Original Paper

Cardiomyopathy-Associated Gene 1-Sensitive PKC-Dependent Connexin 43 Expression and Phosphorylation in Left Ventricular Noncompaction Cardiomyopathy

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Key Words

Left ventricular noncompaction cardiomyopathy (LVNC) • Cardiomyopathy-associated gene 1 (CMYA1) • Connexin 43 (Cx43) • PKC signaling pathway

Abstract

Background/Aims: Cardiomyopathy-associated gene 1 (CMYA1) plays an important role in embryonic cardiac development, postnatal cardiac remodeling and myocardial injury repair. Abnormal CMYA1 expression may be involved in cardiac dysplasia and primary cardiomyopathy. Our study aims to establish the relationship between CMYA1 and Left ventricular noncompaction cardiomyopathy (LVNC) pathogenesis. **Methods:** We explored the effects of CMYA1 on connexins (Cx), which contribute to gap junction intercellular communication (GJIC), and the underlying signaling pathway in human normal tissues, LVNC myocardial tissues and HL1 cells by means of western blotting, RT-qPCR, immunohistochemistry, immunofluorescence, co-immunoprecipitation and scrape loading-dye transfer. **Results:** CMYA1 expression was inversely associated with Cx43 and Cx40 expression, as determined by gap junction PCR array analysis. An increased expression and disordered distribution of CMYA1 at the intercalated discs in LVNC myocardial tissue was also observed. CMYA1 and Cx43 are co-expressed and interact in myocardial cells. CMYA1 expression was positively correlated with p-Cx43 (S368) via the Protein kinase C (PKC) signaling pathway in myocardial tissue and HL1 cells. The diffusion distance of Lucifer Yellow in the HL1 cells in which CMYA1 was over-expressed or knocked down was significantly less or more than that of the control group, respectively. **Conclusion:** Abnormal CMYA1 expression affects the expression and phosphorylation of Cx43 through the PKC signaling pathway, which is involved in the regulation of GJIC. CMYA1 participates in the molecular mechanism of LVNC pathogenesis.

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Published by S. Karger AG, Basel

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Introduction

Left ventricular noncompaction cardiomyopathy (LVNC) is a primary hereditary cardiomyopathy potentially associated with abnormal embryonic development of the heart [1] characterized by numerous prominent trabeculations and deep intertrabecular recesses in the left ventricle. The most common clinical presentations of LVNC are congestive heart failure, cardiac arrhythmia and thromboembolism [2]. Mutations in sarcomere protein, Z-disc, cytoskeletal and mitochondrial genes have been found in LVNC cases [3-8], which only explains a small portion of the causes of LVNC. Therefore, novel pathogenic LVNC genes must be identified and verified.

CMYA1, also named XIRP1, is a member of the cardiomyopathy-associated gene family (CMYA) which was originally discovered by Chinese scholars analyzing differentially expressed genes in chick embryonic heart development [9]. CMYA1 is located on chromosome 3p22.2 and encodes a protein called Xin, which means "heart" in Chinese [10]. Incubation of chick embryos with cCMYA1 antisense oligonucleotides results in abnormal cardiac morphogenesis and altered cardiac looping. The myocardial tissue of affected hearts becomes thickened and tends to form multiple invaginations into the heart cavity. cCMYA1 plays a key role in cardiac morphogenesis and development [11]. CMYA1 knockout during mouse embryonic development demonstrated that CMYA1 also plays a role in mammalian myocardial wall development and morphogenesis [12]. CMYA1 has been shown to be highly expressed in intercalated discs (ICDs) in mouse and pig hearts [12-15], suggesting its potential association with the cyclization process of cardiac development and myocardial contractility [10]. Novel mutations in the conserved region of the CMYA1 gene in patients with LVNC have recently been identified (not published in our preliminary work), and CMYA1 mutations reportedly may lead to the mislocalization of CMYA1 and other ICD components to the ICDs and, consequently, the noncompaction phenotype [16].

ICDs are part of the cardiac muscle sarcolemma, as they contain gap junctions (GJs) and desmosomes [17]. GJs are important determinants of cardiac conduction, and evidence has recently emerged that altered distribution of these junctions and altered expression of their constituent connexins (Cx) may lead to abnormal coupling between cardiomyocytes, likely contributing to myocardial diseases [18]. Twenty-one connexins have been identified in humans. Cx43 is the major subtype of ventricular myocardium [19]. Disorganization of GJ distribution and down-regulation of Cx43 are typical features of myocardial remodeling and may play an important role in the development of arrhythmogenic substrates in human cardiomyopathies [18].

The purpose of this study is to clarify the effects of CMYA1 on the gap junction intercellular communication (GJIC) of cardiomyocytes and the underlying signaling pathway.

