Connexin43 Mutation Causes Heterogeneous Gap Junction Loss and Sudden Infant Death

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Background—An estimated 10% to 15% of sudden infant death syndrome (SIDS) cases may stem from channelopathy-mediated lethal arrhythmias. Loss of the GJA1-encoded gap junction channel protein connexin43 is known to underlie formation of lethal arrhythmias. GJA1 mutations have been associated with cardiac diseases, including atrial fibrillation. Therefore, GJA1 is a plausible candidate gene for premature sudden death.

Methods and Results—GJA1 open reading frame mutational analysis was performed with polymerase chain reaction, denaturing high-performance liquid chromatography, and direct DNA sequencing on DNA from 292 SIDS cases. Immunofluorescence and dual whole-cell patch-clamp studies were performed to determine the functionality of mutant gap junctions. Immunostaining for gap junction proteins was performed on SIDS-associated paraffin-embedded cardiac tissue. Two rare, novel missense mutations, E42K and S272P, were detected in 2 of 292 SIDS cases, a 2-month-old white boy and a 3-month-old white girl, respectively. Analysis of the E42K victim’s parental DNA demonstrated a de novo mutation. Both mutations involved highly conserved residues and were absent in >1000 ethnically matched reference alleles. Immunofluorescence demonstrated no trafficking abnormalities for either mutation, and S272P demonstrated wild-type junctional conductance. However, junctional conductance measurements for the E42K mutation demonstrated a loss of function not rescued by wild type. Moreover, the E42K victim’s cardiac tissue demonstrated a mosaic immunostaining pattern for connexin43 protein.

Conclusions—This study provides the first molecular and functional evidence implicating a GJA1 mutation as a novel pathogenic substrate for SIDS. E42K-connexin43 demonstrated a trafficking-independent reduction in junctional coupling in vitro and a mosaic pattern of mutational DNA distribution in deceased cardiac tissue, suggesting a novel mechanism of connexin43-associated sudden death. (Circulation. 2012;125:474-481.)

Key Words: arrhythmia • connexins • death, sudden • electrophysiology • genetics

Sudden infant death syndrome (SIDS) is defined as sudden infant death in babies <1 year of age that remains unexplained after a thorough case investigation, including medical autopsy, death scene investigation, and detailed review of the clinical history.1 Despite the successes of the national Back to Sleep Campaigns, >2000 infants die each year of SIDS.2 Although SIDS remains poorly understood with a largely unknown origin, recent clinical and molecular evidence has implicated heritable arrhythmia syndromes as a cause of up to 10% to 15% of SIDS.3–8

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GJA1 encodes connexin43 (Cx43), the predominant ventricular gap junction connexin and key protein in the maintenance of synchronous ventricular contraction. Mutations in GJA1 can cause oculodentodigital dysplasia, a disease with a multorgan presentation that in rare cases can include cardiac abnormalities and sudden death.9 Human mutations in both Cx40 and Cx43 have been linked to atrial fibrillation.10,11 Additionally, both global and cardiac-specific Cx43 knockout mice display cardiac abnormalities and sudden death.12,13 Given that Cx43 disruption can result in cardiac arrhythmias and sudden death and that some SIDS cases may stem from cardiac arrhythmias, we hypothesized that mutations in GJA1-encoded Cx43 may cause some cases of SIDS.

Methods

Population-Based Cohort of SIDS

Two hundred ninety-two SIDS cases derived from population-based cohorts and individually referred cases of unexplained infant deaths (114 female infants, 177 male infants, 1 unknown; 203 whites, 76 blacks, 10 Hispanics, 2 Asians, 1 unknown; average age, 2.9±1.9

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months; range, 6 hours–12 months) were submitted to the Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory for postmortem genetic testing. To be defined as SIDS, the death of the infant <1 year of age had to be sudden, unexpected, and unexplained after a comprehensive medicolegal autopsy. Infants whose deaths were due to asphyxia or specific disease were excluded. This study was approved by the Mayo Clinic Institutional Review Board as an anonymous study. Thus, only limited medical information was generally available, including sex, ethnicity, and age at death. Time of day, medication use, and position at death were not available. By definition, the infant’s medical history and family history were negative. In some instances, the DNA Results committee and Institutional Review Board at our institution permitted the reidentification of cases with mutations that may pose a threat to the family. In such cases, participating subjects gave informed consent.

