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ARTICLE

A variant in the carboxyl-terminus of connexin 40 alters GAP junctions and increases risk for tetralogy of Fallot

Valentina Guida[1,11], Rosangela Ferese[1,2,11], Marcella Rocchetti[3], Monica Bonetti[4], Anna Sarkozy[1,12], Serena Cecchetti[5], Vania Gelmetti[1], Francesca Lepri[6], Massimiliano Copetti[7], Giuseppe Lamorte[1], Maria Cristina Digilio[6], Bruno Marino[8,9], Antonio Zaza[3], Jeroen den Hertog[4,10], Bruno Dallapiccola[6] and Alessandro De Luca*[,1]

***GJA5* gene (MIM no. 121013), localized at 1q21.1, encodes for the cardiac gap junction protein connexin 40. In humans, copy number variants of chromosome 1q21.1 have been associated with variable phenotypes comprising congenital heart disease (CHD), including isolated TOF. In mice, the deletion of *Gja5* can cause a variety of complex CHDs, in particular of the cardiac outflow tract, corresponding to TOF in many cases. In the present study, we screened for mutations in the *GJA5* gene 178 unrelated probands with isolated TOF. A heterozygous nucleotide change (c.793C>T) in exon 2 of the gene leading to the p.Pro265Ser variant at the carboxyl-terminus of the protein was found in two unrelated sporadic patients, one with classic anatomy and one with pulmonary atresia. This *GJA5* missense substitution was not observed in 1568 ethnically-matched control chromosomes. Immunofluorescent staining and confocal microscopy revealed that cells expressing the mutant protein form sparse or no visible gap-junction plaques in the region of cell–cell contact. Moreover, analysis of the transfer of the gap junction permanent tracer lucifer yellow showed that cells expressing the mutant protein have a reduced rate of dye transfer compared with wild-type cells. Finally, use of a zebrafish model revealed that microinjection of the *GJA5*-p.Pro265Ser mutant disrupts overall morphology of the heart tube in the 37% (22/60) of embryos, compared with the 6% (4/66) of the *GJA5* wild-type-injected embryos. These findings implicate *GJA5* gene as a novel susceptibility gene for TOF.**
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INTRODUCTION

Tetralogy of Fallot (TOF, MIM no. 187500) is the most common cyanotic heart defect (MIM no. 217095); it is estimated to occur in 2.5–3.5 per 10 000 live births, and accounts for 6.8% of all congenital heart disease (CHD).[1] TOF can arise as the consequence of prenatal infections, maternal illnesses, and maternal therapeutic and non-therapeutic drug exposures. A proportion of TOF cases is associated with chromosome imbalances; ~15% are heterozygous for chromosome 22q11.2 deletion, and nearly 7% have trisomy 21 (Down syndrome).[2,3] Non-syndromic TOF can be caused by dominant mutations in transcriptional regulators (*NKX2.5*,[4,5] *GATA4*,[6,7] *ZFMP2/FOG2*,[8] *FOXH1*[9]) and in genes involved in signal transduction (*NOTCH1*,[10] *JAG1*,[11,12]) *CFC1*,[9] *TDGF1*,[9] *GDF1*,[13] and *NODAL*[14]). However, mutations in these genes have been related also to other CHDs.[8,9,15–17] In addition, large *de novo* copy number variants have been implicated as a cause of non-syndromic TOF.[18] Among these, a copy number variant at chromosome 1q21.1 was found in 1% of non-syndromic sporadic TOF cases.[18] Recently, duplications at chromosome 1q21.1 have been associated with a ~30-fold increase in the risk of TOF.[19] *GJA5* gene (MIM no. 121013), localized at 1q21.1, encodes for the cardiac gap junction protein connexin 40 (Cx40), and is highly expressed in the human outflow tract.[18] A systematic study of the anatomy and histology of Cx40-deleted mice has revealed a variety of complex CHDs in these animals, with the most common malformations being of the conotruncal type, corresponding to TOF in many cases.[20] In humans, abnormalities in the *GJA5* gene have been associated with atrial fibrillation.[21–23]

In the present study, we evaluated whether *GJA5* mutations might be pathogenic in isolated TOF, by gene screening in a series of non-syndromic TOF patients.

