GATA6 loss-of-function mutations contribute to familial dilated cardiomyopathy

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Abstract. Dilated cardiomyopathy (DCM), the most prevalent form of primary heart muscle disease, is the third most common cause of heart failure and the most frequent reason for cardiac transplantation. Mounting evidence has demonstrated that genetic risk factors are crucial in the pathogenesis of DCM. However, DCM is genetically heterogeneous, and the genetic basis of DCM in a large majority of cases remains unclear. In the current study, the coding exons and flanking introns of the GATA6 gene, which encodes a zinc-finger transcription factor essential for cardiogenesis, was sequenced in 140 unrelated patients with DCM, and two novel heterozygous mutations, p.C447Y and p.H475R, were identified in two index patients with DCM, respectively. Analysis of the pedigrees showed that in each family the mutation co-segregated with DCM transmitted in an autosomal-dominant pattern, with complete penetrance. The missense mutations were absent in 400 control chromosomes and predicted to be disease-causing by MutationTaster or probably damaging by PolyPhen-2. The alignment of multiple GATA6 proteins across species revealed that the altered amino acids were completely conserved evolutionarily. The functional assays showed that the mutated GATA6 proteins were associated with significantly reduced transcriptional activation in comparison with their wild-type counterpart. To the best of our knowledge, this is the first study on the association of GATA6 loss-of-function mutations with enhanced susceptibility to familial DCM, which provides novel insight into the molecular mechanism of DCM and suggests potential implications for the antenatal prophylaxis and allele-specific treatment of DCM.

Introduction

Dilated cardiomyopathy (DCM), which is clinically characterized by progressive cardiac chamber enlargement and contractile dysfunction with normal left ventricular wall thickness, is the most prevalent form of primary myocardial disease, with an estimated prevalence of 1/2,500 in the general population (1). It is the third most common cause of heart failure and the most frequent indication for cardiac transplantation in adults and children (1). The entity of DCM has also been recognized as a major cause of sudden cardiac death, accounting for at least 30% of the overall mortality in DCM patients (2). The etiologies underlying DCM are diverse, with both environmental and genetic risk factors involved in the pathogenesis of DCM, although most cases remain idiopathic (3). Approximately 25-50% of DCM individuals had familial forms of the disease, highlighting the important role of genetic defects in the pathogenesis of DCM (4). Pathogenic mutations in >50 genes have been associated with various types of DCM, of which autosomal dominant inheritance is the most common type, although other types, including X-linked, autosomal recessive and mitochondrial inheritance, have also been reported (4-8). Nevertheless, the DCM-related genes identified thus far explain only a minority of DCM patients, and the genetic determinants underpinning DCM in a large majority of cases remain elusive.

The GATA transcription factors are a family of zinc-finger-containing DNA-binding proteins, which preferentially bind to a 5′-(A/T)GATA(A/G)-3′ motif within the regulatory region of target genes (9). Six members of the GATA family have been identified in vertebrates that are parsed into two subfamilies based on their expression profiles. GATA1, GATA2 and GATA3 are prominently expressed in hematopoietic cell lineages, while GATA4, GATA5 and GATA6 are broadly expressed in various mesoderm- and endoderm-derived tissues, particularly in the heart (10). GATA4 and GATA6 are...
highly expressed in the embryonic heart and continue the high expression in the post-natal and adult myocardium, where they function as crucial transcriptional regulators of various key cardiac structural and regulatory genes, including the atrial natriuretic factor (ANF), α- and β-myosin heavy chain, cardiac troponin C, cardiac troponin I, cardiac-restricted ankyrin repeat protein, sodium/calcium exchanger, A1 adenosine receptor, m2 muscarinic receptor, and the myosin light chain 1/3 genes (10). In mice, the overexpression of GATA4 or GATA6 was sufficient to induce cardiac hypertrophy (11,12), whereas the cardiomyocyte-specific conditional deletion of GATA6 significantly reduced the cardiac hypertrophic response to pressure overload stimulation and rapidly led to heart failure, similar to that observed in the mice with heart-specific deletion of GATA4. Furthermore, the combinatorial deletion of GATA4 and GATA6 from the adult heart resulted in DCM and lethality by 16 weeks of age (12-14). In humans, mutations in GATA4 have been associated with various cardiac phenotypes, including congenital heart diseases, atrial fibrillation and DCM (15-25). Similarly, genetic variations in GATA6 are also involved in the pathogenesis of congenital cardiovascular malformations and atrial fibrillation (26-34), rendering it justifiable to screen GATA6 as a prime candidate gene for DCM.

