Novel GATA6 loss-of-function mutation responsible for familial atrial fibrillation

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Abstract. Atrial fibrillation (AF) is the most commonly sustained cardiac arrhythmia, and confers a substantially increased risk of morbidity and mortality. Increasing evidence has indicated that hereditary defects are implicated in AF. However, AF is genetically heterogeneous and the genetic etiology of AF in a significant portion of patients remains unclear. In this study, the entire coding sequence and splice junctions of the GATA6 gene, which encodes a zinc-finger transcription factor crucial for cardiogenesis, were sequenced in 140 unrelated patients with lone AF. The available relatives of the index patient carrying an identified mutation and 200 unrelated ethnically-matched healthy individuals used as the controls were genotyped. The functional characteristics of the mutant GATA6 were assessed in contrast to its wild-type counterpart using a luciferase reporter assay system. As a result, a novel heterozygous GATA6 mutation, p.G469V, was identified in a family with AF inherited in an autosomal dominant pattern. The mutation was absent in the 200 control individuals and the altered amino acid was completely conserved across species. Functional analysis demonstrated that the GATA6 mutation was associated with a significantly decreased transcriptional activity. The findings provide novel insight into the molecular mechanism involved in the pathogenesis of AF, as well as insight into potential therapies for the prevention and treatment of AF.

Introduction

Atrial fibrillation (AF) is the most common type of sustained cardiac arrhythmia, accounting for approximately 1/3 of hospitalizations for heart rhythm disorders (1). The prevalence of AF is approximately 1% in the general population, and is drastically increasing with advancing age, rising from approximately 0.1% in individuals younger than 55 years of age up to almost 10% in those aged 80 years and older (2). The lifetime risk of developing AF is estimated to be 25% for both men and women over 40 years of age (3). AF confers a substantially increased risk of morbidity and mortality. It increases the risk of stroke by 3- to 5-fold, causing a huge economic burden on the health care system and an adverse impact on the quality of life of patients (4). Compared to subjects with sinus rhythm, patients with AF have a 2-fold increased risk of mortality (5). Additionally, AF can also result in other complications, such as adverse hemodynamics, impaired cognitive function or dementia, congestive heart failure and tachycardia-induced cardiomyopathy (6). AF is frequently observed as a complication of various cardiac and systemic conditions, including hypertension, coronary artery disease, valvular heart disease, cardiomyopathies and metabolic disorders. Hence, AF is traditionally regarded as an acquired disorder (1). However, in 30-45% of AF patients, an underlying cause cannot be identified by routine procedures, and such AF is defined as ‘idiopathic’ or ‘lone’ (1), of which at least 15% of patients have a positive family history, so termed familial AF (7). Growing evidence has substantiated the familial aggregation of AF and an increased susceptibility to AF in the close relatives of patients with AF, highlighting the fact that genetic factors play a role in the pathogenesis of AF in a subset of cases (8-14). Studies on human and medical genetics have revealed multiple AF-related genes, including KCNQ1, KCNE2, SCN5A, KCNH2, KCNJ2, GJA5, KCNAs, KCNE3, KCNE5, NPPA, NUP155, SCN1B, SCN2B, SCN3B and GJA1 (15-29). In addition, 4 chromosomal loci linked to AF have been mapped, although the AF-associated genes have not yet been discovered (30-33). Nevertheless, AF is genetically heterogeneous and the genetic determinants for AF in most patients remain unknown (7).

Recently, there is increasing evidence indicating the essential role of several transcription factors, including NKX2-5 and GATA4 in normal cardiogenesis (34-36), and mutations in NKX2-5 and GATA4 have been causally implicated in
congenital cardiovascular abnormalities and AF (37-44). GATA6 is another member of the GATA family, and its expression and function overlap with those of GATA4 during cardiac development, particularly in the regulation of target gene expression synergistically with NKX2-5 (36,45), which implies the potential association of functionally impaired GATA6 with AF.

In this study, in order to evaluate the prevalence of GATA6 mutations in patients with lone AF and characterize the mechanism by which mutated GATA6 predisposes to AF, the coding exons and exon/intron boundaries of GATA6 were sequenced in patients with lone AF in contrast to healthy individuals, and the functional characteristics of the mutant GATA6 were analyzed in comparison with its wild-type counterpart using a luciferase reporter assay system.