MATERIALS AND METHODS

Study subjects

The study cohort included 178 patients with non-syndromic TOF. One hundred and forty-seven cases had TOF with classic anatomy and 31 had

[1]Mendel Laboratory, Casa Sollievo della Sofferenza Hospital, IRCCS, San Giovanni Rotondo, Italy; [2]Department of Experimental Medicine, 'Sapienza' University, Rome, Italy; [3]Biotechnology and Biosciences Department, University of Milan-Bicocca, Milan, Italy; [4]Hubrecht Institute, KNAW and University Medical Centre Utrecht, Utrecht, The Netherlands; [5]Section of Molecular and Cellular Imaging, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Rome, Italy; [6]Medical Genetics, Cytogenetics and Pediatric Cardiology, Bambino Gesù Children Hospital, IRCCS, Rome, Italy; [7]Unit of Biostatistics, Casa Sollievo della Sofferenza Hospital, IRCCS, San Giovanni Rotondo, Italy; [8]Pediatric Cardiology, Department of Pediatrics, 'Sapienza' University, Rome, Italy; [9]Eleonora Lorillard Spencer-Cenci Foundation, Rome, Italy; [10]Institute Biology Leiden, Leiden University, Leiden, The Netherlands
[11]These authors contributed equally to this work.
[12]Current address: Northern Genetic Service, Institute of Human Genetics, Newcastle University, International Center for Life, Newcastle upon Tyne, UK
*Correspondence: Dr A De Luca, Istituto CSS-Mendel, Viale Regina Margherita 261, Rome 00198, Italy. Tel: +39 06 44160510; Fax: +39 06 44160548; E-mail: a.deluca@css-mendel.it
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TOF with pulmonary atresia. All patients were sporadic with the exception of four familial cases with TOF with classic anatomy. All patients underwent clinical assessment, electrocardiogram, echocardiogram, and chest-X-ray studies. Diagnosis of TOF was obtained by echocardiogram in all children. All patients showed viscero-atrial situs solitus, d-ventricular loop, and normally related great arteries. Patients showed neither major nor minor extracardiac features of DiGeorge syndrome/velo-cardio-facial-syndrome, neither deletion of 22q11 and 10p13 DiGeorge syndrome regions, which were excluded using standard molecular protocols. Moreover, these patients had been previously analyzed for mutations in the *GATA4*, *NKX2.5*, *ZFPM2/FOG2*, *GDF1*, *ISLET1,* and *JAG1* genes by denaturing high performance liquid chromatography (DHPLC).[8,12] The study cohort also included 784 unrelated normal control individuals obtained as ethnically matched anonymous samples from the Mendel Institute (Italians of Caucasian origin). Ethical approval for this study was obtained from the Ethics Committees of the participating institutions, and informed consent for the genetic analyses was obtained from all patients or their legal guardians.

Molecular studies

To search for mutations in the *GJA5* gene, the entire coding region of the gene, together with exon–intron boundaries, was PCR amplified from genomic DNA. Primers were designed based on the cDNA in GenBank (NM_005266.5) and the corresponding genomic regions. PCR products were screened by DHPLC by use of the Wave 3500 HT System (Transgenomic, Omaha, NE, USA) at column temperatures recommended by Navigator software, version 1.6.4.12 (Transgenomic). Amplimers with abnormal elution profiles were purified (Microcon PCR (Millipore, Billerica, MA, USA)) and then sequenced bidirectionally using the ABI BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3130xl Genetic Analyzers (Applied Biosystems). Primer pair sequences, as well as PCR and DHPLC analysis settings, are available upon request. The HomoloGene tool of NCBI (http://www.ncbi.nlm.nih.gov/nuccore?Db=homologene&Cmd=Retrieve&list_uids=3856) was used to analyze the level of conservation of sequence variants in orthologous genes. Numbering for the mutation started at the adenine nucleotide (A) in the ATG initiation codon. Genotyping of *GJA5* c.793C>T variant in healthy controls was performed on genomic DNA using a custom TaqMan genotyping assay (Forward primer 5′-CCCCTCTGTGGGCAT AGTC-3′; reverse primer 5′-GCCATTCTCCAGGCACTGATTA-3′; Applied Biosystems), according to manufacturer's protocol on a 7900HT real-time PCR platform (Applied Biosystems). The reactions were cycled with standard TaqMan conditions (2 min at 50°C, 10 min at 95°C, 40 cycles with 15 s at 95°C, and annealing/extension at 60°C for 1 min). The genotypes were called with the SDS 2.2.2 software package (Applied Biosystems).

Statistical analyses

The occurrence of a mutation in a subject was assumed to follow a binomial distribution, for which the power to detect at least one mutation was equal to one minus the probability to observe zero events. Study power for different scenarios according to a set of hypothesized true mutation prevalence was provided (ie, from 2 to 10 per 1000 subjects). According to these scenarios a sample size of 784 subjects has a power >99% to detect at least one mutation with a type I error (α) equal to 0.05, assuming a mutation prevalence of 1% (Supplementary Figure S1).

The prevalence difference of mutation between patients and controls was tested using the Fisher's exact test. A *P*-value <0.05 was considered as statistically significant.

The Fisher's exact test was used to compare the frequency distributions for the morphology of the heart in zebrafish experiments. The significant threshold was set at $P=0.05$.

