx45, respectively, were amplified by PCR from genome DNA using following primer sets.

\[
\text{GJA5 Forward (Cx40-F2)} \quad 5'-\text{TGGAATCCACACATGATAGA-3'} \\
\text{Reverse (Cx40-R2)} \quad 5'-\text{TCAGTTCAGAAAGGCACAGTCT-3'} \\
\text{GJC1 Forward (Cx45-F1)} \quad 5'-\text{GAGCCACCCTACCCAACTGA-3'} \\
\text{Reverse (Cx45-R1)} \quad 5'-\text{ACCAGAGCAATTGTCTACTCA-3'}
\]

The coding regions of KCNQ1, KCNH2, SCN5A, KCNE1, KCNE2, KCNJ2, SCN1B, SCN4B, HCN4, GJA1 (Cx43) were amplified by PCR using exon flanking intronic primers as previously described.\(^1\) Direct DNA sequencing was performed using ABI 3130 genetic analyzer (Applied Biosystems).

2. Plasmid construction

A 1.1-kilobase DNA fragment, encompassing the entire coding region of Cx40, was amplified by PCR from human genomic DNA using the following primers.

\[
\text{Forward (Cx40-F7)} \quad 5'-\text{GAAGATTCACCATGGGCGATGGAGC TTCCT-3'} \\
\text{Reverse (Cx40-R2X)} \quad 5'-\text{GGAAATTCAGCATGGTCTG-3'}
\]

(Underlines represent the restriction recognition sequences for BgIII and EcoRI, respectively)

The PCR fragment was digested with BgIII/EcoRI and subcloned into a bicistronic plasmid pIRE2-EGFP or pIRE2-DsRED2 (Takara Bio), for visual identification of cells expressing...
connexins and green (EGFP) or red fluorescent protein (DsRED2), respectively. Site-directed mutagenesis was performed by QuikChange (Stratagene) as per manufacturer’s instructions. Sequences of PCR-amplified regions were verified for both strands. For EGFP-tagged Cx40 plasmid, the 1.1 kb coding sequence of WT and Q58L Cx40 were PCR-amplified by the following primers.

Forward (Cx40-F2X) 5’-AACAAAGCTTCACCATGGGCAGGCTTCCT-3’
Reverse (Cx40-R5X) 5’-GCGATCCACTGATAGGTCATCTGA-3’

(Underlines represent the restriction recognition sequences for HindIII and BamHI, respectively.) The PCR fragment was digested with HindIII/BamHI and subcloned in frame into the plasmid pEGFP-N1 (Takara Bio), generating fusion constructs (pEGFPN1-Cx40-WT and pEGFPN1-Cx40-Q58L). FLAG-tagged Cx40 plasmids were constructed by replacing the 0.8 kb EGFP fragment of the pEGFPN1-Cx40 plasmids in frame with the FLAG epitope (DYKDDDDK) cDNA at the C-terminal of the Cx40 (pCMV-FLAG-Cx40-WT and pCMV-FLAG-Cx40-Q58L, respectively. EGFP-tag or FLAG-tag did not change the conductance or the gating properties of Cx40 (data not shown).

Bicistronic constructs of WT-Cx40 and Q58L-Cx40 were made using the plasmid pIRES (Takara Bio). The WT-Cx40 (1.1 kb) and EGFP-tagged Q58L-Cx40 (1.8kb) were subcloned either at the upstream or the downstream cloning sites of the IRES (internal ribosomal entry site) (Fig 6B, constructs 3 and 4). Homomeric WT-Cx40 construct and the heteromeric constructs (WT-IRES-Q58L and Q58L-IRES-WT) in Fig 6C were constructed by PCR. WT-Cx40 or Q58L-Cx40 cDNAs were initially PCR-amplified by the primers Cx40-Fa and Cx40-Rb, and the PCR products were digested with NheI/EcoRI and subcloned in the upper multiple cloning sites NheI/EcoRI of pIRES.

Forward (Cx40-Fa) 5’-GCGCTAGCCACCATGGGCATTTGCAGC TTCCT-3’
Reverse (Cx40-Rb) 5’-AGAATTCTCACACTGATAGGTCATCTG-3’
(Underlines represent the restriction recognition sequences for NheI and EcoRI, respectively)

Similarly, WT-Cx40 or Q58L-Cx40 cDNAs were PCR-amplified by the primers Cx40-F8 and Cx40-R3, and the PCR products were digested with XbaI/NotI and subcloned in the lower multiple cloning sites XbaI/NotI of pIRES.