Materials and Methods

Cell culture and treatment

HL1 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (GIBCO) at 37°C and 5% CO₂ in a humidified incubator, and the medium was changed every two days. Cells were serum-starved for 24 h once they reached 70% confluency. They were then either infected with lentiviral particles or transfected with shRNA-CMYA1 plasmids. After treatment with the lentiviral particles or plasmids for 24, 48, or 72 h, the HL1 cells were harvested for the experiments (co-immunoprecipitation, western blotting, RT-qPCR, immunocytochemistry, and scrape loading-dye transfer). The HL1 cells were cultured in serum-free medium for 12 h before being exposed to the protein kinase C (PKC) inhibitor GF109203X or the PKC activator phorbol 12-myristate 13 acetate (PMA).

Tissue samples

Myocardial samples were taken from the LVs of 12 terminal cardiomyopathy patients exhibiting abnormal trabeculation morphology from our heart transplantation program. Non-cardiomyopathy

control hearts were harvested from donors with no history of heart disease who died in accidents. All the participants provided written informed consent for this investigation, which was approved by the Institutional Ethical Review Board of Fuwai Hospital. The investigation also conforms to the principles outlined in the Declaration of Helsinki.

Western blot analysis

Total proteins from the tissues and HL1 cells were analyzed as previously described [20]. Briefly, the frozen tissues and cultured cells were lysed and centrifuged at 15,000 g at 4°C for 10 min. Then, the supernatants were collected and assayed by the BCA protein assay method. Subsequently, the protein samples were subjected to SDS-PAGE (4–12% gradient) and transferred to PVDF membranes. After being blocked with 5% skim milk and incubated with antibodies, the proteins were visualized by enhanced chemiluminescence (ECL) and detected using the Fluor Chem M Multi Fluor system (Cell Biosciences).

The following primary antibodies were used: mouse anti-CMYA1 (Santa Cruz Biotechnology), rabbit anti-Cx43 (Cell Signaling Technology), goat anti-Cx40 (Santa Cruz Biotechnology), rabbit anti-Phospho-Cx43 (Ser368) (Santa Cruz Biotechnology), and rabbit anti-GAPDH (Cell Signaling Technology). Horseradish peroxidase-linked secondary antibodies (Beyotime Biotechnology) were also used.

Real-time quantitative PCR analysis

Total RNA was extracted from the myocardial tissues and cultured cells using TRIzol reagent (Invitrogen) according to a previously published method [21]. Total RNA was subjected to reverse transcription using the Hieff First Strand cDNA Synthesis Kit (Yeast, Shanghai). Real-time quantitative PCR (RT-qPCR) experiments were performed using the 7500 Sequence Detection System (Applied Biosystems). The RT-qPCR procedure consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer sequences of the investigated genes were as follows: 5'-CAGCAGCATCTGTTTGGAGAC-3' (sense) and 5'-CCCTTTAGCTCATCCCTCTG-3' (antisense) for CMYA1; 5'-TCTGCTATGACAAGTCCTTCC-3' (sense) and 5'-GCTTCTCTTCTTCTCATCAC-3' (antisense) for Cx43; 5'-CATCTCCACATTCGCTACTG-3' (sense) and 5'-CAATCCTTCCATTCCTTCT-3' (antisense) for Cx40; 5'-GGTCGGAGTCAACGGATTG-3' (sense) and 5'-ATGAGCCCCAGCCTTCTCCAT-3' (antisense) for GAPDH.

Plasmid construction

The CMYA1 coding region (NM_001198621) was cloned into the pLent6v5 expression vector to obtain an expression construct with amino-terminal EGFP and FLAG tags. The shRNA-CMYA1 plasmid was constructed by inserting a short hairpin double-stranded oligonucleotide targeting CMYA1 (5'-CCGGAGTGCATGCGCTGGATCTTTGCTCGAGCAAAGATCCAGCGCATGCACTTTTTTTTG-3') into the pLKO.1 vector (Addgene). Human Cx43 DNA was amplified from genomic DNA and subcloned into the pDsRed1-N1 vector (with DsRed1 and Myc tags) (Bioworld Technology) [22]. The authenticity of all the sequences was verified by Sanger sequencing.

Gene expression profiling of gap junctions

A Qiagen Gap Junction PCR Array was used to profile the expression of 84 important gap junction-related genes. Total RNA was extracted using an RNeasy Mini kit. According to the manufacturer's instructions, RNA was digested using RNase-free DNase. RNA integrity was confirmed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis. Quantification of human gap junction gene expression levels was determined using an RT2 First Strand kit and RT2 SYBR Green/ROX qPCR Master Mix with a 7500 Sequence Detection System (Applied Biosystems). Target gene expression levels were calculated according to the manufacturer's protocol using the Qiagen RT2 Profiler PCR Array. The relative expression of genes was determined using the comparative cycle threshold ($\Delta\Delta Ct$) method.