Plasmids constructs

The single-base change resulting in the p.Pro265Ser amino-acid substitution was introduced into a full-length cDNA of the wild-type human *GJA5* cDNA cloned into a p-yellow fluorescence protein (YEFP)-N1 tagged plasmid, kindly provided by MH Gollob (Departement of Medicine, University of Ottawa Heart Institute, Ottawa, Canada), by site-directed mutagenesis with the use of the QuickChange Site II-direct mutagenesis kit (Stratagene, La Jolla, CA, USA). Both wild-type and mutated human *GJA5* cDNAs were subsequently subcloned at the C-terminus of pCDNA6/V5HIS plasmid by using primers Cx40 forward (5′-CACACAGGATTCATGGGCGATTGGAGCTTCCTG-3′) and Cx40 reverse (5′-CACACACTCGAGCACTGATAGGTCATCTGACCT-3′), including restriction enzyme site for cloning.

Localization studies

For indirect immunofluorescent staining, murine neuroblastoma (N2A) cells, a gap-junction-deficient cell line, were transfected with *GJA5*-pYEFP-N1 wild-type or mutated plasmid using lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), as indicated by the manufacturer protocol. Cells were blocked and fixed in 4% paraformaldeide at 24–48 h after transfection. Nucleus was highlighted with Hoechst and the cells were examined using a fluorescence microscope (PCM Ecplise TE300, Nikon Instruments, Tokyo, Japan).

For confocal laser scanning microscopy, 3×10^3 N2A cells were seeded on glass coverslips, and transiently transfected with either wild-type or mutated *GJA5*- pCDNA6/V5HIS plasmid, as described below. Cells were fixed after 24–48 h with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with mouse monoclonal anti-V5 antibody (Life Technologies, Paisley, UK), followed by green-fluorescent Alexa Fluor-488 goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA); actin cytoskeleton was detected by red-fluorescent Alexa Fluor-594 phalloidin; (Molecular Probes, Eugene, OR). The extensively rinsed cover glass was then mounted on the microscope slide with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Imaging was performed on a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems, Mannheim, Germany), using excitation spectral laser lines at 405, 488, and 594 nm, tuned with an acousto-optical tunable filter. Signals from different fluorescent probes were taken in sequential scanning mode.

Dye transfer studies

Dye transfer through gap junction channels was investigated using transiently transfected N2A cells; experiments were performed 24 h after transfection with wild-type or mutated constructs. We patch-clamped only adjacent cells with YEFP-positive gap junctions localized at the cell membrane level in both experimental groups. Cells plated on glass coverslips were superfused at room temperature with Tyrode solution containing (mM): 154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-NaOH, 5.5 D-glucose, adjusted to pH 7.35. Lucifer Yellow (LY) CH (2 mM) (Molecular Probes, Eugene, OR, USA) was dissolved in the pipette solution containing (mM): 110 K+-aspartate, 23 KCl, 0.4 $CaCl_2$ (calculated free $Ca_2+=10$–7 M), 3 $MgCl_2$, 5 HEPES KOH, 1 EDTA KOH, 5 ATP-Na salt, pH 7.3. Large patch electrodes (about 1.5 MΩ) were used to facilitate the diffusion of LY in the cytosol of the patched cell after achievement of the whole-cell configuration, detected by monitoring membrane capacitance (Multiclamp 700B, Axon Instruments, Inverurie, Scotland). Measurements were performed with a Nikon TE200 inverted microscope at ×63 magnification (Nikon Instruments). Cells were excited with a xenon lamp (100 W) through suitable bandpass filters and a gray filter to limit dye bleaching. Cell images were acquired once in bright field mode and every minute in fluorescence mode by a MOTIC 2300 camera. Image acquisition and analysis were respectively controlled and performed by ImageJ software (National Institutes of Health, Bethesda, MD, USA); exposure time and gain factor were maintained constant throughout all measurements. Dye permeation through gap-junction channels was investigated using N2A cell pairs. Fluorescence in the injected and recipient cell was monitored at 1 min intervals for 15 min after patch rupture; cell autofluorescence was eliminated by digital subtraction of images acquired before dye dialysis (cell attached configuration). The time course of dye transfer to injected cell and, secondarily, to the recipient one was obtained by measuring fluorescence changes over time. The fluorescence at each time point was expressed as percent of the fluorescence measured at 15 min from the injected cell. Injected cell fluorescence at 15 min, taken as a reference, was assumed to result from equilibration with pipette solution and therefore to represent a LY concentration of 2 mM. LY concentration at each time-point was calculated from the fluorescence normalized to the reference

one. The number of LY molecules (MN_{LY}) corresponding to each LY concentration is: $MN_{LY} = Vol \times C \times N$, where Vol indicates cell volume (assumed 0.75 pL), C is LY concentration and N is Avogadro's number. LY flow rate is expressed as the change over time of MN_{LY} present in the recipient cell. Means were compared by two-way ANOVA. Statistical significance was defined as $P<0.05$. Data are expressed as mean ± SE of independent determinations.

