Evaluating the role of connexin43 in congenital heart disease: Screening for mutations in patients with outflow tract anomalies and the analysis of knock-in mouse models

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ABSTRACT

Background: GJA1 gene encodes a gap junction protein known as connexin 43 (Cx43). Cx43 is abundantly expressed in the ventricular myocardium and in cardiac neural crest cells. Cx43 is proposed to play an important role in human congenital heart disease, as GJA1 knock-out mice die neonatally from outflow tract obstruction. In addition, patients with visceroatrial heterotaxia or hypoplastic left heart syndrome were reported to have point mutations in GJA1 at residues that affect protein kinase phosphorylation and gating of the gap junction channel. However, as these clinical findings were not replicated in subsequent studies, the question remains about the contribution of GJA1 mutations in human congenital heart disease (CHD). Materials and Methods: We analyzed the GJA1 coding sequence in 300 patients with CHD from two clinical centers, focusing on outflow tract anomalies. This included 152 with Tetralogy of Fallot from over 200 patients exhibiting outflow tract anomalies, as well as other structural heart defects including atrioventricular septal defects and other valvar anomalies. Our sequencing analysis revealed only two silent nucleotide substitutions in 8 patients. To further assess the possible role of Cx43 in CHD, we also generated two knock-in mouse models with point mutations at serine residues subject to protein kinase C or casein kinase phosphorylation, sites that are known to regulate gating and trafficking of Cx43, respectively. Results: Both heterozygous and homozygous knock-in mice were long term viable and did not exhibit overt CHD. Conclusion: The combined clinical and knock-in mouse mutant studies indicate GJA1 mutation is not likely a major contributor to CHD, especially those involving outflow tract anomalies.

Key words: Connexin43, congenital heart disease, gap junction, knock-in mouse model, outflow tract, phosphorylations

INTRODUCTION

The gene GJA1 encodes a gap junction protein known as connexin 43 (Cx43). Cx43 is abundantly expressed in the ventricular myocardium, where it plays an important role in electrical conduction in the heart.1,2 Cx43 is also proposed to play a role in congenital heart disease (CHD),
as GJA1 knock-out mice die neonatally from outflow tract obstruction associated with conotruncal heart malformation. These malformations consist of pouches positioned at the base of the pulmonary outflow tract, a region known as the infundibulum. These pouches are comprised of elaborate intraventricular trabeculations forming lacunae and sinusoidal cavities that cause pulmonary outflow obstruction, with neonatal lethality resulting from failure to establish normal pulmonary circulation.\cite{5} These mice also show various coronary artery anomalies\cite{6} associated with dysregulation of coronary vasculogenesis.\cite{7} Previous studies had shown that Cx43 plays an important role in modulating the migratory behavior of cardiac neural crest cells (CNC), and the resulting defects in the deployment of CNCs\cite{8} underlies the conotruncal heart malformation.\cite{9} Other studies in chick, Xenopus and mice suggest that the precise regulation of Cx43 function may be critical in other developmental processes as well.\cite{10-13}

A possible role for GJA1 in CHD has been suggested by the report of GJA1 point mutations in a clinical study of patients with visceroatral heterotaxia and hypoplastic heart syndrome. Six patients with visceroatral heterotaxia\cite{14} and eight patients with hypoplastic left heart syndrome (HLHS)\cite{15} exhibited mutations involving serine and threonine residues that affect protein kinase phosphorylation and gating of the gap junction channel. However, as these findings were not replicated in subsequent clinical studies, the question remains about the contribution of GJA1 mutations in human CHD.\cite{16-20}

To further investigate the role of GJA1 mutations in CHD in this study, we screened for GJA1 mutations by direct sequencing the coding region of Deoxyribonucleic acid (DNA) obtained from 300 patients with CHD, over 200 of which have outflow tract anomalies. We also generated two mouse models to directly address the role of Cx43 phosphorylation in CHD with the targeted knock-in of Cx43 point mutations at serine residues subject to protein kinase C (PKC) or casein kinase 1 (CK1) phosphorylation. We chose to investigate these particular amino acid residues given previous studies showing that they can modulate gap junction conductance and/or trafficking of the Cx43 protein, respectively.\cite{21-23} Both mouse models were found to be viable and fertile, and with no obvious decrease in viability or long term lifespan. The combined results from the mouse and human studies do not support a major role for GJA1 mutations in human CHD, especially those involving the outflow tract.