Immunohistochemistry and immunofluorescence

HL1 cells were cultured on glass cover slips coated with poly-L-lysine. LV myocardial samples were fixed in 10% neutral buffered formalin. Dehydration was accomplished using alcohol and xylene gradients, followed by paraffin embedding. Sections (4 μ m) were fixed for 10 min in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 0.05% H_2O_2 . They were then incubated

with primary antibodies for 1 h at room temperature, washed with PBS for 10 min, incubated with an IgG-peroxidase conjugated secondary antibody for 1 h and washed with PBS for 10 min. All the slides were stained with hematoxylin, dehydrated, and mounted. The immunofluorescence procedure was similar to that described above, except that blocking was achieved with 5% BSA. Mounting and counterstaining were performed with DAPI. The immunohistochemistry images were acquired using a Leica DM750 microscope (magnification 630× in cells and 400× in tissues), and the immunofluorescence images were acquired using a Leica SP8 microscope (magnification 630×). The following antibodies were used: mouse anti-CMYA1 (Santa Cruz Biotechnology), rabbit anti-Cx43 (Cell Signaling Technology), goat anti-Cx40 (Santa Cruz Biotechnology), HRP-conjugated goat anti-rabbit (ZSGB-BIO), HRP-conjugated goat anti-mouse (ZSGB-BIO), Alexa 488-conjugated goat anti-mouse (Beyotime Biotechnology) and Alexa 647-conjugated goat anti-rabbit (Beyotime Biotechnology).

Co-immunoprecipitation

Protein G PLUS-Agarose (Santa Cruz Biotechnology) was washed with 1% bovine serum albumin (BSA) and 10% SDS in PBS. The agarose beads were then washed three times with PBS and 1% BSA to remove the SDS. The HL1 cell lysates were incubated with 2 µg of an anti-FLAG antibody or an anti-Myc antibody (Beyotime Biotechnology) overnight at 4°C, followed by the addition of protein G PLUS-Agarose (Santa Cruz) overnight at 4°C. The beads were washed three times with cold PBS. After 5% loading buffer (Beyotime Biotechnology) was added, the samples were boiled for 5 min. The precipitates were subjected to immunoblotting with an anti-FLAG antibody or an anti-Myc antibody (Beyotime Biotechnology).

GJIC bioassay

HL1 cells were cultured in 6-well plates and treated with either GF109203X or PMA. The cells were scratched with a scalpel when they reached 90% confluency and then incubated with a gap junction-permeable Lucifer yellow (1 mg/ml) dye solution for 5 min at 37°C. The cells were then washed three times with Hank's balanced salt solution (HBSS) and fixed in a 4% paraformaldehyde solution for 10 min. The samples were imaged using a Leica DMI-4000B microscope. In three independent experiments, five fields of view per cell line were imaged, and each field of view was assessed at three places along the scratch for a total of 45 individual measurements for each cell line. The Lucifer yellow dye travel distance was quantified using ImageJ software.

Statistical analysis

All data are presented as the means ± standard deviations. SPSS software 22.0 (IBM Analytics) was used for all statistical analyses. Differences between two groups were compared using ANOVA. In all the analyses, *p < 0.05 and **p < 0.01 were considered statistically significant.

Results

Analysis of the gap junction-related gene expression profiles in heart tissues and HL1 cells

Gene expression in gap junctions isolated from the myocardial samples and HL1 cells was examined using a PCR array employing

Table 1. List of genes down-regulated or up-regulated greater than 2-fold, as determined by PCR array analysis, in LVNC compared to expression in normal hearts

GenBank	Symbol	Description	Fold change
NM_000856	GUCY1A3	Guanylate cyclase 1, soluble, alpha 3	0.27
NM_000165	GJA1	Gap junction protein, alpha 1, 43 kDa	0.46
NM_005343	HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	11.08
NM_000621	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	7.57
NM_032704	TUBA1C	Tubulin, alpha 1c	6.49
NM_005417	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	6.01
NM_002737	PRKCA	Protein kinase C, alpha	3.37
NM_020040	TUBB7P	Tubulin, beta polypeptide 4, member Q, pseudogene	3.14
NM_002514	NOV	Nephroblastoma over-expressed gene	2.76
NM_002524	NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	2.67
NM_002739	PRKCG	Protein kinase C, gamma	2.14