Materials and methods

Study participants. A total of 140 unrelated patients with lone AF were identified among the Han Chinese population. The controls were 200 ethnically-matched unrelated healthy individuals. Peripheral venous blood samples were prepared and clinical data including medical records, electrocardiogram and echocardiography reports were collected. The study subjects were clinically classified using a consistently applied set of definitions (7,43). Briefly, the diagnosis of AF was made by a standard 12-lead electrocardiogram demonstrating no P waves and irregular R-R intervals regardless of the clinical symptoms. Lone AF was defined as AF occurring in individuals <60 years of age without other cardiac or systemic diseases by physical examination, electrocardiogram, transthoracic echocardiogram, and extensive laboratory tests. Familial AF was defined as the presence of documented lone AF in 2 or more 1st- or 2nd-degree relatives. Relatives with AF occurring at any age in the setting of structural heart disease (hypertensive, ischemic, myocardial or valvular) were classified as ‘undetermined’ for having an inherited form of AF. The ‘undetermined’ classification was also used if the documentation of AF on an electrocardiogram tracing was lacking in relatives with symptoms consistent with AF (palpitations, dyspnea and light-headedness), or if a screening electrocardiogram and echocardiogram were not performed, irrespective of the symptoms. Relatives were classified as ‘unaffected’ if they were asymptomatic and had a normal electrocardiogram. Paroxysmal AF was defined as AF lasting for >30 sec that terminated spontaneously. Persistent AF was defined as AF lasting for >7 days and requiring either pharmacological termination or external cardioversion for termination. AF that was refractory to cardioversion or that was allowed to continue was classified as permanent. The study protocol was reviewed and approved by the local institutional ethics committee and written informed consent was obtained from all participants prior to investigation.

Genetic studies. Genomic DNA from all participants was extracted from blood lymphocytes with the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). Initially, the whole coding sequence and splice junctions of the GATA6 gene were screened in 140 unrelated patients with lone AF. Subsequently, genotyping of GATA6 was performed in the available relatives of the index patient carrying an identified mutation and 200 ethnically-matched unrelated healthy individuals used as the controls. The referential genomic DNA sequence of GATA6 was derived from GenBank (accession no. NT_010966). With the help of online Primer 3 software (http://frodo.wi.mit.edu), the primer pairs used to amplify the coding exons (exons 2-7) and intron-exon boundaries of GATA6 by polymerase chain reaction (PCR) were designed as shown in Table I. PCR was carried out using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) on a PE 9700 Thermal Cycler (Applied Biosystems, Foster City, 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 3130XL DNA Analyzer (Applied Biosystems). The sequencing primers were those designed previously for specific region amplifications. 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 and met our quality control threshold with a call rate exceeding 99%.

Alignment of multiple GATA6 protein sequences across species. The multiple GATA6 protein sequences across various species were aligned using the software ClustalW version 2.0 (an interactive program at http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Plasmids and site-directed mutagenesis. The recombinant expression plasmid pcDNA3-hGATA6 was kindly provided by Dr Angela Edwards-Ghatnekar, from the Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, SC, USA. The atrial natriuretic factor (ANF)-luciferase reporter gene, which contains the 2600-bp 5′-flanking region of the ANF gene, namely ANF(-2600)-Luc, was kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan. The identified mutation was introduced into the wild-type GATA6 using a QuikChange 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.

Reporter gene assays. HEK-293 cells were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal calf serum. The ANF(-2600)-Luc reporter construct and an internal control reporter plasmid [pGL4.75 (hRlac/CMV), Promega] were used in transient transfection assays to examine the transcriptional activation function of the GATA6 mutant. HEK-293 cells were transfected with 0.4 µg of wild-type or mutant pcDNA3-hGATA6 expression vector, 0.4 µg of ANF(-2600)-Luc reporter construct, and 0.04 µg of pGL4.75 control reporter vector using PolyFect Transfection Reagent (Qiagen). For 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. A minimum of 3 independent experiments were performed for wild-type and mutant GATA6.

Statistical analysis. Data are expressed as the means ± SD. Continuous variables were examined for normality of distribution and the Student's unpaired t-test was used for the comparison of numeric variables between 2 groups. A comparison of the categorical variables between 2 groups was performed using Pearson's $\chi^2$ test or Fisher's exact test when appropriate. A two-tailed P-value <0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the study subjects. A total of 140 unrelated patients with lone AF and a cohort of 200 ethnically-matched unrelated healthy individuals used as the controls were recruited and clinically evaluated. None of them had overt traditional risk factors for AF. There were no significant differences between the patient and control groups in baseline characteristics including age, gender, body mass index, blood pressure, fasting blood glucose and serum lipid levels, left atrial dimension, left ventricular ejection fraction, heart rate at rest, as well as lifestyle (data not shown). At the time of the present study, 12 patients were also diagnosed with hypertension in accordance with the criterion that the average systolic or diastolic blood pressure (2 readings made after 5 min of rest in the sitting position) was ≥140 or ≥90 mm Hg, respectively, but at the time of the initial diagnosis of AF, their blood pressures were normal. The baseline clinical characteristics of the 140 patients with lone AF are summarized in Table II.

