NKX2-6 mutation predisposes to familial atrial fibrillation

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Abstract. Atrial fibrillation (AF) is the most common form of sustained cardiac arrhythmia and is associated with substantially increased morbidity and mortality rates. Aggregating evidence demonstrates that genetic defects are involved in the pathogenesis of AF and a number of AF-associated genes have been identified. Nevertheless, AF is a genetically heterogeneous disorder and the genetic components underpinning AF in an overwhelming majority of patients remain unclear. In this study, the entire coding exons and splice junction sites of the NK2 homeobox 6 (*NKX2-6*) gene, which encodes a homeodomain transcription factor important for cardiovascular development, were sequenced in 150 unrelated patients with lone AF, and a novel heterozygous NKX2-6 mutation, p.Q175H, was identified in an index patient. Genetic analysis of the available family members of the mutation carrier revealed that the mutation co-segregated with AF transmitted in an autosomal dominant pattern. The missense mutation was absent in the 200 unrelated ethnically matched healthy individuals used as controls and the altered amino acid was completely conserved evolutionarily among species. Due to unknown transcriptional targets of NKX2-6, the functional characteristics of the mutation as regards transcriptional activity were analyzed using NKX2-5 as a surrogate. Alignment between human NKX2-6 and NKX2-5 proteins displayed that the Q175H-mutant NKX2-6 was equivalent to the Q181H-mutant NKX2-5, and the introduction of Q181H into NKX2-5 significantly decreased its transcriptional activity at the atrial natriuretic factor promoter. The present study firstly associates genetically defective NKX2-6 with enhanced susceptibility to AF, providing novel insight into the molecular mechanisms underlying AF and suggesting potential strategies for the antenatal prophylaxis and personalized treatment of AF.

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Introduction

Atrial fibrillation (AF), a supraventricular tachyarrhythmia characterized by chaotic atrial electrical activity and consequently ineffective atrial contraction, is the most common type of cardiac arrhythmia observed in clinical settings, accounting for approximately one-third of hospitalizations for cardiac rhythm disturbances (1). The prevalence of AF is approximately 1% in the general population, and increases strikingly with advancing age, rising from >1% in subjects under 60 years of age to nearly 10% in individuals aged over 80 years (2). According to a report from the Framingham Heart Study, individuals older than 40 years have an approximately 25% chance of developing AF during their lifetime (3). The number of individuals with AF in the United States is presently estimated at 2.3 million and is projected to exceed 15.9 million by the year 2050, mainly due to the aging population and improved cardiovascular survival (4). The condition is associated with severe complications, such as chronic heart failure and stroke, significantly contributing to cardiovascular morbidity and mortality. Compared to sinus rhythm, AF confers a 2-fold increased risk of death (5) and a 5-fold increased risk of thromboembolic stroke (6). Of note, the annual incidence of ischemic stroke resulting from AF increases considerably with age, ranging from 1.5% of adults in their fifties to 23.5% in octogenarians (6). Additionally, AF may result in a degraded quality of life, reduced exercise tolerance, cognitive dysfunction or dementia, worsened renal function, tachycardia-induced cardiomyopathy, myocardial infarction and left ventricular dysfunction or even congestive heart failure (7-16). Therefore, AF represents an increasing public health challenge with profound socioeconomic implications. The appraised cost of treatment for AF is <1% of the health care expenditure (17) and only in the United States, the direct costs of treating nonvalvular AF are in excess of $6.4 billion per year (18). Despite

the high prevalence and substantial clinical significance, the molecular basis underlying AF remains largely unclear.