**MATERIALS AND METHODS**

**Patient recruitment**

Patients were recruited with protocols approved by the Institutional Review Board (IRB) at The Children’s Hospital of Philadelphia (CHOP), and the Fudan University Children's Hospital in China (FUCH). Cardiovascular diagnoses were confirmed by an attending pediatric cardiologist, who reviewed echocardiograms and/or echocardiogram reports, cardiac catheterization reports, and operative notes if applicable.

**Deoxyribonucleic acid extraction, polymerase chain reaction amplification, and DNA sequencing**

For FUCH patients, deoxyribonucleic acid (DNA) was obtained from peripheral blood in all subjects. DNA from patients at CHOP was prepared from blood or lymphoblastoid cell lines. Genomic DNA was extracted using standard techniques. The coding region of GJA1 was polymerase chain reaction (PCR) amplified using primers designed to exclude the GJA1 pseudogene in the human genome\cite{24} [Figure 1a and Table 1]. At FUCH, each DNA sample was subjected to two rounds of nested PCR. In the first round, the whole coding region was amplified using F1 and R3 primers followed by amplification of three overlapping fragments using combination of all 6 pair of primers [Table 1]. At CHOP the whole coding region was amplified using two sets of overlapping primers [Table 1]. At both centres, 25-40 cycles of amplification was carried out by hot start DNA polymerase using annealing temperature of 58-60°C The resulting product was sequenced by Sanger sequencing using standard protocols.

**Gja1 Knock-in mouse models**

Targeting constructs with Gja1, point mutations were made with floxed neomycin selection cassette and either PGK-Neo (S368A) or PGK-Neo-Stop (S330A, S328Y, and S325A) and a thymidine kinase negative selection cassette [Figure 1b]. After electroporation, ES cells were screened with long range PCR using primers amplifying from the homology arms to the floxed neomycin cassette [Table 2], and homologous recombination was confirmed by Southern blotting [Figure 2]. PCR amplifications were carried out with Advantage 2 polymerase (clonetech) with initial annealing temp going down by 0.5°C per cycle starting from 64°C for 20 cycles followed by annealing at 52°C for next 18 cycles.
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**Figure 1:** Genomic deoxyribonucleic acid (DNA) amplification and targeting construct design (a) GJA1 genomic DNA amplification and sequencing. The location of primers used for polymerase chain reaction (PCR) amplification and sequencing of the coding region of GJA1 (black region) are indicated. Primers were designed to avoid amplification of the pseudogene. (b) Gja1 knock-in construct design. The knock-in vectors contained floxed PGK-Neo (S368A) or PGK-Neo-Stop (S330A/S328Y/S325A), a thymidine kinase cassette, and 5' and 3' homology arms (dashed lines). Mutation in the mouse Gja1 knock-in constructs (vertical arrow) consisted of S368A (AGC->GCT) or S330A:S328Y:S325A (TCC->GCA;TCC->TAC;AGC->GCA). Primers used for screening are described in Table 4.

**Table 1:** Primers for polymerase chain reaction amplification of patient deoxyribose nucleic Acid

| Primer sequence | Polymerase chain reaction product |
|-----------------|----------------------------------|
| F1:GAAATACGTGAAACCGTTGG R3:CCTGGTGCACTTTTCTACAGC | 1348bp |
| F1:GAAATACGTGAAACCGTTGG R2:TCTTGTTTCTCTCTCTCTTC | 451bp |
| F2:TATTTGTGTCTGTACACACTCTCTTG T2:CTTTTAACCGATCTTTAAGCGC | 477bp |
| F3:GCTTGTCCTGCGCTCTTCAATAT R3:CTGTGTCCTGCTCTTACAGC | 568bp |
| Fa:AGAATGTAGAATTAGTAAACC Ra: TTCCCTTAACCGATCTTTA | 862bp |
| Fb:GCTGCGAACCCTACATCATCAGTAT Rb: AAGGCTGTTGAGTACCA | 810bp |

†F1-F3 and R1-R3: primers were used at FUCH; Fa-Fb and Ra-Rb: Primers were used at CHOP.

