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GATA4 loss-of-function mutation underlies familial dilated cardiomyopathy

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ABSTRACT

The cardiac transcription factor GATA4 is essential for cardiac development, and mutations in this gene have been implicated in a wide variety of congenital heart diseases in both animal models and humans. However, whether mutated GATA4 predisposes to dilated cardiomyopathy (DCM) remains unknown. In this study, the whole coding region and splice junction sites of the *GATA4* gene was sequenced in 110 unrelated patients with idiopathic DCM. The available relatives of the index patient harboring an identified mutation and 200 unrelated ethnically matched healthy individuals used as controls were genotyped. The functional effect of the mutant GATA4 was characterized in contrast to its wild-type counterpart using a luciferase reporter assay system. As a result, a novel heterozygous GATA4 mutation, p.C271S, was identified in a family with DCM inherited as an autosomal dominant trait, which co-segregated with DCM in the family with complete penetrance. The missense mutation was absent in 400 control chromosomes and the altered amino acid was completely conserved evolutionarily among species. Functional analysis demonstrated that the GATA4 mutant was associated with significantly decreased transcriptional activity and remarkably reduced synergistic activation between GATA4 and NKX2-5, another transcription factor crucial for cardiogenesis. The findings provide novel insight into the molecular mechanisms involved in the pathogenesis of DCM, suggesting the potential implications in the prenatal diagnosis and gene-specific treatment for this common form of myocardial disorder.

1. Introduction

Dilated cardiomyopathy (DCM), the most common form of primary myocardial disorder characterized by ventricular chamber enlargement and contractile dysfunction with normal left ventricular wall thickness, is a major cause of congestive heart failure and sudden cardiac death and is the leading indication for cardiac transplantation in patients worldwide [1]. Approximately 50% of DCM cases are idiopathic, of which 25%–50% are familial, with estimates that vary based on the family members screened [2]. A growing body of evidence demonstrates that genetic risk factors play an important role in the pathogenesis of idiopathic DCM, and a long list of mutations in at least 50 single genes have been linked to familial HCM [2]. Functional characterization of these genetic variations indicates that multiple defects in myocardial structures and cardiac signal molecules may lead to DCM [2]. Despite the seemingly vast number of genes and mutations associated with DCM, the molecular basis for HCM in an overwhelming majority of patients remains unclear.

The zinc-finger transcription factor GATA4 is highly expressed in cardiomyocytes at different developmental stages and continues expression in the adult cardiac myocytes, where it regulates the transcription of several key structural and regulatory genes, including atrial natriuretic factor (ANF), brain natriuretic factor, carnitine palmitoyltransferase Iβ, troponin I, troponin C, α- and β-myosin heavy chain [3–5]. In humans, GATA4 has been demonstrated to be crucial for normal cardiogenesis, as shown by the

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established association of GATA4 mutations with a wide variety of congenital cardiovascular abnormalities, including atrial septal defect (ASD), ventricular septal defect, tetralogy of Fallot, endocardial cushion defect, patent ductus arteriosus, pulmonary stenosis, and hypoplastic right ventricle [6–9]. In mice, homozygous GATA4 deficiency results in early embryonic lethality because of abnormal embryogenesis and heart tube formation [10,11]. In contrast, mice expressing 70% less GATA4 protein died between days 13.5 and 16.5 of gestation, and in these embryos, common atrioventricular canal, double outlet right ventricle and hypoplastic ventricular myocardium were observed [12]. Furthermore, transgenic mice expressing GATA4 mutants showed various cardiac malformations, including septal defects, right ventricular hypoplasia, endocardial cushion defect, tetralogy of Fallot, double outlets of the right ven tricle, and cardiomyopathy, similar to the anomalies seen in humans [8]. More importantly, gene-targeted mice with marked loss of GATA4 protein in the heart survived into adulthood but displayed progressive cardiac enlargement and dysfunction with increased rates of cardiomyocyte apoptosis that was correlated to GATA4 levels [13]. These data highlight the pivotal role of GATA4 in maintaining proper homeostatic remodeling in adult hearts by promoting cell survival and regeneration and inhibiting programmed cell death [14–17].