84 genes relevant to gap junctions. We confined our analysis results to genes with a differential expression of at least 2-fold. We compared the gene expression levels of human gap junction genes of cardiomyopathic tissues and normal control heart tissues; the 9 up-regulated genes were HRAS, NRAS, HTR2A, NOV, PRKCA, PRKCG, SRC, TUBA1C and TUBB7P, and the 2 down-regulated genes were Cx43 and GUCY1A3 (Fig. 1A, Table 1). We identified the altered expression of 12 genes, of which 6 were transcriptionally down-regulated and 6 were up-regulated (Fig. 1B, Table 2), in HL1 cells that over-expressed CMYA1. The up-regulated genes included GJC2, GRB2, NOV, PRKG2, GUCY1A3 and PLCB2, and the down-regulated genes included GJA1, GJA5, GJC3, TJP1, PRKACA and ADRB2. Eleven genes were differentially expressed in HL1 cells in which CMYA1 was knocked down (Fig. 1C, Table 3). The up-regulated genes included GJA1 (Cx43), GJA5 (Cx40), GJA3, TJP1, GJA8, GJC2, GRB2, MAPK3, MAPK7 and PRKCG, whereas the only down-regulated gene was ADCY3. From the integrated analysis of two-group data, three genes (GJA1, GJA5, and TJP1) showed opposite regulation trends in CMYA1 up-regulated or CMYA1 down-regulated HL1 cells. The gene functions were annotated. Two genes, Cx43 and Cx40, were determined to be connexins associated with the gap junction, and TJP1 is a connexin-interacting protein.

Table 2. List of genes down-regulated or up-regulated greater than 2-fold, as determined by PCR array analysis, in plent6v5-CMYA1-transfected HL1 cells compared to expression in the control cells.

GenBank	Symbol	Description	Fold change
NM_175610	TJP1	Tight junction protein 1 (zona occludens 1)	0.19
NM_000165	GJA1	Gap junction protein, alpha 1, 43 kDa	0.24
NM_002730	PRKACA	Protein kinase, cAMP-dependent, catalytic, alpha	0.31
NM_181703	GJA5	Gap junction protein, alpha 5, 40 kDa	0.39
NM_020660	GJD2	Gap junction protein, delta 2, 36 kDa	0.39
NM_000024	ADRB2	Adrenergic, beta-2-, receptor, surface	0.46
NM_000857	GUCY1B3	Guanylate cyclase 1, soluble, beta 3	3.39
NM_000838	GRM1	Glutamate receptor, metabotropic 1	3.01
NM_006259	PRKG2	Protein kinase, cGMP-dependent, type II	2.85
NM_002514	NOV	Nephroblastoma over-expressed gene	2.67
NM_004573	PLCB2	Phospholipase C, beta 2	2.57
NM_020435	GJC2	Gap junction protein, gamma 2, 47 kDa	2.46

Table 3. List of genes down-regulated or up-regulated greater than 2-fold, as determined by PCR array analysis, in shRNA-CMYA1-transfected HL1 cells compared to expression in the control cells.

GenBank	Symbol	Description	Fold change
NM_004036	ADCY3	Adenylate cyclase 3	0.09
NM_005267	GJA8	Gap junction protein, alpha 8, 50 kDa	5.44
NM_002739	PRKCG	Protein kinase C, gamma	4.75
NM_021954	GJA3	Gap junction protein, alpha 3, 46 kDa	3.99
NM_002749	MAPK7	Mitogen-activated protein kinase 7	3.92
NM_080604	TJAP1	Tight junction associated protein 1 (peripheral)	3.34
NM_020435	GJC2	Gap junction protein, gamma 2, 47 kDa	3.31
NM_000165	GJA1	Gap junction protein, alpha 1, 43 kDa	3.22
NM_181703	GJA5	Gap junction protein, alpha 5, 40 kDa	3.03
NM_002746	MAPK3	Mitogen-activated protein kinase 3	2.92
NM_002086	GRB2	Growth factor receptor-bound protein 2	2.91

Fig. 1. Analysis of the gap junction PCR array. Scatter plot of PCR array analysis: 84 genes were observed to be up-regulated or down-regulated in the human hearts with LVNC (A) compared to expression in the normal hearts and in plent6v5-CMYA1-transfected (B) or shRNA-CMYA1-transfected (C) HL1 cells compared to expression in the control cells. The gray lines indicate the desired threshold of a 2.0-fold change in gene expression.