**GJA1 Mutational Analysis**

Genomic DNA was extracted from frozen necropsy tissue with the Qiagen DNeasy Tissue Kit (Qiagen, Inc, Valencia, CA) or from autopsied blood with the Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). Using polymorphic primer sets, detecting high-performance liquid chromatography, and direct DNA sequencing, we performed open reading frame/splice site mutational analysis on GJA1 (chromosome 6q22.31, 1 coding exon) as previously described. Polymerase chain reaction and denaturing high-performance liquid chromatography conditions are available on request. One thousand ethnically matched adult reference alleles were examined for the absence of identified mutations. Paternity was confirmed with the ABI Verification Set (Life Technologies Corp, Carlsbad, CA).

**Plasmid Construction of Cx43 Expression Vectors**

For electrophysiological studies, full-length coding sequence for rat Cx43 (GenBank accession No. NW_047601), which is >97% homologous to human, was subcloned into pIRESS2-dsRED (Clontech, Mountain View, CA). The recombinant plasmid expressed both Cx43 and dsRED as a bicistronic mRNA, allowing connexin-expressing cells to be selected directly for electrophysiological studies. For fluorescent microscopy, rat Cx43 was subcloned into either mCherry or green fluorescent protein (gift from Dr David C. Sprat, Albert Einstein College of Medicine, Bronx, NY), linking the fluorescent protein to the C terminus of Cx43. All mutations were incorporated via site-directed mutagenesis using a method adapted from the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA), and vectors were sequenced to confirm the appropriate mutation. Analysis of Cx43-containing plaques in N2A cells was done by counting plaques from all images (n = 3 transfections), and statistical analysis (1-way ANOVA with Tukey highest-significant-difference correction) was performed with Statistica 6.0. N2A cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Electrophysiological Recordings for Wild-Type and Mutant Cx43**

Electrophysiological recordings of gap junction currents were obtained from N2A cell pairs transiently transfected with wild-type (WT)–Cx43, and/or E42K–Cx43, or S272P–Cx43. Junctional conductance was measured between cell pairs with a dual whole-cell voltage clamp with 1-dimensional patch-clamp amplifiers (Axopatch; Axon Instruments, Foster City, CA) at room temperature. The bath solution contained 135 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CsCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 5 mmol/L HEPES, 5 mmol/L dextrose, 2 mmol/L pyruvate, and 1 mmol/L BaCl2, pH 7.4. Patch electrodes had resistances of 3 to 5 MΩ when filled with internal solution (125 mmol/L CsCl, 10 mmol/L EGTA, 0.5 mmol/L CaCl2, and 10 mmol/L HEPES, pH 7.2). Currents were filtered at 0.5 kHz and sampled at 2 to 5 kHz. Data were acquired with appropriate software (PCLAMP8; Axon Instruments) and analyzed (PCLAMP8 [Axon Instruments] and ORIGIN 6.0 [Microcal Software, Northampton, MA]). Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from 0 to −20 mV were applied to 1 cell to establish a transjunctional voltage gradient, and junctional current was measured in the second cell (held at 0 mV). A t test was performed for relevant comparisons.