Materials and methods

Study participants. A cohort of 140 genetically unrelated patients with DCM was prospectively enlisted from the Han Chinese population. The available relatives of the index patients were also recruited. The controls comprised 200 ethnically matched, unrelated healthy individuals. Prior to enrollment in the study, all the subjects were evaluated for detailed individual and familial histories, underwent complete 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 requiring any these procedures. Medical records were reviewed in all cases of deaths thought to be related to DCM. Diagnosis of DCM was made based on the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathy. The criteria included a left ventricular end-diastolic diameter >27 mm/m² and a left ventricular ejection fraction <40% or fractional shortening <25% in the absence of abnormal loading conditions, coronary artery disease, congenital heart lesions and other systemic diseases (24,35). Exclusion criteria were insufficient echocardiographic image quality, or coexistent conditions that may lead to cardiac contractile dysfunction, such as uncontrolled systemic hypertension, coronary artery or valvular heart disease. A diagnosis of familial DCM was assigned when occurring in at least two closely related family members (36). Individuals were classified as healthy when found to be well with normal echocardiographic parameters. Peripheral venous blood samples from all the participants were collected. The clinical studies were conducted with investigators blinded to the results of genetic testing. The study was performed in accordance with the principles outlined in the 1964 Declaration of Helsinki and its later amendments as well as the ethics laws of China, and the study protocol was approved by the local institutional ethics committee (of Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China). Written informed consent was obtained from each participant prior to enrollment in the study.

Mutational screening of GATA6. Genomic DNA was extracted from each subject from whole blood leukocytes using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The coding regions and splice junction sites of GATA6 were sequenced initially in 140 unrelated patients with DCM and subsequently in the available relatives of the index patients carrying the identified mutations and the 200 control individuals using the same method. The reference genomic DNA sequence of GATA6 was derived from GenBank at the National Center for Biotechnology Information (NCBI; accession no. NT_010966; http://www.ncbi.nlm.nih.gov/nucleotide). The primer pairs used to amplify the coding exons and flanking introns of GATA6 by polymerase chain reaction (PCR) were designed as described in a previous study (32). The PCR was performed using HotStar Taq DNA Polymerase (Qiagen GmbH, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA), with standard conditions and concentrations of reagents. Amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified with QIAquick Gel Extraction kit (Qiagen GmbH). The two strands of each PCR product were sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). The sequencing primers were identical to those designed for the amplification of specific regions of GATA6. The DNA sequences were viewed and assessed using DNA Sequencing Analysis Software v5.1 (Applied Biosystems). A sequence variation was verified by re-sequencing an independent PCR-generated amplicon from the same subject and met our quality control thresholds with a call rate of >99%. Additionally, for an identified sequence variant, the single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) and human gene mutation (HGM; http://www.hgmd.org) databases were queried to confirm its novelty.

Comparison of amino acid sequences of GATA6 proteins from various species. Amino acid sequences of GATA6 protein from human (NP_005248.2) were aligned with those from rhesus monkey (XP_002800933.1), cattle (XP_002697773.1), mouse (NP_034388.2), rat (NP_062058.1), fowl (NP_990751.1), and zebrafish (NP_571632.1) using the online program of MUSCLE, version 3.6 (http://www.ncbi.nlm.nih.gov).

Prediction of the disease-causing potential of the GATA6 sequence variations. The causative potential of a GATA6 sequence variation was assessed using MutationTaster (an online program at http://www.mutationtaster.org), which provides a probability for the variation to be a pathogenic mutation or a benign polymorphism. The p-value utilized at this point is the probability of the correct prediction as opposed to the probability of error as used in t-test statistics (i.e. a value close to 1 indicates a high ‘security’ of the prediction). The online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was also used to assess the pathogenic similarity of an amino acid variation.
Plasmids and site-directed mutagenesis. The expression vector pcDNA3-hGATA6 used in the present study was generously provided by Dr Angela Edwards-Ghatnekar, from the Division of Rheumatology and Immunology, Medical University of South Carolina (Charleston, SC, USA). The ANF-luciferase reporter plasmid, which contains the 2600-bp 5'-flanking region of the ANF gene, i.e., ANF(-2600)-Luc, was kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine (Chuo-ku, Chiba, Japan). Each identified mutation was introduced into the plasmid containing the wild-type human GATA6 cDNA to generate the mutant expression vector using a QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers, which was verified by direct sequencing.