Fig. 2. Expression of CMYA1, Cx43 and Cx40 in human cardiac tissues. (A) Representative images of CMYA1, Cx43 and Cx40 protein expression in human hearts with LVNC and normal hearts, as determined by immunohistochemistry. The slide was counterstained with hematoxylin. Brown spots represent the CMYA1-, Cx43- and Cx40-positive cells that are localized at the ICDs of human hearts (arrows) (magnification 400×). (B) Western blot analysis showing increased protein levels of CMYA1 and reduced protein levels of Cx43 and Cx40 in the human hearts with LVNC. (C) Bar graphs showing the relative levels of the CMYA1, Cx43 and Cx40 proteins (n=3 for normal hearts and n=12 for LVNC hearts, ** p<0.01). (D) RT-qPCR data showing the increased mRNA levels of CMYA1 and reduced mRNA levels of Cx43 and Cx40 (n=3 for normal hearts and n=12 for LVNC hearts, ** p<0.01).

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Expression and localization of CMYA1, Cx43 and Cx40

To investigate the relationship between the CMYA1 protein and the identified connexins (Cx43 and Cx40), we performed immunocytochemistry, western blot analysis and RT-qPCR in the human cardiac tissues and HL1 cells. Cx43 and Cx40 were localized to the cytoplasm and membranes in the cardiac tissues and HL1 cells (Fig. 2A and Fig. 3A). Localization of the

Fig. 3. The effect of CMYA1 on Cx43 and Cx40 expression in HL1 cells. (A) Representative images of Cx43 and Cx40 protein expression in HL1 cells transfected with plent6v5-CMYA1 or shRNA-CMYA1, as determined by immunocytochemistry with an anti-Cx43 antibody and an anti-Cx40 antibody. The brown spots represent the Cx43 and Cx40 protein that localized to the cytoplasm and membrane of HL1 cells. The slides were counterstained with hematoxylin (blue-purple) (magnification 630×). (B) Western blotting was used to determine the expression of CMYA1, Cx43 and Cx40 in HL1 cells transfected with plent6v5-CMYA1 or shRNA-CMYA1 and control cells. (C) Bar graphs showing densitometric analysis of the 130 kDa CMYA1 protein, the 43 kDa Cx43 protein and the 40 kDa Cx40 protein normalized to the density of the 37 kDa GAPDH band from (B). The relative expression of each band = the density of each band/density of the GAPDH band (n=3 for each group, ** p<0.01). (D) RT-qPCR was used to determine the mRNA levels of all the above mentioned cells (n=3 for each group, * p<0.05, ** p<0.01).

calized to the cytoplasm and membrane of HL1 cells. The slides were counterstained with hematoxylin (blue-purple) (magnification 630×). (B) Western blotting was used to determine the expression of CMYA1, Cx43 and Cx40 in HL1 cells transfected with plent6v5-CMYA1 or shRNA-CMYA1 and control cells. (C) Bar graphs showing densitometric analysis of the 130 kDa CMYA1 protein, the 43 kDa Cx43 protein and the 40 kDa Cx40 protein normalized to the density of the 37 kDa GAPDH band from (B). The relative expression of each band = the density of each band/density of the GAPDH band (n=3 for each group, ** p<0.01). (D) RT-qPCR was used to determine the mRNA levels of all the above mentioned cells (n=3 for each group, * p<0.05, ** p<0.01).

CMYA1, Cx43 and Cx40 proteins in the human cardiac tissues is indicated by arrows. CMYA1, Cx43 and Cx40 protein expression was concentrated at the ICDs of normal cardiomyocytes and at the disordered ICDs of diseased cardiomyocytes (Fig. 2A). Western blot analysis confirmed the increased cardiac CMYA1 abundance and decreased cardiac connexin abundance in the left ventricular myocardial tissues of patients with LVNC compared with their abundance in normal control heart tissues. Cx43 protein expression was higher than that of Cx40 in the ventricular myocardium (Fig. 2B). The expression of Cx43 and Cx40 in the HL1 cells in which CMYA1 was over-expressed was consistent with the results in the left ventricular myocardial tissues of patients with LVNC. Compared with that in the control group, Cx43 and Cx40 expression was increased in the HL1 cells in which CMYA1 expression was low (Fig. 3B).

The interaction of CMYA1 with Cx43

Immunofluorescence revealed a strict colocalization of CMYA1 with Cx43 at the ICDs in the myocardial tissues (Fig. 4A). The ICD structures showed a disordered morphology in the human hearts with LVNC, suggesting that CMYA1 and Cx43 may have a functional relationship. To verify the protein interactions of CMYA1 and Cx43 in HL1 cells, we observed HL1 cells co-transfected with plent6v5-CMYA1 and pDsRed1-N1-Cx43 with a confocal laser

Fig. 4. Binding of

CMYA1 and Cx43.