GATA4 regulates cardiac gene expression by forming complexes with other transcriptional factors, including NKX2-5, TBX5, SRF, SMAD1, SMAD4, and JARID2 [5]. NKX2-5 is another critical regulator of cardiac development with expression and functions that overlap with GATA4 during embryogenesis [9]. Moreover, GATA4 and NKX2-5 physically interact and have been shown to cooperatively regulate the expression of multiple essential cardiac target genes, including those encoding ANF, T- and L-type Ca^{2+} channels, connexin40, α-actin, ID2 and LRRC10 [5]. Targeted knockout of NKX2-5 in mice gives rise to impaired cardiac growth and chamber formation, deranged gene regulatory network, and early embryonic death, while cardiac-specific deletion of NKX2-5 causes progressive cardiomyopathy and complete heart block [1–20]. Mutations in the human NKX2-5 gene have been related to a diverse range of congenital heart diseases, including atrial and ventricular septal defects, tetralogy of Fallot, hypoplastic left heart, transposition of the great arteries, valvular malformations, left ventricular contractile dysfunction, and DCM [21–23]. These results justify screening GATA4 as a prime candidate gene for DCM.

2. Materials and methods

2.1. Study population

A total of 110 unrelated patients with idiopathic DCM were recruited from the Han Chinese population. The available relatives of the index patients were also enrolled. The controls were 200 ethnically-matched unrelated healthy individuals. All participants were evaluated by detailed history, physical examination, chest radiography, electrocardiogram, echocardiography, and exercise performance testing. Cardiac catheterization, angiography, endomyocardial biopsy, and cardiac magnetic resonance imaging were performed only if there was a strong clinical indication. Medical records were also reviewed in the case of deceased or unavailable relatives. Diagnosis of idiopathic DCM was made in accordance with the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathy: a left ventricular end-diastolic diameter >27 mm/m^2 and an ejection fraction <40% or fractional shortening <25% in the absence of abnormal loading conditions, coronary artery disease, congenital heart lesions, and other systemic diseases [24]. Individuals were excluded if they had insufficient echocardiographic image quality, or coexistent conditions that may lead to contractile dysfunction, such as uncontrolled systemic hypertension, coronary artery disease, or valvular heart disease. Familial DCM was defined as having two or more first-degree relatives with idiopathic DCM. Peripheral venous blood specimens from the study subjects and control individuals were prepared. All clinical studies were performed with investigators blinded to the results of genetic testing. This study conformed to the principles of the Declaration of Helsinki and the study protocol was approved by the local institutional ethics committee. Written informed consent was obtained from all participants prior to investigation.

2.2. Genetic studies

Genomic DNA was extracted from blood lymphocytes of all participants with Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The whole coding region and splice junction sites of the *GATA4* gene was sequenced in 110 unrelated patients with idiopathic DCM. Genotyping GATA4 in the available relatives of the proband carrying an identified mutation and 200 unrelated healthy controls was performed. The referential genomic DNA sequence of GATA4 derived from GenBank (accession No. NC_000008). The primer pairs used to amplify the coding exons and intron–exon boundaries of GATA4 by polymerase chain reaction (PCR) were designed as described previously [25]. The PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a PE 9700 Thermal Cycler (Applied Biosystems, Foster, CA, USA) with standard conditions and concentrations of reagents. Amplified products were purified with the QIAquick Gel Extraction Kit (Qiagen). Both strands of each PCR product were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was validated by resequencing of an independent PCR-generated amplicon from the subject. In addition, for an identified sequence variant, the single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) and Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) databases were queried to confirm its novelty.

2.3. Multiple sequence alignments

Multiple GATA4 protein sequences across various species were aligned using the online MUSCLE program, version 3.6 (http://www.ncbi.nlm.nih.gov/).

2.4. Plasmids and site-directed mutagenesis

The recombinant expression plasmids GATA4-pSSRa, NKX2-5-pEFSA, and ANF-luciferase reporter (ANF-luc), which contains the 2600-bp 5′-flanking region of the ANF gene, were kindly provided by Dr. Ichiro Shiojima from Chiba University School of Medicine, Japan. The identified mutation was introduced into the wild-type GATA4 using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

2.5. Reporter gene assays

Hela cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega) were used in transient transfection assays to examine the transcriptional activity of the GATA4 mutant. Hela cells were transfected with 0.4 μg of wild-

type or mutant GATA4-pSSRa, 1.0 μg of ANF-luc, and 0.04 μg of pGL4.75 using PolyFect Transfection Reagent (Qiagen). For co-transfection experiments, 0.2 μg of wild-type GATA4-pSSRa, 0.2 μg of mutant GATA4-pSSRa, 1.0 μg of ANF-luc, and 0.04 μg of pGL4.75 were used. Firefly luciferase and Renilla luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) 48 h after transfection. The activity of the ANF promoter was presented as fold activation of Firefly luciferase relative to Renilla luciferase. Three independent experiments were performed at minimum for wild-type and mutant GATA4.