Dual-luciferase reporter assay. HEK-293 cells plated onto 12-well plates at an initial density of 2x10^4 cells/well were cultured in Dulbecco's modiﬁed Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂, and grown to a confluence of ~80%. Transient transfection was subsequently performed using Lipofectamine® 2000 Transfection Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The ANF(-2600)-Luc reporter vector and an internal control reporter plasmid pGL4.75 (hRluc/CMV; Promega) were used in transfection assays to evaluate the transcriptional activation function of the GATA6 mutants. HEK-293 cells were transfected with 0.4 µg of empty vector pcDNA3, or 0.4 µg of wild-type or mutant pcDNA3-hGATA6 expression plasmid, together with 0.4 µg of ANF(-2600)-Luc reporter construct and 0.04 µg of pGL4.75 control reporter vector. For the co-transfection experiments, 0.2 µg of wild-type pcDNA3-hGATA6, 0.2 µg of mutant pcDNA3-hGATA6, 0.4 µg of ANF(-2600)-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 and normalized to the cells transfected with empty vector. The activity of the ANF promoter was presented as the fold activation of normalized Firefly luciferase relative to normalized Renilla luciferase. Three independent experiments were performed at minimum for wild-type and mutant GATA6. Experiments were performed in triplicate.

Statistical analysis. Data are expressed as means ± SD. Continuous variables were tested for normality of distribution, and the 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 χ² test or Fisher's exact test when appropriate. A two-tailed p<0.05 was considered to indicate a statistically significant result.

Results

Clinical characteristics of the study population. A cohort of 140 genetically unrelated patients with DCM (76 males, mean age 53.2±12.8 years) was clinically evaluated in contrast to a total of 200 ethnically matched, unrelated healthy individuals (105 males, mean age 54.6±10.7 years) that were used as controls. Of the 140 DCM patients, 51 had a positive family history of DCM, while no family history of DCM was confirmed in the 200 control individuals. Compared with those in the control group, blood pressure and left ventricular ejection fraction were statistically decreased whereas the heart rate, left ventricular end-diastolic diameter and left ventricular end-systolic diameter were significantly increased in the patient group. The baseline clinical characteristics of the 140 unrelated DCM patients are shown in Table I.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=140)</th>
<th>Controls (n=200)</th>
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<tr>
<td>Age (years)</td>
<td>53.2±12.8</td>
<td>54.6±10.7</td>
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<tr>
<td>Male (%)</td>
<td>76 (54.3)</td>
<td>105 (52.5)</td>
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<tr>
<td>Family history of DCM (%)</td>
<td>51 (36.4)</td>
<td>0 (0)</td>
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<tr>
<td>SBP (mmHg)</td>
<td>115.8±14.6</td>
<td>126.2±11.5</td>
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<tr>
<td>DBP (mmHg)</td>
<td>75.2±8.7</td>
<td>82.5±7.3</td>
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<tr>
<td>HR (bpm)</td>
<td>92.6±10.1</td>
<td>75.8±9.6</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>62.1±8.5</td>
<td>48.1±6.2</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>51.5±7.2</td>
<td>32.7±6.0</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>38.9±5.3</td>
<td>64.2±6.5</td>
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NYHA function class (%)

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<td></td>
<td>25 (17.9)</td>
<td>47 (33.6)</td>
<td>52 (37.1)</td>
<td>16 (11.4)</td>
</tr>
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DCM, 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; NA, not applicable or not available. *P<0.0001 when compared with the controls.