**Immunostaining for Intercalated Disk Proteins**

Glass-mounted sections of formalin-fixed, paraffin-embedded myocardial samples (5–7 μm) were prepared by the Mayo Clinic Tissue and Cell Molecular Analysis core facility using a Leica RM2255 rotary microtome. The sections were deparaffinized, dehydrated, and rehydrated by immersion as follows: 5 minutes in xylene, 2 minutes in xylene, 2 minutes in 100% ethanol, 2 minutes in 75% ethanol, 2 minutes in 50% ethanol, and 5 minutes in distilled water. For antigen retrieval, sections were placed in citrate buffer (pH 6.0), brought to a boil (11 minutes of microwaving), left to cool to room temperature, and washed in PBS (5 minutes). To reduce background autofluorescence, samples were simultaneously blocked with 3% goat serum, 1% BSA, and 0.5% Triton X 100 in PBS for 40 minutes at room temperature. Primary antibody incubation (diluted in blocking buffer) occurred at 4°C overnight. After equilibrating at room temperature, sections were washed 3 times in PBS for 5 minutes and incubated with Cy3-conjugated goat anti-mouse or anti-rabbit IgG (H+L) secondary antibodies (1:400 in PBS; Jackson Immunolabs, West Grove, PA) for 2 hours at room temperature. Samples were washed 3 times in PBS for 5 minutes and mounted in 50% glycerol, 50% PBS, and 0.1% propyl gallate.

**Chemical Reagents**

Antibodies were as follows: rabbit polyclonal anti-Cx43 (1:400; Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti–Pan cadherin (1:400; Sigma), mouse monoclonal anti-plakoglobin (1:1000; Sigma), mouse monoclonal anti-plakophilin2 (2a+2b, undiluted; Fitzgerald Industries International, Acton, MA), mouse monoclonal anti-desmoplakin (1:10; Fitzgerald), and rabbit polyclonal anti–ZO-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA).

**Results**

**Identified Mutations**

Overall, mutational analysis of GJA1 revealed 2 novel missense mutations in 2 of 292 SIDS victims (<1%). E42K-Cx43 was identified in a 2-month-old white boy (Figure 1A), and S272P-Cx43 was identified in a 3-month-old white girl (Figure 1B). Both mutations were absent in >1000 ethnically matched control alleles and involved residues highly conserved across a variety of species (Figure 1C). E42K-Cx43 localized to the extracellular side of the first transmembrane domain, whereas S272P-Cx43 localized to the C terminus (Figure 1D). The common polymorphism A253V-Cx43 was similarly found in both cases and controls.

**E42K Case Description**

In the case of the E42K-positive infant, we were able to unblind the case and obtain more information. The E42K-positive SIDS victim was born at 4 lb, 11 oz at 34 weeks’ gestation along with a fraternal twin brother and spent a few days in the neonatal intensive care unit after birth. No ECG was available from this stay. Over the next 2 months, his mother noticed that he sometimes would be very sleepy or fall asleep immediately after feedings, whereas his twin brother would remain wide awake. His parents also noticed times when he was sleeping that his skin became slightly pale and almost translucent. During the last 2 weeks of his life, his parents had to constantly wake him or use a cold compress to
keep him awake long enough to eat. Interestingly, the family history is positive for previous infant deaths in twins, but these deaths were 3 generations removed from the current victim. Neither parent has cardiac abnormalities, and both were negative for the mutation (Figure 1A). Paternity was confirmed. The other 3 siblings of the victim remain in good health. Poor tissue quality precluded our ability to extract DNA from other tissue types to confirm mosaicism.

**Trafficking of Cx43 Mutations**

Given that loss of trafficking is a common pathogenic mechanism in connexin mutations, we investigated the effects of E42K-Cx43 and S272P-Cx43 on Cx43 trafficking. To address this, we transfected Cx43-negative neuroblastoma (N2A) cells with WT-Cx43, E42K-Cx43, or S272P-Cx43 tagged with either green fluorescent protein or mCHERRY and assessed the number of plaques formed between the cells. No defects in trafficking with mutant Cx43 alone were identified (data not shown). To determine the effects of the mutants on WT-Cx43 trafficking in an effort to recapitulate the heterozygous state of the infant, we cotransfected N2A cells with WT and mutant Cx43 together. The number of gap junctional plaques did not differ in cells containing either of the Cx43 mutations compared with WT, and cotransfection of WT-Cx43 together with E42K-Cx43 or S272P-Cx43 did not alter the rate of plaque formation, indicating that there are other mechanisms independent of trafficking by which these mutations might affect cardiac conduction (Figure 2).