For the analysis of the synergistic transcriptional activation between GATA4 and NKX2-5 [26], COS-7 cells were grown and transfected with 0.2 μg of wild-type or mutant GATA4-pSSRa, alone or together with 0.2 μg of wild-type NKX2-5-pEFSA, 1.0 μg of ANF-luc, and 0.04 μg of pGL4.75 using PolyFect Transfection Reagent (Qiagen). When both wild-type and mutant GATA4-pSSRa were co-transfected, 0.1 μg of each GATA4-pSSRa was used.

2.6. Statistical analysis

Data are expressed as means ± SD. Continuous variables were tested for normality of distribution and student's unpaired *t* test was used for comparison of numeric variables between two groups. Comparison of the categorical variables between two groups was performed using Pearson's χ^2 test or Fisher's exact test when appropriate. A 2-tailed *p* value of <0.05 indicated statistical significance.

3. Results

3.1. Characteristics of the study subjects

A total of 110 unrelated patients with idiopathic DCM were clinically evaluated in contrast to 200 control individuals. None of them had overt traditional risk factors for DCM. All the patients manifested with typical DCM phenotype as described previously [24]. The control individuals had no evidence of organic cardiac diseases, and their echocardiogram results were normal. The baseline clinical characteristics of the study subjects are summarized in Table 1.

3.2. Novel GATA4 mutation

By direct sequencing of the *GATA4* gene, a heterozygous mutation was identified in 1 out of 110 unrelated patients with idiopathic DCM, with a mutational prevalence of approximately 0.91%. Specifically, a substitution of C for G in the second nucleotide of codon 271 (c.812G >C), predicting the transition of cysteine (C) into serine (S) at amino acid position 271 (p.C271S) was detected in the proband from family 1. The sequence chromatograms showing the observed heterozygous GATA4 mutation of c.812G >C compared with its control sequence are shown in Fig. 1A. A schematic diagram of GATA4 depicting the structural domains and location of the mutation identified in this study is presented in Fig. 1B. The missense mutation was neither found in the control population nor reported in the SNP and EVS databases. The genetic scan of the family showed that the mutation was present in all affected living family members, but absent in unaffected family members examined. Analysis of the pedigree demonstrated that the mutation co-segregated with DCM transmitted as an autosomal dominant trait in the family with complete penetrance. The pedigree structure of the family is illustrated in Fig. 1C. The phenotypic characteristics of the affected living family members are listed in Table 2.

Additionally, the proband's son had also first-degree atrioventricular conduction block. Individual III-7 had congenital ASD as well as paroxysmal atrial fibrillation (AF), and his son also had congenital ASD.

Table 1
The baseline clinical characteristics of the study subjects.

	Patients (*n* = 110)	Controls (*n* = 200)
Age (years)	51.8 ± 13.7	52.1 ± 11.4
Male (%)	68 (61.8)	120 (60.0)
Family history of DCM (%)	42 (38.2)	0 (0)
SBP (mmHg)	113.5 ± 16.1	118.3 ± 12.9
DBP (mmHg)	72.2 ± 9.4	80.5 ± 6.3
HR (bpm)	108.6 ± 15.2	76.7 ± 10.8
LVEDD (mm)	70.1 ± 8.4	48.6 ± 6.7
LVESD (mm)	57.5 ± 9.1	35.3 ± 6.5
LVEF (%)	35.2 ± 10.6	64.3 ± 7.0
NYHA function class (%)		
I	18 (16.4)	NA
II	42 (38.2)	NA
III	38 (34.5)	NA
IV	12 (10.9)	NA

DCM indicates dilated cardiomyopathy; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; and NA, not applicable.

3.3. Alignment of multiple GATA4 protein sequences across species

A cross-species alignment of GATA4 protein sequences showed that the altered amino acid was completely conserved evolutionarily (Fig. 2).

3.4. Transcriptional activity of the GATA4 mutant

As shown in Fig. 3, the same amount (0.4 μg) of wild-type and mutant GATA4 activated the ANF promoter by ~10-fold and ~2-fold, respectively. When the same amount of wild-type GATA4 (0.2 μg) was cotransfected with mutant GATA4 (0.2 μg), the induced activation of the ANF promoter was ~5-fold. These results suggest that the GATA4 mutant has a significantly reduced activation activity compared with wild-type counterpart.