AF often occurs secondary to various cardiovascular and systemic diseases and surgical procedures, such as coronary artery disease, congenital heart disease, valvular heart disease, cardiac surgery, cardiomyopathy, myocarditis, pulmonary heart disease, left ventricular dysfunction, hypertension, hyperthyroidism, diabetes mellitus and electrolyte imbalance. Other potential risk factors responsible for the development of AF include age, male gender, obesity, high-level physical training, prehypertension, increased pulse pressure, diastolic dysfunction, obstructive sleep apnea, hyperuricemia, kidney disease, systemic inflammation, pericardial fat, tobacco use and alcohol consumption (19-23). However, in 30-45% of the total number of patients, AF is diagnosed in the absence of the above-mentioned associated diseases or predisposing factors, a condition referred to as idiopathic AF or lone AF, and up to 15% of these patients with lone AF have a clearly established positive family history, and are thus defined as familial AF (1). Aggregating epidemiological studies have demonstrated the pronounced clustering of AF in families and the markedly increased incidence of AF in the close relatives of AF patients, indicating a pivotal role of genetic factors in the pathogenesis of familial AF (24-31). In previous studies, genome-wide genetic linkage analysis with highly polymorphic microsatellite markers mapped AF-susceptibility loci on human chromosomes 10q22-24, 11p15.5, 6q14-16, 5p13, 10p11-q21 and 5p15, of which AF-causing mutations in 2 genes, including potassium voltage-gated channel, KQT-like subfamily, member 1 (*KCNQ1*) on chromosome 11p15.5 and nucleoporin 155 kDa (*NUP155*) on chromosome 5p13, were identified and functionally deciphered (32-37). The direct analysis of candidate genes has identified an increasing number of AF-related genes, including potassium voltage-gated channel, Isk-related family, member 1 (*KCNE1-5*), potassium voltage-gated channel, subfamily H (eag-related), member 2 *(KCNH2*), potassium voltage-gated channel, shaker-related subfamily, member 5 (*KCNA5*), potassium voltage-gated channel, Shal-related subfamily, member 3 (*KCND3*), potassium inwardly-rectifying channel, subfamily J *(KCNJ2), KCNJ8,* gap junction protein, alpha 1, 43 kDa (*GJA1*), gap junction protein, alpha 5, 40 kDa (*GJA5*), atrial natriuretic peptide (*ANP*), sodium channel, voltage-gated, type V, alpha subunit (*SCN5A*) and sodium channel, voltage-gated, type I, beta subunit *(SCN1B-4B)* (38-63). Nevertheless, these well established AF-associated genes only explain a small fraction of cases of AF, and in an overwhelming majority of patients, the genetic determinants for AF remain unknown.

Recent studies indicate that abnormal embryonic development and structural remodeling of the cardiovascular system, particularly the pulmonary veins and the atria, create a major anatomic substrate liable to AF (64,65). A growing body of evidence substantiates the crucial role of several cardiac transcription factors, including NK2 homeobox (NKX2)-5, GATA binding protein (GATA)4, GATA5, GATA6 and paired-like homeodomain 2 (PITX2)c in normal cardiovascular morphogenesis (66-87), and multiple mutations in these transcription factors have been causally linked to AF (88-101). NKX2-6 is another member of the NK2-family of transcription factors and its expression profile and functional roles partially overlap with those of NKX2-5 during cardiovascular development (102-105), which warrants the screening of *NKX2-6* as a preferred candidate gene for the development of AF.

Materials and methods

Study subjects. A cohort of 150 unrelated patients with lone AF was enrolled from the Han Chinese population. The available relatives of the index patients were also recruited. A total of 200 ethnically-matched unrelated healthy individuals were enlisted as the controls. All participants were evaluated by detailed medical history, physical examination, an electrocardiogram and echocardiography. Cardiac catheterization, angiography, a chest X-ray 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. The diagnosis and classification of AF was made in accordance with the guidelines for the management of patients with AF (1,88). Briefly, AF was diagnosed by a standard 12-lead electrocardiogram demonstrating no P-waves and irregular R-R intervals regardless of 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 termed when lone AF existed in an additional 2 or more first- or second-degree relatives. Relatives with AF occurring at any age in the presence 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 documentation of AF on an electrocardiogram tracing was lacking in relatives with symptoms consistent with AF (palpitation, 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 >30 sec that terminated spontaneously. Persistent AF was defined as AF lasting >1 week and requiring either pharmacological therapy or electrical cardioversion for termination. AF that was refractory to cardioversion or that was allowed to continue was classified as permanent AF. Peripheral venous blood samples were obtained from all the participants. The clinical studies were performed with investigators blinded to the results of the genotypes. This study conformed to the principles of the Declaration of Helsinki and the study protocol was approved by the local Institutional Ethics Committee of Shanghai Chest Hospital, Shanghai Jiao Tong University (the ethical approval number for cases and controls: KS1101; the date of the approval: April 12, 2011). Written informed consent was obtained from all participants prior to enrollment.