(A) Colocalization of CMYA1 and Cx43 in human hearts. Confocal laser scanning microscope images of fluorescent double-immunohistochemical staining of CMYA1 (green) and Cx43 (red) proteins in human hearts with LVNC and normal hearts (magnification 400×). CMYA1 and Cx43 colocalized at the ICDs of human hearts (yellow). The nuclei were stained by DAPI (blue). (B, C) HL1 cells were co-transfected with plent6v5-CMYA1 (with EGFP and FLAG tag) and

pDsRed1-N1-CX43 (with DsRed1 and Myc tag) alone or in combination. (B) EGFP is visualized in green, DsRed1 is visualized in red, and colocalization at the membranes is visualized in yellow. The nuclei were stained with DAPI (blue) (magnification 630×). (C) Extracted proteins from all the above mentioned cells were untreated (input) or precipitated by an anti-FLAG or a Myc antibody and then separated by 4-12% SDS-PAGE. The transferred membrane was immunoblotted with an anti-FLAG or an Myc antibody. Co-immunoprecipitation (Co-IP) of the cell lysates showing CMYA1 binding with Cx43.

scanning microscope. The CMYA1 protein (green) colocalized with Cx43 (red) at the cell membranes, and the merged images are shown in yellow (Fig. 4B).

The co-immunoprecipitation assay was performed to assess the protein interactions between CMYA1 and Cx43. Two plasmids, plent6v5-CMYA1-FLAG and pDsRed1-N1-CX43-Myc, were co-transfected into HL1 cells (Fig. 4C, lane 3). The cell lysates were immunoprecipitated with anti-Myc (for Cx43), and the immunoprecipitants were revealed with an anti-FLAG antibody (for CMYA1) (Fig. 4C, line 2). Conversely, the cell lysates were immunoprecipitated with anti-FLAG (for CMYA1), and the immunoprecipitants were revealed with an anti-Myc antibody (for Cx43) (Fig. 4C, line 4). The input lane showed that both CMYA1 and Cx43 were expressed after cell transfection (Fig. 4C, line 5 and 6). These results indicate that CMYA1 co-immunoprecipitates with Cx43 in HL1 cells.

Function of GJIC

To investigate Cx43 expression and GJIC in HL1 cells in which CMYA1 was over-expressed or knocked down, scalpel loading-dye transfer (SL-DT) and immunofluorescence assays were used. Lucifer yellow (LY) and Cx43 were observed in fewer diffusely fluorescent HL1 cells in which CMYA1 was over-expressed and in more diffusely fluorescent HL1 cells in which CMYA1 was knocked down (Fig. 5). At the same time, we also tested the phosphorylation level of Cx43 in the myocardial tissues and HL1 cells and determined the ratio between

Fig. 5. The effect of CMYA1 on GJIC in HL1 cells. (A) HL1 cells were transfected with plent6v5-CMYA1 or shRNA-CMYA1 and control vectors, followed by Lucifer yellow (LY) scrape loading-dye transfer assays and fluorescent immunohistochemical staining of Cx43 (magnification 200×). Scalpel cuts through the cell monolayers, leading to cell wounding and dye uptake, are depicted by dashed lines. (B) Quantitative analyses of the diffusion distance of Lucifer Yellow and the fluorescence intensity of Cx43, representative of the GJIC efficiency and the Cx43 expression level (n=3 for each group, * p<0.05, ** p<0.01).

phosphorylated Cx43 and total Cx43 using immunoblots. The phosphorylation levels of Cx43 were significantly increased in the left ventricular myocardial tissues of patients with LVNC and in the HL1 cells in which CMYA1 was over-expressed (Fig. 6A, 6C). The phosphorylation levels of Cx43 were significantly decreased in the HL1 cells in which CMYA1 was knocked down (Fig. 6C).

PKC signaling pathway activation is responsible for Cx43 phosphorylation

When treated with GF109203X, HL1 cells showed an increased diffusion distance of Lucifer Yellow, enhancing the permeability of the GJ channel. When treated with PMA, HL1 cells showed a reduced diffusion distance of Lucifer Yellow, weakening the permeability of the GJ channel (Fig. 7). These results indicate that gap junction function is related to Cx43 phosphorylation. The expression levels of PKCα and P-Cx43 (S368) were significantly increased, and the expression level of Cx43 was significantly decreased in HL1 cells in which CMYA1 was over-expressed (Fig. 8). These results indicate that abnormal CMYA1 expression affects the expression and phosphorylation of Cx43 via the PKC signaling pathway, which is involved in the regulation of GJIC.

Discussion

To clarify the relationship between CMYA1 and LVNC pathogenesis, we studied the function of CMYA1 in human myocardial tissues and HL1 cells. In this study, we explored the expression and localization of CMYA1 and gap junction proteins, the interaction of CMYA1 with Cx43 and the effect of CMYA1 on the phosphorylation of Cx43 and the function of GJIC in cardiomyocytes.