3.5. Synergistic transcriptional activity between GATA4 mutant and NKX2-5

As shown in Fig. 4, in the presence of 0.2 μg of wild-type NKX2-5, the same amount (0.2 μg) of wild-type and mutant GATA4 activated the ANF promoter by ~22-fold and ~8-fold, respectively. When the same amount of wild-type GATA4 (0.1 μg) was cotransfected with mutant GATA4 (0.1 μg), the induced activation of the ANF promoter was ~15-fold, indicating that the GATA4 mutant has a significantly decreased synergistic transactivational activity with NKX2-5.

4. Discussion

In the current study, a novel heterozygous GTA4 mutation of p.C271S was identified in a family with DCM. The missense mutation co-segregated with DCM in the family and was absent in the 400 reference chromosomes from an ethnically matched control population. A cross-species alignment of multiple GATA4 protein sequences displayed that the altered amino acid was completely conserved evolutionarily. Functional analysis revealed that the mutant were associated with significantly decreased transcriptional activity alone or in synergy with NKX2-5. Therefore, it is very likely that genetically compromised GATA4 contributes to the DCM in these mutation carriers.

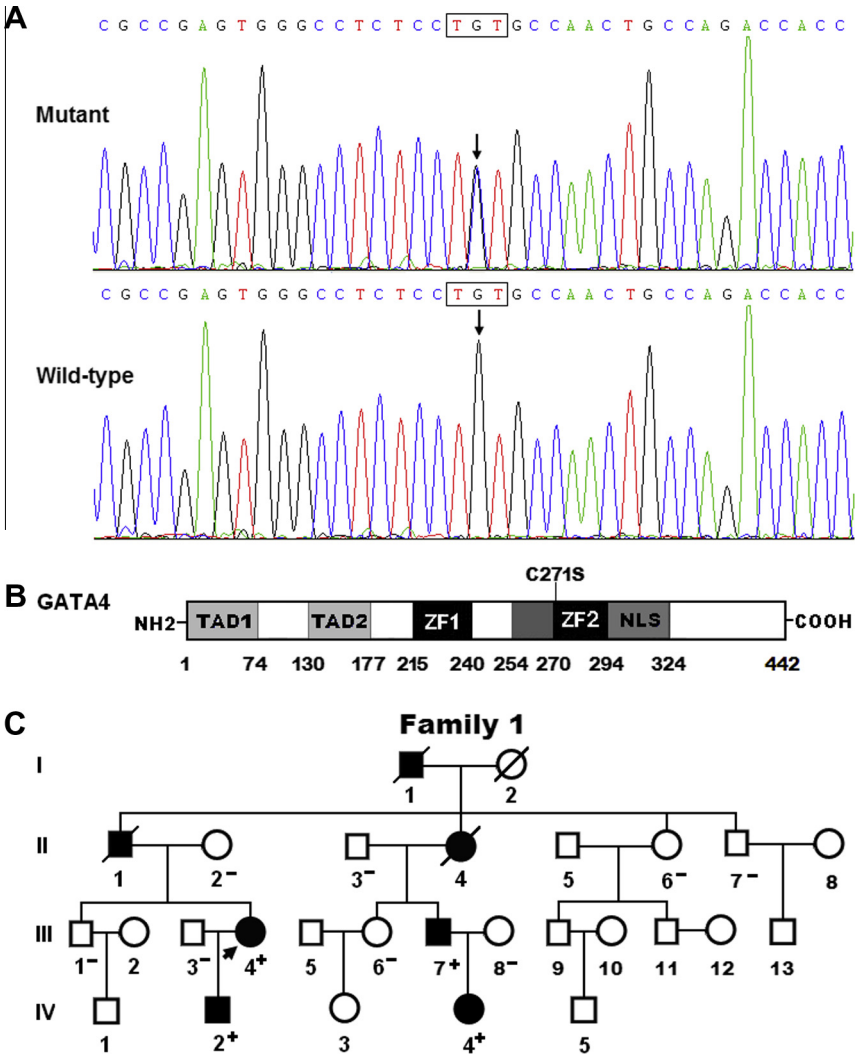

Fig. 1. GATA4 C271S mutation associated with dilated cardiomyopathy. (A), sequence electropherogram showing the GATA4 variation compared with its control. The arrow indicates the heterozygous nucleotides of C/G in the proband (mutant) or the homozygous nucleotides of G/G in the corresponding control individual (wild-type). The rectangle denotes the nucleotides comprising a codon of GATA4. (B), schematic diagram of GATA4 protein structure with the dilated cardiomyopathy related mutation indicated. The mutation found in patients with dilated cardiomyopathy is shown above the structural domains. NH_2 means amino-terminus; TAD, transcriptional activation domain; ZF, zinc finger; NLS, nuclear localization signal; COOH, carboxyl-terminus. (C), pedigree structure of the family with dilated cardiomyopathy. Family members are identified by generations and numbers. Square indicates male family member; circle, female member; symbol with a slash, the deceased member; closed symbol, affected member; open symbol, unaffected member; arrow, proband; "+", carrier of the heterozygous missense mutation; and "−", non-carrier.