**Electrophysiological Properties of Cx43 Mutations**

To further evaluate the functional integrity of the mutant gap junctions, we performed dual whole-cell patch clamp. Cx43 constructs were expressed in N2A cells, and junctional currents were recorded from cell pairs 48 hours after transfection. Cell pairs expressing WT-Cx43 robustly coupled (27.06±6.32 nS; n=24; Figure 3A and 3C). However, cells expressing the E42K-Cx43 mutation displayed a complete absence of electric coupling (Gj values were 0.02±0.02 nS; n=15; P<0.001 versus WT-Cx43; Figure 3B and 3C). To assess whether mutant Cx43 decreased WT junctional currents, cells were cotransfected with WT-Cx43 and E42K-Cx43. Gj values were 0.1±0.04 (n=19; P<0.001 versus...
WT-Cx43), indicating a complete failure of WT-Cx43 to rescue the phenotype brought about by E42K (Figure 3B and 3C). Additional electrophysiological experiments confirmed that the green fluorescent protein and mCHERRY tags did not alter the E42K effects on the connexin protein (data not shown). In contrast to the E42K phenotype, S272P-expressing cells demonstrated WT coupling (P>0.05 versus WT-Cx43; Figure 3C).

**Immunohistochemical Staining of SIDS-Associated Cardiac Tissue**

Given its abnormal function in vitro, we performed immunohistochemistry on myocardial tissue from the proband to investigate the effects of E42K on the localization of Cx43 and on the distribution patterns of further intercalated disk components (including N-cadherin, desmoplakin, plakoglobin, plakophilin-2, and ZO-1). Ventricular samples obtained at autopsy from an adult, a 7-month-old child, and a 2-month-old child with no clinical or pathological evidence of heart disease were subjected to the same immunostaining protocol and served as controls. Intercalated disks showed strong immunoreactive signal for Cx43 in both the adult and 7-month-old myocardial samples (Figure 4A and 4B). In contrast, Cx43 showed a different, rather polygonal distribution pattern in the 2-month-old myocardial specimen, staining the entire cardiac myocyte membrane uniformly instead of clustering at intercalated disk sites (Figure 4C). Our observations are in agreement with previous observations.18 In marked contrast to the control samples, the E42K-Cx43 SIDS case demonstrated a patchy distribution pattern for Cx43. Certain areas seemed to express Cx43 at control levels, whereas adjacent areas showed no immunoreactive signal for the gap junction protein (Figure 4D–4F). These observations suggest somatic mosaicism. The signal for both the nondesmosomal adhesion molecule N-cadherin and the plakin protein demoplakin was strong and indistinguishable from controls, demonstrating an adult-like signal clustering at intercalated disk sites (Figure 5). Immunoreactive signal for plakoglobin, plakophilin-2, and ZO-1 was absent from both the SIDS sample and the 2-month-old control sample (data not shown). It is noteworthy that a lack of immunoreactive signal does not necessarily indicate the absence of protein in...
the myocardium at this stage. It suggests, however, that the expression levels and/or localization patterns are such that the protein cannot be detected by this approach.

**Discussion**

This is the first study to report a SIDS-associated mutation in *GJA1*-encoded Cx43 that causes formation of a biophysically nonfunctional gap junction channel. We discovered 2 novel Cx43 mutations in 2 unrelated SIDS victims, with both mutations involving highly conserved residues and absent in >1000 ethnically matched controls. The first, E42K, localizes to the transmembrane region near the extracellular loops, which are important for proper formation of fully functioning connexons. S272P localizes to the cytoplasmic C terminus, near a region of multiple serine phosphorylation sites. However, functional studies for S272P showed WT trafficking and gap junctional coupling, whereas E42K demonstrated a profound reduction in junctional conductance for cells expressing the E42K-Cx43 mutation either alone or together with WT, similar to the phenotype of other disease-associated connexin mutations. In addition, immunostaining of the decedent’s tissue revealed patchy loss of Cx43, suggestive of somatic mosaicism similar to that seen in cases of atrial fibrillation induced by somatic Cx40 and Cx43 mutations and highly predictive of an arrhythmogenic substrate. Therefore, we predict that although the death of the E42K-positive victim may be due directly to mutation-induced pathology, the death of the S272P-positive victim remains unexplained because our investigations demonstrated WT junctional physiology in this second case.