Fig. 6. The effect of CMYA1 on p-Cx43 (S368). (A) Western blot showing the increased phosphorylation levels of Cx43 (S368) in the normal human hearts and human hearts with LVNC. (B) Bar graphs showing the ratio of p-Cx43 (S368)/Cx43 in the human hearts with LVNC (n=3 for normal hearts and n=12 for LVNC hearts, **p<0.01). (C) Western blot analysis of p-Cx43 (S368) in HL1 cells in which CMYA1 was over-expressed (HL1/plent6v5-CMYA1) or knocked down (HL1/shRNA-CMYA1) and control cells. GAPDH served as the quantity control. (D) Bar graphs showing the ratio of p-Cx43(S368)/Cx43 (n=3 for each group, **p<0.01).

Fig. 7. Modulation of GJIC by CMYA1 through the PKC signaling pathway in HL1 cells. (A) HL1 cells in which CMYA1 was over-expressed or knocked down and control cells were cultured with treatment with 30 nM GF109203X for 48 h, 100 nM PMA for 0.5 h, or without any treatment (magnification 200×). Scalpel cuts through the cell monolayers leading to cell wounding and dye uptake are depicted by dashed lines. Inter-cellular communication was determined by measuring the diffusion distance of Lucifer Yellow. (B) Quantitative analyses of the diffusion distance of Lucifer Yellow, representative of GJIC efficiency (n=3 for each group, * p<0.05, ** p<0.01).

Fig. 8. The effect of activation and inhibition of the PKC signaling pathway on p-Cx43 (S368) in HL1 cells. (A) Western blotting was used to determine the expression of CMYA1, PKC α , Cx43 and the phosphorylation of Cx43 (S368) in HL1 cells in which CMYA1 was over-expressed or knocked down and control cells with or without treatment. (B) Bar graphs showing the densitometric analysis of CMYA1, PKC α , Cx43 and p-Cx43 (S368) (n=3 for each group, **p<0.01).

Fig. 9. Scheme of CMYA1 participating in LVNC pathogenesis via the PKC signaling pathway. CMYA1 over-expression affects the expression and phosphorylation of Cx43 via the PKC signaling pathway, which is involved in the regulation of GJIC. CMYA1 participates in the molecular mechanism of LVNC pathogenesis.

In this study, we monitored the differential expression of a panel of 84 genes known to be associated with gap junctions. According to the distribution and function of the genes, two genes associated with cardiomyopathy were screened out, Cx43 and Cx40. We demonstrated that abnormal CMYA1 expression can cause abnormal Cx40 and Cx43 expression at the mRNA and protein levels in HL1 cells. We also observed the increased expression and disordered distribution of CMYA1 at the ICDs and decreased expression of Cx40 and Cx43 in the LVNC myocardial tissues, potentially revealing the key role of CMYA1 in the association between connexin expression and LVNC pathogenesis. CMYA1 over-expression can lead to decreased Cx43 expression and influence its transport, localization and structure, which may result in decreased GJIC in HL1 cells. This results in adjacent cells being unable to communicate information or regulate cell growth and proliferation, leading to cell lesions [23]. We found that CMYA1 and Cx43 are co-expressed in myocardial cells, suggesting that CMYA1 may be involved in the functional regulation of gap junctions. Cx43 is the most important connexin in ventricular myocytes [24], and it is synthesized on the reticulum and transferred to the Golgi apparatus, finally aggregating to form GJs on cell membranes [25]. In a study on Cx43 gene-deficient mice, when the expression of ventricular Cx43 was reduced by 50%, the conduction velocity of the ventricle was reduced by 38%, indicating that Cx43 plays a decisive role in the conduction velocity of the ventricle [26]. Changes in the structure and function of gap junctions can cause arrhythmia [27-30]. The incidence of arrhythmias in

patients with LVNC is very high. The common types of arrhythmias in patients with LVNC are ventricular arrhythmias, bundle branch blockage, atrioventricular blockage, and atrial fibrillation. The mechanism of arrhythmia in patients with LVNC may be related to the irregular branches and connections of the muscle bundle. Decreased conduction velocity and conduction blockage are important causes of arrhythmia, which can be caused by the abnormal excitability of cardiomyocytes or by the disordered electrical coupling function of the GJ channel. By studying the effects of membrane currents and GJ channels on the velocity of ventricular tachycardia, a reduction in the number of GJ channels could result in a slower myocardium conduction velocity and decreased cell membrane excitability. A reduced number of GJ channels can result in the excitability of various regions of the heart being nonuniform, resulting in conduction blockage [31].