**Table 2:** Primers for screening knock-in mice

| Primer sequences | Polymerase chain reaction product |
|------------------|----------------------------------|
| 5HF:GCAAGATCCTGCTGCTGAAAAACC 5HR:CTGACTGTTGGCTTTTGGTTG | 2.6kb |
| 3HF:ACTTGCGCTGGGAAAAGCGGCCTC 3HR:AACCGGCTTTCTGATGCTCTC | 5.9kb |
| LPF:CTCACAGCTCTATCTCTCTCTCTC LPR:CTGACAGAGCTCTACTACCATCGA | WT: 461bp |
| MF:ACTCTCACATATGTCTCTCTCTC MR:CCCTACTTTTGCCTAGCTA | KI -Neo: 550bp |
| | KI +Neo: >2kb |
| | 519bp |
Table 3: Congenital heart defects in patient cohorts

| Outflow tract defects                      | Valvar defects                       | Other defects                      |
|--------------------------------------------|-------------------------------------|-----------------------------------|
| Tetralogy of fallot (152)                  | Aortic atresia (3)                  | Coarctation of aorta (7)          |
| Pulmonary atresia (6)                      | Tricuspid atresia (9)               | Visceroatrial heterotaxy (11)     |
| Truncus arteriosus (6)                     | Mitral atresia (3)                  | Single atrium (3)                 |
| Transposition of great arteries (31)       | Atrio-ventricular septal defect (22)| Single ventricle (5)              |
| Double-outlet right ventricle (29)         |                                     | Interrupted aortic arch (1)       |
|                                            |                                     | Hypoplastic right heart syndrome (4)|
|                                            |                                     | Total/Partial anomalous pulmonary venous drainage (4) |
|                                            |                                     | Hypoplastic left heart syndrome (4) |

† Number in parenthesis equal to number of patients recruited in the study

Immunoblotting and immunohistochemistry

Western immunoblotting was performed as previously described and quantified using a Li-Cor Biosciences Odyssey infrared imaging system and associated software (inverted images are presented). Cx43 was detected with our Cx43NT1 antibody and the antibody against vinculin was obtained from Sigma Chemical Co. (V4505).

Immunohistochemistry was performed on formalin-fixed paraffin sections of hearts from wild-type, CK1 and S368A mice. Briefly, the de-paraffinized and hydrated histologic sections were subjected to heat-induced epitope retrieval for 20 minutes, blocked with 5% normal goat serum and 1% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes, and then incubated with rabbit anti-Cx43 antibody (1:500) for 1
hour at room temperature.\textsuperscript{[26]} Sections were washed in PBS and incubated with Alexa fluor 647 goat anti-rabbit secondary antibody (Invitrogen) for 30 minutes. Sections were washed three times and then were embedded in Prolong Gold Antifade reagent (Invitrogen). Fluorescence images were acquired using a TissueFax-System (TissueGnostics, Vienna, Austria) with far-red (Cy5, HyQ filter set: Excitation 590-650, emission 673-762) Zeiss PlanApo 20X/OD objective. After thresholding, the number of pixels in junctions (i.e., area) and intensity of the pixels were quantified using the public domain National Institutes of Health (NIH) Image program (developed at the United States National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**RESULTS**

**Sequencing for \textit{GJA1} mutations in patients with congenital heart disease**

We analyzed 300 patients with CHD recruited at FUCH and CHOP, including 224 patients with outflow tract anomalies, 152 of which had tetralogy of Fallot (TOF) that included the full range of pulmonary valve morphologies (stenosis, atresia, “absent”) [Table 3]. Approximately 50\% of the patients from both cohorts were less than 1 year of age [Table 4]. Double stranded DNA sequencing showed no amino acid altering mutations in these 300 patients. Two novel synonymous nucleotide substitutions were found in several TOF patients: G717A in one patient from FUCH, and C555T (1 patient) and G717A (6 patients) from the CHOP cohort. As \textit{GJA1} mutations were suggested to arise \textit{de novo} in somatic tissues in patients with CHD,\textsuperscript{[13]} we also analyzed genomic DNA from surgically resected right ventricular outflow tract myocardium for 26 of the patients from FUCH; however, no difference in the DNA sequence was found.

\textit{Gja1} knockin mouse mutants show no overt congenital heart defects

Given limitation in the patient sample size, we made two \textit{Gja1} knock-in constructs to assess whether specific \textit{Gja1} mutations at important regulatory sites in Cx43 can cause CHD in mice. These included a construct [Figure 1b] containing S368A substitution at a PKC phosphorylation site in Cx43 that reduces channel conductance,\textsuperscript{[21]} and a construct with S325A/S328Y/S330A (CK1) mutations at sites phosphorylated by casein kinase 1 known to regulate Cx43 assembly into a gap junction structure.\textsuperscript{[22,23]} From previous work, we know that elimination of S368 or S325/S328/S330 would change the conductance properties of the resulting gap junction channels.\textsuperscript{[22,27-29]} Mice from both knock-in constructs were heterozygous/homozygous viable [Figure 3; data not shown], with apparently normal lifespan (mice could be routinely aged > 12 months) and no obvious CHD. Surprisingly, immunostaining of mutant mice