Genetic scanning of NKX2-6. Genomic DNA was extracted from the blood lymphocytes of each participant using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The coding exons and exon-intron boundaries of the *NKX2-6* gene were sequenced in the 150 unrelated patients with lone AF. The available relatives of the index patient harboring an identified *NKX2-6* mutation and the 200 unrelated control individuals were genotyped for *NKX2*-6. The referential genomic DNA sequence of *NKX2-6* was derived

Table I. The intronic primers used to amplify the coding exons and exon-intron boundaries of *NKX2-6*.

Exon	Forward primer	Reverse primer	Amplicon
1	5'-GAC AAG ACG GGA AGT TCA GG-3'	5'-TCG AAC CCA GGA GAT AGG AG-3'	447 bp
2-a	5'-CCA GGG AGA GGA AAG TCT TG-3'	5'-CAG GAC GGG CAC AGC TAC TC-3'	454 bp
2-b	5'-AGA ACC GAC GCT ACA AAT GC-3'	5'-GAG ATC CCT CCG GAA AGA AG-3'	500 bp

NKX2-6, NK2 homeobox 6; bp, base pairs.

from GenBank (accession no. NG_030636), a gene sequence database at the National Center for Biotechnical Information (NCBI; http://www.ncbi.nlm.nih.gov/). With the help of online Primer 3 software (http://frodo.wi.mit.edu/), the primer pairs used to amplify the coding regions and flanking splice junction sites of *NKX2-6* by polymerase chain reaction (PCR) were designed as described in Table I. PCR was carried out using HotStar TaqDNA Polymerase (Qiagen, Hilden, Germany) on a Veriti 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 amplicon were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit under an ABI PRISM 3130 XL DNA Analyzer (both from Applied Biosystems). The sequencing primers were the same as those mentioned above for the specific region amplifications. DNA sequences were viewed and analyzed using DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was validated by re-sequencing of an independent PCR-generated amplicon from the same subject. In addition, 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.

Alignment of multiple NKX2-6 protein sequences. The conservation of the amino acid altered by missense mutation was appraised by aligning human NKX2-6 to chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish and frog NKX2-6 using the HomoloGene and Show Multiple Alignment links on the NCBI website (http://www.ncbi.nlm.nih.gov/homologene).

Prediction of the causative potential of an NKX2-6 sequence variation. The disease-causing potential of an *NKX2-6* sequence variation was predicted using an online program, MutationTaster (http://www.mutationtaster.org), automatically yielding a probability for a variation to be either a pathogenic mutation or a benign polymorphism. Notably, the P-value used here is the probability of the correct prediction rather than the probability of error as used in t-test statistics (i.e., a value close to 1 indicates a high accuracy of the prediction). Additionally, another online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was also used to evaluate the pathogenic likeliness of an amino acid substitution.

Expression plasmids and site-directed mutagenesis. The recombinant expression vector NKX2-5-pEFSA and the ANF-luciferase (ANF-luc) reporter plasmid, which contains the 2,600 bp 5'-flanking region of the ANF gene, were kindly provided by Dr. Ichiro Shiojima from Chiba University School of Medicine, Chiba, Japan. Owing to unknown downstream genes of NKX2-6, NKX2-5 was used as a surrogate in transcriptional analysis to assess the functional consequences of the Q175H homeodomain substitution (104). Alignment between the human NKX2-6 and NKX2-5 proteins illustrated that Q175H-mutant NKX2-6 was equivalent to Q181H-mutant NKX2-5 (data not shown). The c.543G>C transition, which was predicted to generate the p.Q181H mutation, was introduced into wild-type NKX2-5 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.