Table 2
Phenotypic characteristics of the affected living pedigree members.

Individual	Gender	Age (years)	Cardiac phenotype	LVEDD (mm)	LVESD (mm)	LVEF (%)	ECG findings
III-4	F	52	DCM	74.6	62.4	36.3	
III-7	M	48	DCM, ASD	65.7	53.9	41.0	AF
IV-2	M	26	DCM	58.8	48.0	47.5	AVB
IV-4	F	23	DCM, ASD	60.2	49.5	38.6	

M denotes male; F, female; DCM, dilated cardiomyopathy; ASD, atrial septal defect; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; AF, atrial fibrillation; AVB, atrioventricular conduction block; NA, not available or not applicable.

Previous experiments have substantiated that GATA4 is a transcriptional activator of multiple genes expressed during cardiac development including ANF gene [5]. GATA4 may bind to target DNA in conjunction with other partners including transcription factor NKX2-5, and the synergistic transcriptional activation mediated by NKX2-5 has been verified [6,26]. Hence, the functional characteristics of the GATA4 mutation may be explored by analysis of the transcriptional activity of the ANF promoter in cells expressing GATA4 with or without NKX2-5. In the present study, the functional effect of the novel p.C271S mutation of GATA4 identified in our familial DCM patients was investigated by transcriptional activity assays and the results showed decreased transcriptional activity and diminished synergistic activation with NKX2-5 on a target gene. These findings imply that haploinsufficiency or dominant-negative effect resulting from GATA4 mutation is potentially an alternative pathological mechanism underling DCM.

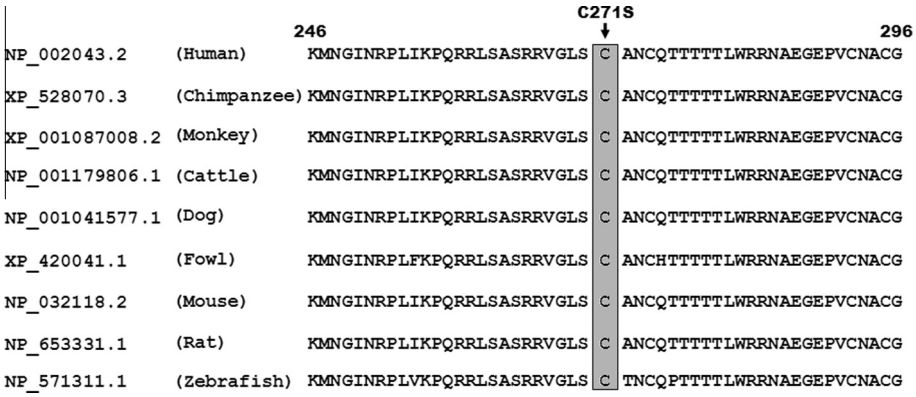

Fig. 2. Alignment of multiple GATA4 protein sequences across species. The altered amino acid of p.C271 is completely conserved evolutionarily among species.