Zebrafish and *in situ* hybridization experiments

Zebrafish were kept and the embryos were staged as described before.[24] All procedures involving experimental animals were performed in compliance with local animal welfare laws, guidelines, and policies. *In situ* hybridizations were done essentially as described[25] using probe specific for myocardial marker cardiac myosin light chain 2.[26] Embryos were cleared in methanol and mounted in 100% glycerol before pictures were taken. Phenylthiourea was added to suppress pigmentation in developing embryos at 20 hpf.

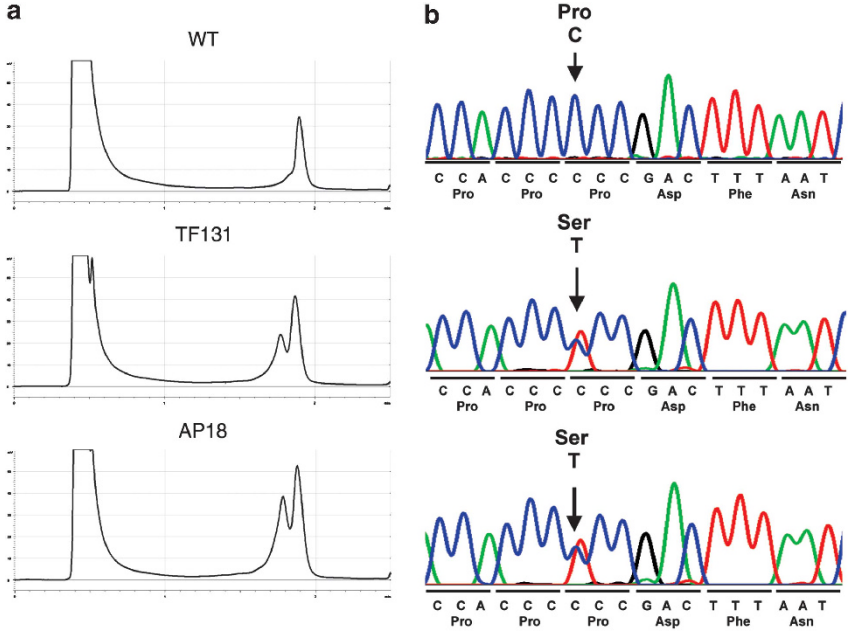
RNA and injections

pCS2+ −*Gja5* constructs were linearized with *Not*I enzyme and capped mRNA prepared with the Message Machine kit (Ambion, Austin, TX, USA). mRNA was diluted in nuclease-free water, and 1 nl per embryo was injected at the 1- to 2-cell stage.

RESULTS

Mutation analysis

One hundred and seventy-eight unrelated TOF probands (either sporadic ($n=174$) or familial ($n=4$)) with isolated TOF with ($n=31$) or without ($n=147$) pulmonary atresia were screened for mutations in *GJA5* gene by DHPLC. Amplicons bearing heteroduplexes were then analyzed by DNA sequencing. A heterozygous nucleotide change (c.793C>T) in exon 2 of the gene leading to the p.Pro265Ser variant was found in two unrelated sporadic patients with TOF, one with classic anatomy (TF131) and one with pulmonary atresia (AP18) (Figures 1a and b). None of them disclosed any conduction defect at electrocardiogram. The p.Pro265Ser substitution was found to alter an amino-acid residue that was highly conserved across multiple species, including chimpanzee, dog, cows, mouse, rat, chicken, and zebrafish (GenBank accession numbers: XP_001156907.1, NP_001017442.1, NP_001071490.1, NP_032147.1, NP_062153.1, NP_990835.1, NP_001007214.1, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Figure 1c). Family members were unable to be contacted to verify if p.Pro265Ser had *de novo* occurrence or was transmitted by parents. The p.Pro265Ser missense change was not observed in 784 unaffected, unrelated, ethnically matched control subjects (Italians of Caucasian origin) (1568 control chromosomes), a sample with a power >99% to detect a variant with 1% prevalence such as p.Pro265Ser substitution. Significantly, the prevalence of this substitution in patients was statistically different from that found in controls (2/178 (1.12) *versus* 0/784 (0.00), $P=0.03$). In addition to p.Pro265Ser variant, mutation analysis disclosed four previously reported *GJA5* polymorphisms (c.-188C>T (rs1775479); c.-185A>G (rs1692140); c.531C>T, p.Tyr123Tyr (rs2232191); c.*53A>G (rs1692141)).