Forward (Cx40-F8) 5’-GCTCTAGACACCATGGCGATTGGAGCTCCT-3’
Reverse (Cx40-R3) 5’-ATAAGATGCCGCGCTCACACTGATAGGTCATCTG-3’
(Underlines represent the restriction recognition sequences for XbaI and NotI, respectively)

Translation rate of the upstream cloned gene is generally greater than that cloned at the downstream site. Expression levels of WT-Cx40 (40 kDa) and Q58L-Cx40-GFP (67 kDa) are determined by western blotting using ant-Cx40 antibody.

3. **Cell culture and transfection**

Connexin 40 constructs were introduced into connexin-deficient HeLa cells or mouse neuroblastoma (N2A) cells, maintained in F-12 or Minimum Essential Medium, respectively, supplemented with 10% fetal bovine serum. HeLa and N2A cells were transfected with plasmids using Lipofectamine LTX or Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol.

4. **Electrophysiology**

Gap junction currents from heterologously expressed N2A cell pairs were recorded using whole-cell double patch clamp techniques as previously described. Recordings were carried out independently in each cell of a pair using two Axopatch 200B amplifiers (Axon Instruments). Current signals were filtered at 100-200 Hz and digitally sampled at 1-2 KHz.
using an analog-to-digital interface (Digidata 1322A, Axon Instruments). The data were analyzed using Clampfit 9.2 (Axon Instruments) and Origin 7.5 (Origin Lab). The external solution contained (in mmol/L) 160 NaCl, 10 CsCl, 2 CaCl₂, 0.6 MgCl₂ and 10 HEPES, at pH 7.4. The intracellular (pipette) solution contained (in mmol/L) 130 CsCl₂, 0.5 CaCl₂, 10 HEPES, 10 EGTA, 2 Na₂ATP and 3 MgATP (added daily), (pH = 7.2). Pipette resistance was 5-10 MΩ. Octanol was added directly to the external solution at the final concentration of 1 mmol/L at each experiment. Experiments were carried out at room temperature (20-22 °C). All the chemicals were purchased from Sigma or Wako (Tokyo, Japan).

Gap junction channel conductance (gⱼ) was determined by conventional methods. Briefly, both cells in the pair (cell₁ and cell₂) were independently voltage-clamped at the same holding potential (-40 mV). Cell₁ was then stepped to a new voltage, thus creating a potential difference across the junction (Vⱼ). The current in cell₂ was considered equal and opposite to the junctional current (Iⱼ), and gⱼ was measured from the ratio Iⱼ/Vⱼ. The pulses were 2 or 5 sec in duration with an interpulse interval of 15 sec. Unitary conductance was obtained from pairs where only one or two functional channels were spontaneously detected. In some cases, cells were uncoupled by exposure to 1 mmol/L octanol. Histograms of events were obtained from channels recorded during repetitive 10-20 sec steps to Vⱼ= +60 mV. To measure unitary conductance, only junctional current traces with events that lasted for longer than 20 ms were included. ⁹,¹⁰ All-points histograms of digitized current traces and the frequency distribution histograms were constructed using Origin 7.5.

To analyze the electrophysiological properties of heterotypic gap junctions consisting of Cx40-WT and Cx40-Q58L, N2A cells were transiently transfected with either Cx40-WT (pIRES2-EGFP plasmid) or Cx40-Q58L (pIRES2-DsRED2 plasmid). Sixteen hours later, both cells were split with trypsin/EDTA and co-cultured. On the following day, the
heterotypic cell pairs of Cx40-WT (green) and Cx40-Q58L (red) were visually identified under fluorescent microscopy. Experiments were carried out at room temperature (20-22 °C).

5. Immunocytochemistry

HeLa cells were cultured on a glass-bottom dish (Asahi Techno Glass, Chiba, Japan) and transfected with the fusion plasmids of pEGFPN1-Cx40-WT, pCMV-FLAG-Cx40-Q58L, or both. Next day, the cells were washed with phosphate-buffered saline (PBS), fixed in PBS containing 2% formaldehyde for 30 min at 4 °C, and permeabilized with 0.05% Triton X-100 for 30 min at 4 °C. After blocking with PBS containing 4% bovine serum albumin for 1 h at room temperature, the cells were stained with anti-FLAG M2 antibody (mouse monoclonal, 1:200, Sigma) for 1 h at room temperature. Protein reacting with antibody was visualized with Alexa 546-labeled secondary antibody (goat, 1:300, Invitrogen). EGFP and Alexa 546 fluorescence images were recorded with a FluoView FV1000 confocal microscope (Olympus Co, Tokyo) with a 60x oil immersion objective.