Functional gap junction channels are formed by 2 hemichannels (connexons) in adjacent cells (each consisting of a hexamer of 1 or more types of connexin protein subunits), allow intercellular communication via the passage of electric impulses between myocytes, and thus facilitate synchronous contraction of the myocardium. Cx43 is the major gap junction protein in ventricular tissue. Global deletion of the *GJA1* gene in mice results in death shortly after birth, caused primarily by structural abnormalities in the cardiac outflow tract present well before birth. In contrast, Cx43 conditional knockout mice (CKO) show no significant differences in their phenotype or behavior compared with their littermate controls and yet die suddenly at 81.4 ± 3.3 days. As shown by immunohistochemistry and Western immunoblotting studies, by 45 days, Cx43 expression in the ventricles was reduced to 18% in the CKO animals compared with controls. High-powered views showed areas where cells formed rare plaques alternating with clusters of cells stained positively for Cx43. The QRS amplitude was progressively decreased in the CKO animals and paralleled the loss of Cx43 expression. Programmed electric stimulation induced sustained polymorphic ventricular tachycardia in 8 of 10 CKO animals at 45 days, whereas only nonsustained VT was induced in one third of the control mice. Yet, despite the increased susceptibility of CKO mice to lethal arrhythmias, hemodynamic parameters and ventricular contractility of CKO mice at all time points were no different from those of controls.

Moreover, chimeric Cx43 mice, which were generated by introducing Cx43-deficient embryonic stem cells in WT recipient blastocysts, also demonstrated conduction delay and spontaneous ventricular tachycardia in the absence of structural changes. Gap junction expression in those hearts was highly abnormal, with foci of Cx43-deficient myocytes interspersed throughout an otherwise well-coupled myocardial syncytium. Telemetry recordings in the chimeric animals showed increased frequency of premature ventricular contractions compared with control littermates. Moreover, multiple morphologies of ventricular ectopy were recorded, suggesting the presence of multiple arrhythmogenic foci. It is true that the chimeric animals showed decreased ventricular systolic function (fractional shortening was 33.2 ± 2.08% in the chimeric animals compared with 41.0 ± 1.27% in con-
However, although the left ventricular systolic pressures were decreased in the chimeras, there were no differences in the diastolic pressures between chimeric and control animals. Moreover, there was no evidence of ventricular dilatation or hypertrophy. Taken together, these results suggest that gap junction remodeling predisposes to arrhythmias but does not obligate any significant mechanical dysfunction. It is likely that the cardiac tissue in our decedent mimics this latter instance, and such findings in murine models suggest why no cardiac abnormalities were detected at autopsy to render a non-SIDS diagnosis and why the infant was able to survive 2 months before death.

Loss of ventricular gap junction function underlies the formation of an arrhythmogenic substrate in animal models. However, as the previously discussed studies support, it is likely that heterogeneous Cx43 loss, not reduction of Cx43 per se, creates an arrhythmogenic substrate whereby safe, albeit slow, conduction is dissipated and disrupted by sinks of well-coupled cells. Thus, the spontaneous ventricular tachyarrhythmias and sudden death noted in cardiac-restricted knockout mice may actually stem from low residual mosaic expression of Cx43. The proarrhythmic nature of heterogeneous Cx43 expression is further supported by the observation that heterogeneous populations of neonatal murine ventricular myocytes have proarrhythmic impulse propagation.