c

Homo	240	--KPRQHMAKCQL-SGP-SVGIVQSCTPP**P**DFNQCLEN	273
Pan	240	--KPRQHMAKCQL-SGP-SVGIVQSCTPP**P**DFNQCLEN	273
Canis	240	--KSGQGMAECQL-PGP-SAGIVQNCTPP**P**DFNQCLEN	273
Bos	241	--KSQPGVGECQL-PGP-SAGRVQSCTPP**P**DFNQCLEN	274
Mus	240	--KSRQGVDKHQL-PGP-PTSLVQSLTPP**P**DFNQCLKN	273
Rattus	239	--KSRQG-DKHQL-LGP-STSLVQGLTPP**P**DFNQCLKN	271
Gallus	246	AYKPSPSTAPRRLESAP-QVERAQMYTPP**P**DFNQCL-A	281
Danio	238	ASKQRPNTPSTMPALSPNPSTPNRACTPP**P**DFNQCLTS	275

Figure 1 DHPLC elution profiles (**a**) and sequence electropherograms (**b**) of the novel *GJA5* variant p.Pro265Ser. The position of the mutated nucleotide is indicated by an arrow. (**c**) Phylogenetic conservation analysis of *GJA5* p.Pro265Ser variant. The mutant amino-acid residue 265 is highlighted in yellow.

Protein localization studies

To determine if the p.Pro265Ser variant had an effect on protein localization, N2A cells deficient in gap junctional communication were transiently transfected with either the wild-type or the p.Pro265Ser mutant constructs directly 'tagged' with YEFP at the carboxyl-terminus (CT) end of the protein and visualized using both indirect immunofluorescent staining and confocal microscopy. Following 24 h incubation, cells expressing wild-type Cx40 showed cytoplasmic punctate staining and aggregation at the plasma membrane of large gap-junction plaques in the region of cell–cell contact (Figure 2a). A different cellular distribution was observed for the p.Pro265Ser variant. Cells expressing this variant formed sparse or no visible gap-junction plaques and had intracellular retention of Cx40 within a wide array of punctuate structures (Figure 2b). Similar staining patterns were obtained using confocal microscopy (Figures 2c and d).

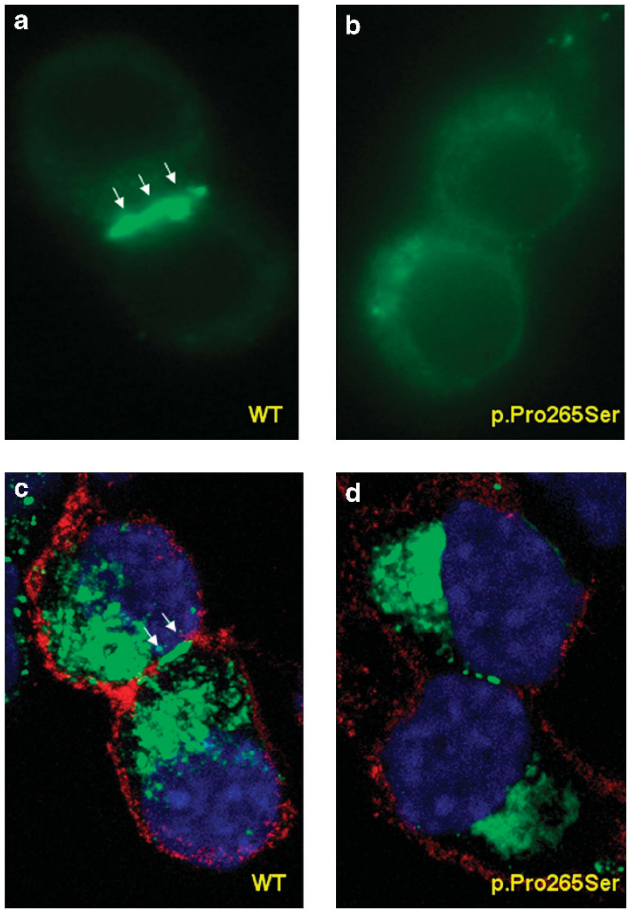


Figure 2 Differential localization of the wild-type and *GJA5* p.Pro265Ser mutant proteins in N2A cells. N2A cells were transfected with plasmids encoding either wild-type *GJA5* or the *GJA5* mutant p.Pro265Ser, tagged with YEFP at the CT. In cells expressing YEFP-tagged *GJA5* wild-type gap-junction-like structures were readily seen at cell–cell interfaces (white arrows) (**a**). However, cells expressing YEFP-tagged p.Pro265Ser mutant failed to assemble gap junctions or formed sparse gap junctions (**b**). Confocal microscopic analysis of the wild-type and *GJA5*- p.Pro265Ser mutant proteins in N2A cells. N2A cells expressing wild-type *GJA5* formed large gap-junction plaques between adjacent cells (**c**). In contrast, N2A cells transfected with the p.Pro265Ser mutant showed formation of punctuate gap-junction-like structures at cell–cell interfaces (**d**).