6. Western blotting

N2A cells maintained in a 6 well dish were transiently transfected with 3 μg Cx40 plasmids. Two days after transfection, cells were washed with PBS, and total cell lysate was extracted with lysis buffer including 50 mM Tris (pH7.5), 150 mM NaCl, 1% TritonX-100, 0.1 μg/ml aprotinin, 1x complete protease inhibitor (Roche Applied Science). Lysates precleared by centrifugation at 15,000 xg for 10 min were subjected to SDS-PAGE and immunoblotting with rabbit anti-Cx40 antibody (Millipore). Proteins reacting with primary antibodies were visualized by ECL system (GE Healthcare). The membrane was reprobed by anti-GAPDH antibody (Sigma).
7. Surface biotinylation

HeLa cells plated on 100 mm dishes were transiently transfected with 11 μg of pEGFPN1-Cx40-Q58L using Lipofectamine 2000 (Invitrogen). Surface biotinylation was performed 48 hours after transfection using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific, #89881) as per the manufacturer’s protocol. Briefly, after 30 min of biotin labeling reaction at 4 °C, cell were lysed, mixed with NeutrAvidin agarose, and loaded on a column. The biotinylated proteins were eluted with the elution buffer. Fractions of the flowthrough, elute, and input lysate (1:1 diluted with lysate buffer) were subjected to a 4-12% gradient SDS-PAGE and immunoblotting with anti-Cx40 antibodies (Cx40-A, 1:50 dilution, Alpha Diagnostic International). Proteins reacting with primary antibodies were visualized by LI-COR infrared imaging technology. Detection was done using anti-rabbit IRDye 800CW (1:10,000) antibodies (LI-COR Biosciences, # 926-32213).

8. Functional evaluation of novel SCN5A mutations associated with PFHB1

Three novel SCN5A mutations associated with PFHB1 were identified; a missense mutation F777L, a compound heterozygous frame shift mutation p.P701fsX710 plus p.P2006fsX2037, and a frame shift mutation p.V1764fsX1786. These mutations were not found in 400 unaffected control alleles. Functional properties of these mutations were evaluated by whole-cell patch clamp. The mammalian expression plasmids encoding the mutations were constructed by site-directed mutagenesis as we described previously using a human Na channel α subunit (Nav1.5) cDNA. The human cell line tsA-201 was transiently transfected together with Na channel β1 subunit, and the whole-cell Na currents were recorded as we previously described. Electrode resistance ranged from 0.8 to 1.5 MΩ. Data
acquisition was carried out using an Axopatch 200B patch clamp amplifier and pCLAMP10 software (Axon Instruments). Currents were filtered at 5 kHz (–3 dB; 4-pole Bessel filter) and digitized using an analog-to-digital interface (Digidata 1440A; Axon Instruments). Experiments were carried out at room temperature (20–22°C). Voltage errors were minimized using series resistance compensation (generally 80%). Cancellation of the capacitance transients and leak subtraction were performed using an online P/4 protocol. The pulse protocol cycle time was 10 s. The data were analyzed using Clampfit 10 (Axon Instruments) and SigmaPlot 11 (SPSS Science). The holding potential was –120 mV. The bath solution contained (in mmol/l): 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.35 (adjusted with NaOH). The pipette solution (intracellular solution) contained (in mmol/l): 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.35 (adjusted with CsOH). The time from establishing the whole-cell configuration to onset of recording was consistent cell-to-cell to exclude the possible time-dependent shift of steady-state inactivation.

To determine activation parameters, the current-voltage relationship was fit to the Boltzmann equation

\[ I = (V - V_{rev}) \times G_{max} \times (1 + \exp[ V - V_{1/2} ] / k)^{-1} \],

where \( I \) is the peak Na current during the test pulse potential \( V \). The parameters estimated by the fitting are \( V_{rev} \) (reversal potential), \( G_{max} \) (maximum conductance), and \( k \) (slope factor) (Supplemental Figure 1B). Steady-state availability for fast inactivation was measured with a standard double-pulse protocol (Supplemental Figure 1C, left inset), and the data were fit with the Boltzmann equation

\[ I/I_{max} = (1 + \exp[(V - V_{1/2}) / k])^{-1} \],

where \( I_{max} \) is the maximum peak Na current, to determine the membrane potential for \( V_{1/2} \) and \( k \). Functional properties of other mutations in SCN5A or SCN1B were previous reported (Supplemental table S1).
Supplemental Figure S1