Identification of GATA6 mutations in DCM patients. By analysis of the protein coding sequence of GATA6 in the 140 unrelated DCM patients, two heterozygous missense mutations were identified in 2 of 140 patients, respectively, with a mutational prevalence of ~1.43%. Specifically, a substitution of adenine for guanine at amino acid position 447 of the GATA6 gene (c.1340G>A), equivalent to the transition of cysteine to tyrosine at amino acid 447 (p.C447Y), was identified in the index patient from family 2. The sequence chromatograms showing the detected heterozygous GATA6 variations in contrast to the corresponding control sequences are shown in Fig. 1. A schematic diagram of GATA6 showing the structural domains and the locations of the identified mutations is presented in Fig. 2. The
two variants were not observed in 400 control alleles or identified in the SNP and HGM databases, which were consulted again on January 21, 2014.

A genetic screen of family members of the mutation carriers demonstrated that in each family, the variation was present in all the affected family members available, but absent in the unaffected family members examined. Analysis of the pedigrees showed that in each family the variation co-segregated with DCM transmitted in an autosomal dominant pattern with complete penetrance. The pedigree structures of the two families are shown in Fig. 3. In family 1, the proband's father (I-1) and two brothers (II-3 and II-8) also had an electrocardiogram documented atrial fibrillation. The phenotypic characteristics and results of genetic screening of the affected pedigree members are shown in Table II.

**Figure 1.** Sequence electropherograms showing the *GATA6* mutations in contrast to their corresponding controls. The arrow indicates the heterozygous nucleotides of G/A in the probands from families (A) 1 and (B) 2, respectively, (mutant) or the homozygous nucleotides of (A) G/G and (B) A/A in the corresponding control individuals (wild-type). The rectangle denotes the nucleotides constituting a codon of GATA6.

**Figure 2.** Schematic diagram showing GATA6 protein structures with the dilated cardiomyopathy (DCM)-related mutations indicated. The mutations identified in patients with familial DCM are shown above the structural domains. NH2, amino-terminus; TAD, transcriptional activation domain; ZF, zinc finger; NLS, nuclear localization signal; COOH, carboxyl-terminus.

Multiple alignments of *GATA6* protein sequences among various species. A cross-species alignment of multiple GATA6 protein sequences showed that the affected amino acids were highly conserved from human to zebrafish, indicating that these amino acids are functionally important (Fig. 4).

**Disease-causing potential of GATA6 sequence variations.** The *GATA6* sequence variations of c.1340G>A and c.1424A>G were predicted to be disease-causing, with an identical p-value of 1.00000. No SNPs in the altered regions were identified in the MutationTaster database. The amino acid substitutions...
of C447Y and H475R in GATA6 were also predicted by PolyPhen-2 to be damaging, with scores of 1.000 (sensitivity 0.00; specificity 1.00) for p.C447Y and 0.972 (sensitivity 0.77; specificity 0.96) for p.H475R, respectively.

Reduced transcriptional activity of the GATA6 mutants. The wild-type GATA6, C447Y-mutant GATA6 and H475R-mutant GATA6 activated the ANF promoter by ~12-, ~2- and ~3-fold, respectively. When wild-type GATA6 was co-expressed with the same amount of C447Y-mutant GATA6 or H475R-mutant GATA6, the induced activation of the ANF promoter was the same as ~6-fold. These results showed that the two GATA6 mutants are associated with significantly reduced transcriptional activity compared with their wild-type counterpart (Fig. 5).

Discussion

In the current study, two novel heterozygous GATA6 mutations, p.C447Y and p.H475R, were identified in two families with DCM. In each family the mutation co-segregated with DCM transmitted in an autosomal dominant mode with complete penetrance. The two mutations, which were absent in 400 referential chromosomes from an ethnically matched control population, altered the amino acids that were completely conserved evolutionarily and were predicted to be pathogenic. Functional assays showed that the mutant GATA6 proteins were associated with significantly reduced transcriptional activity. Therefore, it is likely that functionally compromised GATA6 contribute to DCM in these families.