Dye transfer studies

In order to explore whether functional intercellular channels are formed, in particular by the mutant proteins that traffic to the plasma membrane, the transfer of the gap junction permeant tracer LY between cells transfected either with the wild-type or mutant construct was investigated. Dye transfer efficiency was quantified by measuring the rate of dye transfer between adjoining cells. As shown in Figure 3a, LY dialyzed into a cell transfected with wild-type *GJA5* cDNAs diffused easily to the adjacent cell reaching equilibration in about 13–15 min. As LY does not diffuse through the N2A cell membrane, the intercellular diffusion of the dye suggests the presence of permeable gap junctions, in this case formed by Cx40. The rate of fluorescence increment in injected cells was similar between cells transfected with mutant and wild-type ($n = 14$ for both) constructs thus their results were pooled. On the other hand, the rate of fluorescence increment in the recipient cell was lower steep in the presence of mutated gap junctions ($P < 0.05$). For instance, at 13 min the relative fluorescence dye intensity was $18.9 \pm 3.4\%$ (corresponding to 2.9×10^5 dye molecules per second) and $9.2 \pm 2.7\%$ (corresponding to 1.3×10^5 dye molecules per second) with wild-type and mutated constructs, respectively, ($P < 0.05$, Figure 3b).

Zebrafish experiments

Previous data have shown that Cx40 has an important role in cardiomyocytes of the atria, cardiac conduction system, and endothelial cells of large arteries. It has already been demonstrated that Cx40 knockout mice exhibit cardiac electrophysiological and morphological phenotypes.[27] We used the zebrafish embryo model system to elucidate the role of mutant *GJA5* in heart patterning *in vivo*. To this end, we injected synthetic mRNA encoding mutant *GJA5*-p.Pro265Ser or as a control wild-type *GJA5* into zebrafish embryos at the 1- to 2-cell stage and assessed the morphology of the heart. Aberrant heart morphology was apparent in embryos-expressing mutant *GJA5* at 24 h post fertilization. *In situ* hybridization analysis for cardiac myosin light chain 2, a cardiac myosin marker, showed that the overall morphology of the heart tube was disrupted in the 37% (22/60) of *GJA5*- p.Pro265Ser-injected embryos, compared with the 6% (4/66) of *GJA5*-wild-type-injected embryos (Fisher's exact test, $P < 0.0001$) (Figure 4). Noteworthy, the mutant *GJA5*-injected embryos did not display gross developmental defects and had normal body size compared with wild-type *GJA5*-injected embryos. These data demonstrate that mutant *GJA5*-p.Pro265Ser has a dominant effect over endogenous wild-type *GJA5* and disrupts normal heart morphology.

DISCUSSION

Deficient or improper gap junction channel function caused by mutations in the genes encoding connexin proteins has been associated with a variety of diseases, such as peripheral neuropathy, oculo-dento-digital dysplasia, sensorineural deafness, skin disorders, and cataracts.[28] This is the first report of *GJA5* variant in TOF patients and in general in CHDs. Various *GJA5* defects other than p.Pro265Ser were previously associated with familial and sporadic atrial fibrillation.[21–23] Similarly, different mutations of cardiac transcription factor *NKX2.5* have been identified in kindreds with conduction abnormalities (atrioventricular block) and concurrent congenital heart malformations, primarily secundum atrial septal defect, as well as in patients with sporadic TOF.[4,15,29] In the present study, variant p.Pro265Ser was found in ~1% of TOF patients. The low incidence of p.Pro265Ser variant in the cohort we studied may be consistent with the high degree of heterogeneity of this CHD.