![Figure 3: Genotype distribution of Gja1 knock-in mice litters. Shown are the genotype distribution recovered in three litters of mice obtained from the mating of heterozygous Gja1 knock-in mice with the S330A:S328Y:S325A (CK1) mutation. The genotype obtained (blue) is not significantly different from the expected distribution (red).](image-url)

\begin{table}[h]
\centering
\caption{Patients with complex heart defects from Children’s Hospital of Philadelphia (CHOP) and Fudan University Children’s Hospital in China (FUCH)}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Gender} & \textbf{Fudan University Children’s Hospital in China} & \textbf{Age} & \textbf{Children’s Hospital of Philadelphia} & \textbf{Age} \\
\hline
\textbf{Gender} & \textbf{< 28D} & \textbf{28D < 1Y} & \textbf{1Y < 3Y} & \textbf{>3Y} & \textbf{Unknown} \\
\hline
\textbf{Male} & 14 cases & 51 cases & 17 cases & 33 cases & \\
\textbf{Female} & 6 cases & 20 cases & 18 cases & 23 cases & \\
\textbf{% each age period} & 11.0\% & 39.0\% & 19.2\% & 30.8\% & \\
\hline
\end{tabular}
\end{table}
showed no significant difference in Cx43 localization gap junctions; and, the density of Cx43 immunostaining did not appear to be significantly changed when compared to wildtype hearts [Figure 4]. The integrated densities of the area of junctional staining in arbitrary units were: Wild type-Cx43 (WT) = 18742±5191; CK1 = 17957±3851; PKC = 17352±1415.

When heart tissue was analyzed by Western immunoblotting, we observed the typical reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migration of Cx43 due to its phosphorylation at sites such as S325/328/330 in the wild type mouse tissues that was much less prominent in heart tissue from the CK1 mice [Figure 5]. We had previously shown that phosphorylation at S368 does not affect SDS-PAGE migration. The PKC1 mice, however, also showed a loss of these slower migrating species. Quantitation of the Western blot showed the Cx43 protein expression level (when normalized to vinculin) was reduced by more than 70% in the PKC mutant and nearly 30% in the CK1 mutant mouse hearts [Table 5]. Although, these mice could potentially show altered responses to acute signaling events, we did not detect any developmental defects or changes in their long-term viability or lifespan.

**DISCUSSION**

We found no amino acid altering mutations in *GJA1* gene in 300 patients with CHD, including 224 patients with outflow tract anomalies. However, two synonymous sequence variants were found - one is a novel Single Nucleotide Polymorphism (SNP, C555T) and another is a known variant in National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism database (dbSNP, G717A, rs57946868). Given the neonatal lethality of the *Gja1* knockout mouse, it is possible that the disruption or perturbation of the Cx43 function may not be postnatal viable. We note that in both cohorts, half of the patients with complex CHD were less than 1 year of age.

Mutations in *GJA1* have been reported in patients with oculodentodigital dysplasia (ODDD), a disorder causing craniofacial and limb dysmorphisms, neurodegeneration and deafness. Cardiac abnormalities were rare and consisted of conduction anomalies, with one ODDD individual exhibiting ventricular tachycardia and atrioventricular block associated with a Ile130Thr mutation, and another with atrioseptal defects associated with a Gly21Arg mutation. We noted a mutant mouse model with a N-ethyl-N-nitrosourea-induced point mutation in *Gja1* exhibited phenotypes similar to ODDD. Also
in these patients, there were no structural heart defects except for some cases of patent foramen ovale.

Surprisingly, our studies showed Gja1 knock-in mice with point mutations at important regulatory serine residues Gja1S368A or Gja1S325A/S328Y/S330A were homozygous, viable and fertile. We chose to examine the S328Y mutation since early findings indicated that this site might be modified in a CHD patient,[14,18] although subsequent studies indicated this was not the case.[16-20] Findings from the previous in vitro studies would have predicted that these mice should show functional impairment for Gja1 due to defects in Cx43 trafficking and or gating of the Cx43 gap junction channel. These unexpected findings show the importance of animal model studies to validate in vitro findings.[21,22] Together, our results indicate that GJA1 mutations are not commonly associated with outflow tract anomalies. These findings do not preclude a role for GJA1 mutations in a more complex or severe CHD condition associated with prenat al/neonatal lethality, a clinical population difficult to survey.

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