Human GATA6 gene maps to chromosome 18q11.1-q11.2, coding for a protein with 595 amino acids (37). The functional domains of GATA6 comprise two transcriptional activation domains (TAD1, amino acids 1-215; TAD2, amino acids 280 ‑351), two adjacent zinc fingers (ZF1, amino acids 388-413; ZF2, amino acids 443-467), and one nuclear localization signal (NLS, amino acids 427-497). The two TADs are important for the appropriate transcriptional activity of GATA6. The N-terminal ZF2 is responsible for DNA sequence recognition and binding to the consensus motif, while the
C-terminal ZF1 is crucial for the sequence specificity and stability of protein-DNA binding. The NLS is required for the sub-cellular trafficking and distribution of GATA6 (33). The GATA6 mutations p.C447Y and p.H475R identified in the present study are located in ZF2 and NLS, respectively, and may therefore exert impact on the transcriptional activity of GATA6 by interfering with the binding to target DNA or nuclear distribution of GATA6.

GATA6 has been found to mediate the expression of several target genes during embryogenesis and cardiac morphogenesis, including the genes that encode atrial natriuretic factor (ANF), brain natriuretic peptide, α- and β-myosin heavy chain, and gap junction protein Cx40 (10,38). Thus, the functional effect of the GATA6 mutation may be investigated by assay of the transcriptional activity of a target gene promoter in tool cells. In the present study, the functional characteristics of the novel GATA6 mutations identified in the DCM patients were determined by transcriptional activation analysis and the results showed a significantly decreased transcriptional activity on the downstream gene, ANF. These findings suggest that haplo-insufficiency or a dominant-negative effect resulting from GATA6 loss-of-function mutation is potentially an alternative molecular mechanism underpinning DCM.

Association of genetically defective GATA6 with increased vulnerability to DCM has been established in animals. In zebrafish, embryos depleted of GATA6 developed variable cardiac morphogenetic defects including cardiaco bifida, partially fused tube, and fused but non-looping tube (39). By contrast, zebrafish embryos depleted of GATA4 and GATA6 were heartless and restoring either gene product was sufficient to rescue cardiomyocyte specification (40). In mice, among the mammalian GATA factors that have been identified thus far (GATA1-GATA6), GATA6 was the earliest expressed during embryonic development, and GATA6-deficient embryos died shortly after implantation (41). Although the mice heterozygous for a GATA4 or GATA6 null allele were normal, compound heterozygosity of GATA4 and GATA6 resulted in embryonic lethality accompanied by a spectrum of cardiovascular defects, including thin-walled myocardium, ventricular septal defect, persistent truncus arteriosus, double outlet right ventricle, myocardial hypoplasia, reduced prolife-
ration of cardiomyocytes, and the impaired differentiation of vascular smooth muscle cells (42,43). The mice with a cardiomyocyte-specific conditional deletion of GATA6, which lacked >95% of GATA6 protein in the heart, were viable and survived into adulthood, but they were predisposed to progressive deterioration in cardiac function and enlargement of heart in adulthood, a phenotype similar to that of GATA4 heart-specific deleted mice. Furthermore, the combinatorial deletion of GATA4 and GATA6 from the adult murine heart led to DCM (12). By contrast, the overexpression of GATA6 was sufficient to induce myocardial hypertrophy in vitro and in vivo, both alone and in combination with GATA4 (11,12). These experimental results emphasize the pivotal role of GATA6 in the development and remodeling of the heart.

GATA6 has been confirmed to regulate the expression of multiple key cardiac genes alone or in cooperation with its transcriptionally synergistic partners, such as GATA4, NKX2-5 and Tbx20, and in humans, an increasing number of mutations in such target molecules as α-actin, α-myosin heavy chain, troponin C, and troponin I, as well as in the transcriptional cooperative partners of GATA6, including GATA4, NKX2-5 and Tbx20, are involved in the pathogenesis of DCM (10,24,25,44,45). These findings suggest that functionally compromised GATA6 predisposes to DCM probably by reducing the expression of genes essential for cardiac structure and function.

Atrial fibrillation was documented in three DCM patients from family 1, consistent with previous studies on the association of GATA6 mutations with atrial fibrillation (32-34). Similarly, mutations in other cardiac transcriptional factor genes, such as GATA4, GATA5, NKX2-5 and PITX2c, were also associated with atrial fibrillation (21-23,46-53). These observations support the hypothesis that a subset of atrial fibrillation may have developmental origin.

In conclusion, to the best of our knowledge, this is the first study to connect GATA6 loss-of-function mutations with enhanced susceptibility to DCM, providing novel insight into the molecular mechanism of DCM, suggesting potential implications for the antenatal prophylaxis and allele-specific treatment of DCM.

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