GJIC, a common mode of communication between adjacent cells, is involved in cell intercellular exchange and electrical signal transmission. Gap junction-mediated intercellular communication has been recognized in cells from different tissues of various organisms and has been implicated in a variety of cellular functions and dysfunctions. A variety of factors, such as pH, Ca²⁺ concentration, voltage, and cyclic nucleotides, can affect the permeability of GJ channels, mostly by changing the phosphorylation levels of GJ proteins. Some studies have shown that changes in the function of GJ channels are not consistent with changes in these factors [32, 33]. In other words, the important factors regulating GJ channels may still be unknown. Protein-protein interaction is an important basis for many cellular functions. Signal transduction, cell cycle regulation, RNA transcription, DNA replication, and protein translation are all dependent on protein-protein interactions. Identifying which proteins have direct or indirect interaction with GJs is an important way to adequately elucidate GJ function and regulation. Immunoprecipitation experiments showed that CMYA1 binds to Cx43 in HL1 cells. The SL-DT assays showed that the function of GJIC was significantly decreased in the HL1 cells in which CMYA1 was over-expressed and significantly improved in HL1 cells in which CMYA1 was expressed at low levels. According to the immunofluorescence results in HL1 cells, Cx43 expression was weaker in HL1 cells in which CMYA1 was over-expressed and stronger in HL1 cells in which CMYA1 was expressed at low levels. These findings suggest that CMYA1 interacts with Cx43 and promotes the phosphorylation of Cx43, leading to GJ channel closure. Phosphorylation affects almost the entire life cycle of connexins, from synthesis, assembly, and function to degradation. The phosphorylation level of Cx43 is the most important factor affecting the permeability and function of GJ channels [34].

Studies have shown that multiple residues in the carboxyl-terminal region of Cx43 in the myocardium can be phosphorylated. Phosphorylation of Cx43 at the S368 site in the myocardium is involved in the regulation of gap junction permeability, which is dependent on phosphorylation of the PKC signaling pathway. In the myocardium, phosphorylation of Cx43 (S368) is stimulated by PMA and is indispensable for the reduction of gap junctions. In contrast, when treated with PKC inhibitors, the phosphorylation of Cx43 (S368) decreased, and the permeability of the gap junction channel was enhanced. The phosphorylation of Cx43 (S368) caused by the PKC signaling pathway in the myocardium leads to a conformational change in the protein, which can decrease the permeability of the gap junction channel [35]. Activation of the PKC signaling pathway resulted in high levels of Cx43 phosphorylation at the Ser368 site, decreased permeability of the GJ channel, and reduced GJ function [36]. We found that the phosphorylation level of Cx43 (S368) was significantly increased in LVNC tissues and HL1 cells in which CMYA1 was over-expressed. LY transmission occurred within minutes after loading in communication-competent cells, indicating the communication phenomenon of gap junctions [37]. According to the SL-DT assay results, the PKC inhibitor GF109203X and the PKC activator PMA can effectively inhibit or activate the phosphorylation of Cx43 (S368), destroying and inhibiting the structure and function of GJIC [38, 39]. This indicates that CMYA1 is involved in the PKC signaling pathway, which may regulate the phosphorylation and expression of Cx43.

We acknowledge that there are several limitations in this study. First, because human myocardial samples are very valuable, the number of tissue samples was small. Second, due

to the progress of the test, the PKC signaling pathway was not discussed in depth. Thus, more research is needed to confirm our findings. In conclusion, our findings in human myocardial tissues and HL1 cells have indicated that abnormal CMYA1 expression affects the phosphorylation of Cx43 through the PKC signaling pathway, which is involved in the regulation of GJIC. Fig. 9 depicts a model for the CMYA1 molecular mechanism underlying LVNC via the PKC signaling pathway. CMYA1 participates in the molecular mechanism of LVNC pathogenesis.

Abbreviations

CMYA1 (Cardiomyopathy-associated gene 1); Cx43 (Connexin 43); CT (Cycle threshold); DAB (3, 3'-Diaminobenzidine); ECL (Enhanced chemiluminescence); GJ (Gap junction); GJIC (Gap junction intercellular communication); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); ICD (Intercalated disc); LVNC (Left ventricular noncompaction cardiomyopathy); LY (Lucifer yellow); PKC (Protein kinase C); PMA (Phorbol 12-myristate 13 acetate); PBS (Phosphate buffered saline); PCR (Polymerase Chain Reaction); SL-DT (Scalpel loading-dye transfer); TJP1 (Tight junction protein ZO-1).

Acknowledgements

Y.X. and Y.W. prepared figures and wrote the main manuscript text; Y.X. performed the experiments. All authors reviewed the manuscript.

This study was supported by CAMS Innovation Fund for Medical Sciences (No. CIFMS, 2016-I2M-1-015) and PUMC Youth Fund and the Fundamental Research Funds for the Central University (No. 33320140167).

Disclosure Statement

The authors declare that they have no competing interests.

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