Moreover, Boulaksil et al demonstrated increased heterogeneity of Cx43 in both congestive heart failure patients with documented ventricular tachycardias and a heart failure mouse model that showed inducible polymorphic ventricular tachycardia in the setting of heterogeneous Cx43 loss. In this case, whether the heterogeneity of Cx43 is related to the polymorphic morphology of the tachycardias remains unsolved. Finally, a Cx43 somatic mutation was recently found to underlie lone atrial fibrillation in a patient. Immunohistochemistry of the patient’s atrial tissue showed a mosaic pattern of intracellular retention of Cx43 and a mosaic pattern of aberrant gap junction formation, very much like the images we obtained from our case. Expression of the mutant Cx43 form in oocyte pairs from the genus Xenopus showed a dramatic decrease in cell-cell coupling conductance.

We hypothesize, given the immunostaining pattern of the decedent, that mutant E42K-Cx43, although trafficking properly in an in vitro setting, does not properly interact with adjacent Cx43 to form fully functional gap junctions, forming an arrhythmogenic substrate. We know that other cardiac disorders trigger a reduction in Cx43 expression, and it is likely that such changes may not be detected at levels
assessed by our plaque formation assay. Furthermore, our immunostaining assay is optimized to detect high levels of Cx43 at gap junctions. A change in expression may reduce Cx43 to a level undetectable by our present assay. This may explain why our in vitro data in N2A cells demonstrate proper trafficking, whereas intact decedent heart tissue demonstrates a mosaic pattern.

Because this infant was not wearing a Holter monitor at the time of his tragic demise, it is impossible to verify our postulated exit rhythm of ventricular fibrillation. Nevertheless, E42K-Cx43 was identified in a sudden death cohort in a gene with known association with cardiac disease, was located in a highly conserved region of the gene, and was absent in >1000 ethnically matched alleles. In addition, the heterogeneous staining pattern of the infant’s cardiac tissue offers strong evidence that this mutation conferred a sudden death–predisposing arrhythmogenic phenotype. Furthermore, this deceased infant’s loss-of-function E42K-Cx43 mutation and likely somatic mosaicism essentially represent the human equivalent of the chimeric Cx43 mouse. The S272P-Cx43 mutation had a WT phenotype in our investigations; therefore, although rare and highly conserved, it cannot at this time be considered a sudden death–predisposing mutation.

Conclusions
This is the first study to implicate a mutation in a connexin protein in the pathogenesis of SIDS. E42K-Cx43 demonstrated a severe loss-of-function phenotype and is located in a crucial conserved region of the protein. Although such SIDS-associated Cx43 mutations are rare, this study provides evidence for the contribution of Cx43 dysregulation to sudden death and contributes to the growing body of literature implicating cardiac arrhythmias as the cause of a subset of deaths caused by SIDS.

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Disclosures
None.

References

Figure 5. Normal N-cadherin and desmoplakin staining in a control and a case. Confocal images of control and E42K-connexin43 (Cx43) myocardium immunostained for N-cadherin (top) and desmoplakin (bottom). A and C, Two-month-old control myocardium staining. B and D, Two-month-old E42K-Cx43 case myocardium staining. Scale bars = 10 μm.

N-cadherin

Desmoplakin
Sudden infant death syndrome (SIDS) claims the lives of >2000 infants every year in the United States. By definition, SIDS is characterized by the unexplained death of an infant, but recent evidence has implicated mutations in genes associated with heritable arrhythmia syndromes in up to 10% to 15% of SIDS cases. GJA1 encodes the connexin43 (Cx43) protein, a crucial gap junction protein involved in the maintenance of synchronous ventricular contractions. Recently, genetic mosaicism in the GJA1 gene has been implicated in the pathogenesis of atrial fibrillation, and global and cardiac-specific Cx43 knockout mice display cardiac abnormalities and sudden death, making GJA1 a candidate gene for SIDS pathogenesis. In this study, we assessed the spectrum and prevalence of GJA1 genetic variation in nearly 300 SIDS victims and identified a missense mutation, E42K, in a 2-month-old white boy (JBS, Ew). GJA1 variants, mutations, and connexin 43 dysfunction as it relates to the occludentodigital dysplasia phenotype. *Hum Mutat.* 2009;30:724–733.


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