GATA6 mutation. Direct sequencing of the coding exons and exon-intron boundaries of the GATA6 gene was carried out after PCR amplification of genomic DNA from each of the 140 patients with lone AF. A heterozygous GATA6 mutation was identified in 1/140 unrelated patients, with a prevalence of approximately 1.71% for GATA6 mutation. Specifically, A substitution of T for G in the second nucleotide of codon 469 (c.1406G>T), predicting the transition of glycine (G) into valine (V) at amino acid 469 (p.G469V) was identified in a patient with a positive family history. A representative 12-lead electrocardiogram of the index patient with AF is recorded in Fig. 1. The sequence chromatograms showing the detected heterozygous GATA6 sequence mutation of c.406G>T compared with
the corresponding control sequence are shown in Fig. 2. The missense mutation was not found in either the control population or reported in the SNP database (http://www.ncbi.nlm.nih.gov/SNP). Genetic scan of the available family members displayed that the mutation was present in all affected living family members, but absent in unaffected family members.
examined. Pedigree analysis demonstrated that the mutation cosegregated with AF transmitted in an autosomal dominant pattern in the family with complete penetrance. The pedigree structure of the family is illustrated in Fig. 3. The phenotypic characteristics and results of genetic screening of the affected family members are listed in Table III. Congenital atrial septal defect was confirmed by medical records of previous catheter-based repairs in 2 AF patients (II-3 and III-4), indicating that AF may share a common genetic origin with congenital heart disease.

Alignment of multiple GATA6 protein sequences. A cross-species alignment of GATA6 protein sequences showed that the altered amino acid was completely conserved, as presented in Fig. 4, suggesting that the amino acid is functionally important.

Transcriptional activity of the GATA4 mutants. The transcriptional characterization of the mutated GATA6 in HEK-293 cells was examined using one of its direct cardiac downstream target genes, ANP, as a luciferase reporter, and the activity of the ANP promoter was presented as the fold activation of Firefly luciferase activity relative to Renilla luciferase activity. Equal amounts of wild-type (0.4 µg) and G469V-mutant GATA6 (0.4 µg) activated the ANP promoter by ~11- and ~2-fold, respectively. When the same amount of wild-type GATA4 (0.2 µg) was co-transfected with G469V-mutant GATA4 (0.2 µg), the induced activation of the ANP promoter was ~6-fold. These results suggest that the GATA6 mutation has a significantly reduced activation activity compared with its wild-type counterpart (Fig. 5).

Discussion

In the present study, a novel heterozygous GTA6 mutation of p.G469V identified in a family with lone AF is reported. This missense mutation of GATA6 was present in all the affected family members examined but was absent in the unaffected family members available and 400 normal chromosomes from a matched control population. A cross-species alignment of
LI et al: NOVEL GATA6 MUTATION IN AF

788

Functionally, GATA6 consists of 2 transcriptional activation domains (TADs), 2 adjacent zinc fingers (ZFs), and 1 nuclear localization signal (NLS). The 2 TADs are essential for the transcriptional activity of GATA6. The C-terminal ZF is required for DNA sequence recognition and binding to the consensus motif, while the N-terminal ZF is responsible for stability and sequence specificity of protein-DNA binding as well as the transcriptional activation by GATA factors. The majority of the protein-protein interactions of GATA factors are mediated by its C-terminal ZF. The NLS sequence is associated with the subcellular trafficking and distribution of GATA6 (36). The GATA6 mutation, p.G469V, identified in this study is located in the C-terminal ZF; therefore, it may be expected to exert influence on the transcriptional activation of GATA6 by interfering with the binding to the downstream target DNA.

It has been substantiated that GATA6 is an upstream regulator of multiple genes transcribed during embryogenesis and cardiac morphogenesis, including the genes that encode the atrial natriuretic peptide (ANP), brain natriuretic peptide, α-myosin heavy chain, β-myosin heavy chain and cardiac troponin C and I (36). Hence, the functional characteristics of the GATA6 mutations may be explored by analysis of the transcriptional activity of the ANP promoter in cells transfected with the GATA6 mutant in contrast to its wild-type counterpart. In this study, the functional effect of the novel p.G469V mutation of GATA6 identified in our familial AF patients was characterized by transcriptional activity assays and the results demonstrated a significantly decreased transcriptional activity on a downstream gene, consistent with the loss-of-function effects of other GATA6 mutations underlying congenital cardiovascular malformations on the transcriptional activity of the ANP promoter (47,48). These findings indicate that the haploinsufficiency or loss-of-function effect resulting from GATA6 mutations is potentially an important pathophysiological mechanism involved in AF, although the functional roles of the recently reported AF-related GATA6 mutations remain to be ascertained (49).