Functional properties of the novel SCN5A mutations

Panel A shows whole-cell Na currents recorded from tsA201 cells expressing wild type (WT) Nav1.5 (left) or Nav1.5 mutant F777L (right). Currents were elicited by step pulses from -90 mV to +60 mV (10 mV step) from a holding potential of -120 mV. Bars: 5 msec and 2 nA. Non-inactivated late currents were not observed. Panel B shows current-voltage relationship. Average peak current density was significantly reduced in F777L (p<0.001). WT: 391.7±47.1 pA/pF, n=15 (open circles). F777L: 301.2±30.0 pA/pF, n=9 (closed circles). Panel C shows that the voltage-dependence of activation of F777L channels (closed circles) was not different from control, whereas steady-state inactivation curve was significantly shifted in the hyperpolarizing direction in F777L (WT: $V_{1/2} = -87.1 \pm 0.5$ mV, n=25; F777L: $V_{1/2} = -92.4 \pm 1.3$ mV, n=9; p<0.001). These biophysical properties suggest a decrease in the number of functional (conductive) sodium channels during the action potential upstroke consequent to the mutation. Previous studies have revealed that mutations A1180V\textsuperscript{12} and D1275N\textsuperscript{13}, also found in our series (see supplemental Table S1), exhibit minor functional abnormalities when expressed in cultured cells, though more drastic changes are observed when the channels are expressed in cardiomyocytes\textsuperscript{14}. Cells expressing compound heterozygous mutations p.P701fsX710 and p.P2006fsX2037, or a frame shift mutation pV1764fsX1786, exhibited no Na current, suggesting haploinsufficiency of cardiac Na current in the afflicted population.

Supplemental Figure S2

Exercise stress test of the proband’s mother
Electrocardiographic recording obtained from the probands’s mother during a treadmill exercise stress test at the age of 16. A heart rate of 177 bpm was achieved after 9 min 20 sec of exercise test by Bruce protocol. During the recovery phase at 1 min 17 sec, superior axis narrow QRS ventricular tachycardia with a rate of 110 bpm was observed (upper panel). Ventricular tachycardia was spontaneously terminated at 20 min 23 sec of the recovery phase (lower panel).

**Supplemental Figure S3**

**Co-expression of Cx40-WT and Cx40-Q58L in N2A cells.**

Panels A-C show fluorescence images from a cell pair recorded from cells co-transfected with pIRES2-EGFP-Cx40-WT and pIRES2-DsRED2-Cx40-Q58L (0.5 μg each). Notice expression of both the green (A) and the red marker (B), giving a yellow color in the overlay (C). Calibration bar: 20 μm. Panel D: Junctional conductance recorded from cell pairs as that shown in panels A-C was 18.9±5.4 nS (n=6). This number was not statistically different from that obtained from pairs expressing WT-GFP and Q58L-GFP (average Gj= 13.0±2.4 nS; n=17).

**Supplemental Figure S4**

**Surface biotinylation of Q58L-Cx40 expressed in HeLa cells.**

HeLa cells transfected with pEGFPN1-Cx40-Q58L were surface-labeled with biotin, and lysed. Cell lysate was mixed with NeutrAvidin agarose and loaded on a column. Flowthough, elute (biotin-labeled membrane fraction) and the input lysate (1:1 diluted with lysate buffer) were subjected to SDS-PAGE and immunoblotting. A single 67KDa band of similar intensity
was detected in both elute and the input lysate, but not in the flowthrough. These data indicate that mutation Q58L did not prevent surface expression of the Cx40 protein.
SUPPLEMENTAL REFERENCES


VT initiation at 1'17'' (recovery phase)

Spontaneous VT termination at 20'23''
Flow  Elute  Lysate
Through

75  

37  

→ Q58L- GFP
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GJA5: connexin 40, SCN5A: cardiac voltage-gated Na channel α subunit, SCN1B: voltage-gated Na channel β1 subunit
†: Patients 1 and 3 are sudden cardiac death victims
*: Patient 3 is a compound heterozygous carrier of SCN5A mutations

Supplemental Table S2. ECG parameters of the family members

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<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>QTc (ms)</th>
<th>Axis (degree)</th>
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*: advanced AV block