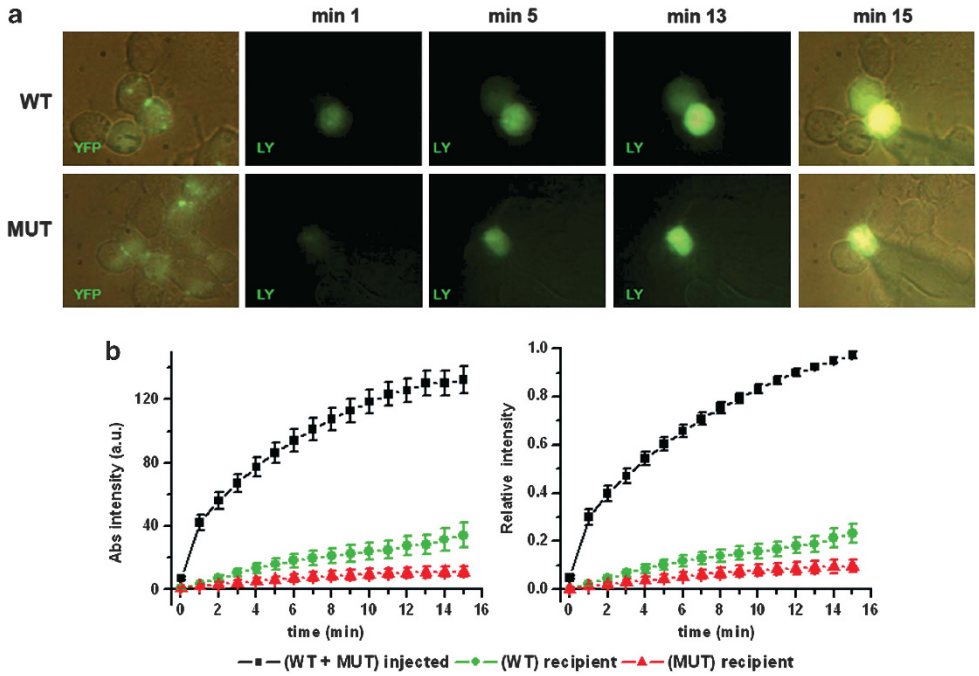


Figure 3 Dye transfer (LY) through gap-junction channels in N2A cell pairs transfected with either wild-type (WT), or mutated (MUT) constructs. (**a**) Examples of the intercellular diffusion of the dye at different time points after patch rupture (time 0); the bright field image and the fluorescence one at 15 min after patch rupture are superimposed. (**b**) Average time course of absolute (left panel) and relative (right panel) fluorescence; in each panel the upper curve refers to the injected cell (data from WT and MUT were pooled) and the lower ones to WT and MUT recipient cells, respectively.

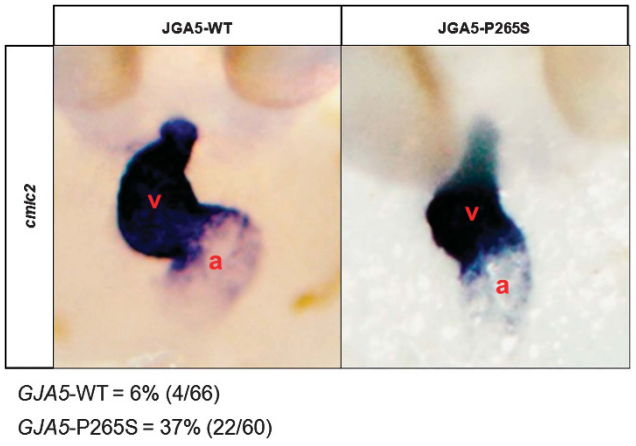


Figure 4 Expression of mutant *GJA5*-p.Pro265Ser disrupts zebrafish heart morphology in zebrafish *in vivo*. *In situ* hybridization staining for cardiac myosin (cardiac myosin light chain 2) in 48 hpf zebrafish embryos that were injected at the 1- to 2-cell stage with wild-type *GJA5* (left) or mutant *GJA5*-p.Pro265Ser (right). The overall morphology of the heart tube is disrupted in *GJA5*-p.Pro265Ser-injected embryos compared with *GJA5*-wild-type-injected embryos. Dorsal views are shown with anterior to the top. A, atrium; v, ventricle.

On analysis of 230 patients with both syndromic and non syndromic TOF, genetic abnormalities were identified in the 18% of patients.[30] The most common genetic alteration was 22q11.2 deletion associated with DiGeorge/VCFS syndrome. Single-gene mutations in *JAG1*, *NKX2.5*, and *TBX1* were found in 1.3%, 0.9%, and 0.4% of the cohort, respectively. In a parallel study, we found *JAG1*, *NKX2.5*, and *ZPFM2/FOG2* mutations in 2.7, 1.1, and 0.6% of sporadic patients with isolated TOF.[8,12] Clearly, TOF is a heterogeneous CHD, with small contributions from different loci. Present results further expand the genetic heterogeneity of this defect.

GJA5 gene mutated in TOF maps to the 1q21.1 chromosomal region. Microdeletion 1q21.1 (del 1q21.1) and the reciprocal microduplication 1q21.1 (dup 1q21.1) are newly recognized genomic disorders, characterized by developmental delay, neuropsychiatric abnormalities, dysmorphic features, and a variety of congenital malformations, including CHDs.[31–33] In addition, both del 1q21.1 and dup 1q21.1 have been found in patients with non-syndromic CHD.[18,34,35] A recent study of 512 patients with apparently isolated sporadic TOF using an array designed to detect deletions or duplications revealed that 5/512 individuals had a deletion or duplication of chromosome 1q21.1, encompassing *GJA5* gene.[18] A more recent study further defined the relationship between 1q21.1 rearrangements and isolated TOF showing that 1q21.1 duplications confer an odds ratio for TOF of ~30.[19] Furthermore, within the 1q21.1 region, the same authors identified three smaller overlapping duplications in three patients with TOF in which *GJA5* gene was the only gene in common. Moreover, they found a *GJA5* triplication in one patient with pulmonary atresia.[19] Similarly, we identified *GJA5* p.Pro265Ser variant in one patient with TOF and one with TOF and pulmonary atresia, which is considered the extreme end of the anatomic spectrum of TOF and is a cardiac defect with a very high genetic involvement.[36,37] Taken together, present and previous findings strongly suggest that *GJA5* is the key gene in the etiology of cardiovascular diseases found in patients with 1q21.1 chromosome imbalances.