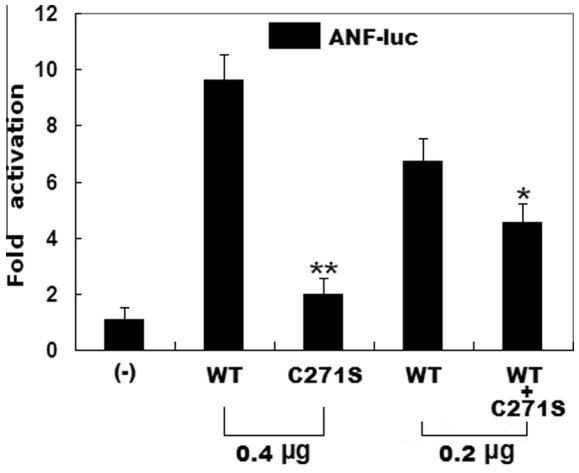

Fig. 3. Functional defects associated with GATA4 mutation. Activation of atrial natriuretic factor promoter driven luciferase reporter in Hela cells by wild-type (WT) or mutant GATA4, alone or in combination, showed significantly decreased transactivational activity by the mutant protein. Experiments were performed in triplicate, and mean and standard deviations are shown. ** and * represent $p < 0.001$ and $p < 0.005$, respectively, when compared with wild-type GATA4.

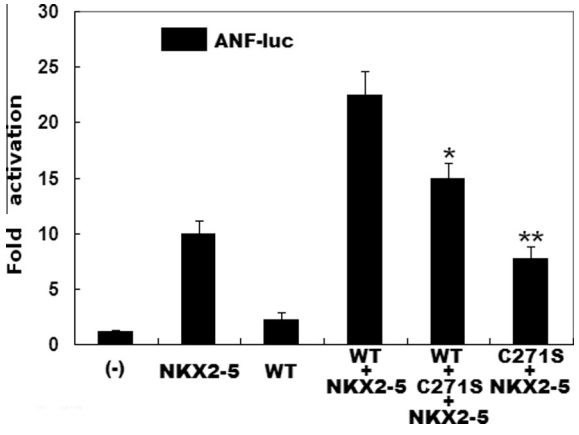

Fig. 4. Reduced synergistic transcriptional activation with NKX2-5 caused by GATA4 mutation. The synergistic activation of the atrial natriuretic factor promoter in COS-7 cells by NKX2-5 and mutant GATA4 was significantly reduced compared with that by NKX2-5 and wild-type GATA4. Experiments were performed in triplicate, and mean and standard deviations are shown. ** represents $p < 0.001$ and * represents $p < 0.01$, when compared with NKX2-5 and wild-type GATA4.

The findings that GATA4 loss-of-function mutation confers susceptibility to DCM may be partially attributed to the developmental and regenerative defects of the myocardium as well as abnormal heart remodeling. During embryogenesis, GATA4 is critical for activation and maintenance of the core cardiac regulatory network, and GATA4-null mutations compromise cardiomyocyte specification and maturation, leading to death [27]. GATA4 is also pivotal for postnatal maturation and homeostasis of cardiomyocytes, and adult heart function and adaptation [13]. The mice with cardiomyocyte-specific deletion of GATA4 were viable and survived into adulthood, but they presented with a progressive deterioration in cardiac function and dilation in adulthood. Moreover, in this mouse model pressure overload or exercise stimulation failed to induce cardiac hypertrophy, but induced rapid decompensation, precipitous heart failure and increased apoptosis [13]. In contrast, overexpression of GATA4 in the heart was sufficient for inducing cardiac hypertrophy [28]. In humans, mutations in the transcriptionally cooperative partners of GATA4, including NKX2-2 and TBX20, have been associated with familial DCM [23,29]. Taken together, these data support that GATA4 mutation predisposes to DCM.

Additionally, some important cardiac genes were activated by GATA4, and mutations in multiple target molecules have been implicated in DCM, including α-actin, α myosin heavy chain, troponin C, and troponin I [2,5]. Therefore, mutated GATA4 probably enhances vulnerability to DCM by reducing expression of target genes.

Similar with previous reports [6–9,30–32], in this study, congenital heart disease was observed in two patients bearing the GATA4 mutation, and one mutation carrier had also AF. The phenotypic variability within this family may be explained by the following reasons. Firstly, AF may occur as rarely as a few times in a lifetime for some patients and a longer duration of electrocardiographic monitoring is needed to record paroxysmal AF. Next, AF occurs more commonly in older patients and some carriers may not be old enough to develop AF. Thirdly, some congenital cardiac structural defects may close spontaneously, hence we cannot rule out the possibility that some mutation carriers with DCM had minor cardiac defects that closed shortly after birth on their own. Finally, different genetic backgrounds and environmental risk factors may be responsible for the variable penetrance of the phenotype.

In conclusion, the current study firstly links GATA4 loss-of-function mutation to DCM and provides novel insight into the molecular mechanisms implicated in the pathogenesis of DCM, implying potential implications in early prophylaxis and gene-specific treatment of this common cardiomyopathy.

Acknowledgments

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