The findings that functionally compromised GATA6 confers susceptibility to AF may be partially ascribed to the abnormally developed pulmonary vein myocardium (50-52). The pulmonary venous vessels are ensheathed by a layer of

multiple GATA6 protein sequences exhibited that the altered amino acid was completely conserved. Functional analysis demonstrated that the p.G469V mutation of GATA6 was associated with a significantly decreased transactivation by mutant protein. Experiments were performed in triplicate and the mean and standard deviations are shown. *P<0.001 and **P<0.005, respectively, when compared with wild-type GATA6.

GATA transcription factors are a group of DNA binding proteins characteristic of preferential binding to the consensus DNA sequence GATA of target gene promoters. The GATA family comprises 6 members (GATA1-GATA6), of which GATA4, GATA5 and GATA6 are expressed in various mesoderm and endoderm-derived tissues, particularly in the embryonic and adult heart (36). GATA6 maps to human chromosome 18q11.1-q11.2 by fluorescence in situ hybridization, which encodes a predicted 449-amino-acid protein (46).

Figure 4. Alignment of multiple GATA6 protein sequences across species. The altered amino acid of p.G469 is completely conserved across species.

Figure 5. Functional impairments resulted from GATA6 mutation. Activation of ANP-luciferase reporter in HEK-293 cells by GATA6 wild-type (WT) or mutant (G469V), alone or in combination, demonstrated significantly reduced transactivation by mutant protein. Experiments were performed in triplicate and the mean and standard deviations are shown. *P<0.001 and **P<0.005, respectively, when compared with wild-type GATA6.
myocardium termed pulmonary myocardial sleeve, which has been demonstrated to be responsible for the initiation and perpetuation of AF by several potential arrhythmogenic mechanisms, including intrinsic pacemaker activity and properties facilitating re-entrance (50-52). Genetic labeling lineage-tracing studies have shown that NKX2-5 is expressed in the atrial and pulmonary myocardium and is essential for the localized formation of the sinoatrial node during embryonic development. NKX2-5 can act as a suppressor of the sinoatrial node lineage gene program, which limits pacemaker activity to the sinus and atrioventricular node. When the NKX2-5 protein level was lowered in a hypomorphic model, the pulmonary cardiomyocytes switched to connexin40-negative, HCN4-positive cells, a nodal-like phenotype with pacemaker activity (51). In NKX2-5-deficient mouse embryos, HCN4 was activated in the entire embryonic heart tube, whereas connexin40 expression was lost, and the ectopic expression of pacemaker cells was observed throughout the heart tube (53). In humans, AF has been reported as an isolated phenotype or a part of compound phenotypes in patients harboring NKX2-5 mutations (40,54,55). Therefore, as a transcriptionally cooperative partner of NKX2-5, GATA6, when a loss-of-function mutation occurs, may conduces to the formation of the pulmonary myocardium sleeve and the shift of the pulmonary myocardium to a sinoatrial node-like phenotype by reducing the expression of NKX2-5, hence producing an atrial electrophysiological substrate favoring AF.

There are a large number of downstream genes transactivated by GATA6, and mutations in several target genes have been associated with AF, including β-myosin heavy chain, ANP and connexin40 (24,25,56-58). Therefore, it is highly likely for mutated GATA6 to predispose to AF by decreasing the expression of target genes.

Of note, congenital atrial septal defect was documented in 2 AF patients carrying the p.G469V mutation of GATA6. Of note, congenital atrial septal defect was documented in 2 AF patients carrying the p.G469V mutation of GATA6. There are a large number of downstream genes transactivated by GATA6, and mutations in several target genes have been associated with AF, including β-myosin heavy chain, ANP and connexin40 (24,25,56-58). Therefore, it is highly likely for mutated GATA6 to predispose to AF by decreasing the expression of target genes.

In conclusion, the results from the present study link a novel mutation in the cardiac transcription factor, GATA6, to familial AF and provides novel insight into the molecular mechanism involved in AF, as well as insight into potential therapies for the prevention and treatment of this common type of arrhythmia.

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