In agreement with a pathogenic role of Cx40 mutations in CHDs, previous studies on animal models have demonstrated that this gene has a major role in the cardiac conduction system and in heart morphogenesis. *Cx40* gene is strongly expressed in both atria and ventricles in early stages of development.[38] Kirchhoff *et al*[39] have

shown that Cx40-deficient mice may have cardiac hypertrophy in conjunction with common atrioventricular junction or a ventricular septal defect. In a subsequent study, Gu *et al*[20] have shown that both Cx40 heterozygous and homozygous-null mice exhibit complex cardiac malformations, such as endocardial cushion defects and conotruncal malformations. In particular, over one third of the heart from homozygous-null mice showed either DORV or TOF. Consistent with results obtained in mice, we demonstrated that expression of *Cx40*-p.Pro265Ser mutant in zebrafish disrupts the formation of heart tube in over one third of the cases. Taken together, present and previous results provide reliable evidence that Cx40 participates in early cardiac morphogenesis and its absence, altered expression, or abnormal functioning increases the probability of cardiac malformations.

Present data have shown that overexpression of *Cx40*-p.Pro265Ser mutant in N2A cells, which are gap junction- and connexin-deficient, reduces dye transfer through gap junctions. Previous studies in mouse aortic endothelium deficient in Cx40 showed a similar reduction in dye transfer rate compared with wild-type aortas. On the basis of protein localization results, we hypothesize that reduced intercellular communication in cells expressing p.Pro265Ser variant may be the consequence of a reduction in membrane associated Cx40, and the fact that we do see some punctuate staining and even plaque formation in these cells might explain residual dye transfer capacity of mutant-expressing cells. Perhaps, *Cx40*-p.Pro265Ser variant is a hypomorphic allele with reduced level of protein expression, or more likely, an allele acting in a dominant-negative fashion, as this latter hypothesis would better explain the dominant effect over the endogenous wild-type protein observed in zebrafish embryos micro-injected with the mutant transcript. It might be possible that the mutant protein forms nonfunctional chimeric connexons with wild-type Cx40, which are not properly inserted into the cell membrane.

The *GJA5*-p.Pro265Ser variant identified here affects a highly conserved proline located in the CT-domain. Connexins-CT usually function as vital regulatory elements of channel gating, and may have influences on the rates of connexin trafficking and the synthesis, assembly, and degradation of gap-junction channels. Nuclear magnetic resonance (NMR) data have shown that the Cx40CT domain is intrinsically disordered in structure.[40] In general, intrinsically disordered proteins have been identified as having important roles in molecular recognition, molecular assembly, protein modifications, and entropic chains.[41] In the case of membrane proteins, like Cx40, intrinsically disordered domains seem to have an important role in cell signaling events, acting as components of macromolecular processes and substrates for regulatory events such as phosphorylation.[42] In this regard it is intriguing that phylogenetically conserved proline265 is contained within a stretch of 11 amino acids (VQGLTPPPDFN) that is highly affected upon binding of the c-Src SH3 domain. Therefore, it might be that an SH3-binding site is lost as a result of the p.Pro265Ser mutation, leading to dysregulation of Cx40. c-Src is a plasma membrane-associated tyrosine kinase, and c-Src induced phosphorylation of Cx43 has been correlated with an inhibition of gap junction intercellular communication. It was also shown that Src activation may lead to phosphorylation of tyrosine residues as well as indirectly serine phosphorylation (perhaps MAPK-mediated),[43] Moreover, it was seen that tyrosine phosphorylation by Src occur through binding to proline-rich-residues.[44] The Cx40CT sequence does not contain a consensus PXXP SH3-binding motif, but contains two peptides rich in prolines and/or positively charged residues.[45] One of these proline-rich domain is the Cx40CT domain affected by SH3 corresponding to the peptide between Val258 to Asn268, and containing Pro265 residue mutated in our TOF patients. NMR analyses have also shown that binding by Src may have an important role in regulating interactions at the CT with other molecular partners of Cx40, in example zonula occludens-1 (ZO-1).[46,47] This might be relevant as interaction with ZO-1 was found to influence the formation[47] and size of the Cx43 gap-junction plaque,[48] as well as the internalization and remodeling of Cx43 upon changes in the intracellular environment.[46,49]

Future studies should be designed to investigate the role of p.Pro265Ser in regulating interactions of Cx40-CT with its molecular partners and biological consequences.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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