Reporter gene assays. COS-7 cells from our cell bank 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) was used in transient transfection analyses to evaluate the transcriptional activity of the *NKX2-5* mutant. The COS-7 cells were transfected with 0.4 μg of wild-type or mutant NKX2-5-pEFSA, 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 NKX2-5-pEFSA, 0.2 μg of mutant NKX2-5-pEFSA, 1.0 μg of ANF-luc and 0.04 μg of pGL4.75 were used. Firefly luciferase and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega) 48 h after transfection. The activity of the *ANF* promoter was presented as the fold activation of firefly luciferase relative to *Renilla* luciferase. Three independent experiments were performed at minimum for wild-type and mutant *NKX2-5*.

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

Results

Clinical characteristics of the study subjects. A total of 150 unrelated patients with lone AF were clinically evaluated in contrast to 200 control individuals. None of them had underlying comorbidities or traditional risk factors for AF. There was

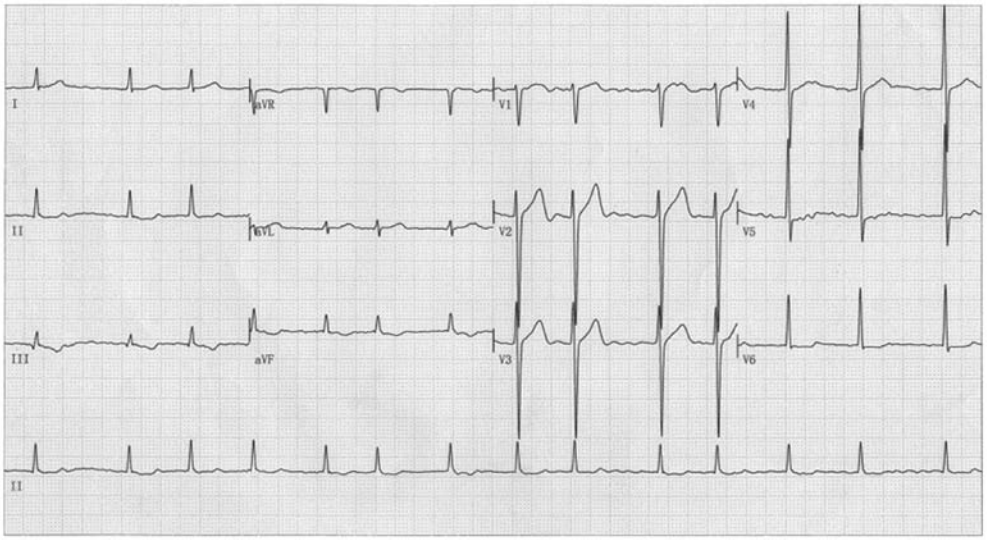

Figure 1. Standard 12-lead surface electrocardiogram of the proband from family 1. The electrocardiogram shows atrial fibrillation.

Table II. The baseline clinical characteristics of the 150 patients with lone atrial fibrillation.

Variables	Statistics
Baseline demographics	
Age at first diagnosis of atrial fibrillation (years)	51.4±10.2
Male (n, %)	82 (54.7)
Body mass index (kg/m^2)	23.4±3.8
Left ventricular ejection fraction (%)	64.2±6.1
Left atrial diameter (mm)	37.0±4.5
Personal history of atrial fibrillation (n, %)	
Type of atrial fibrillation at presentation	
Paroxysmal	89 (59.3)
Persistent	36 (24.0)
Permanent	25 (16.7
History of cardioversion	113 (75.3)
Positive family history of atrial fibrillation	58 (38.7)
Medical history (n, %)	
History of syncope	17 (11.3)
History of pacemaker)	12 (8.0)
Thromboembolic complication	6 (4.0)
Medications (n, %)	
Amiodarone	105 (70.0)
Warfarin	91 (60.7)
Aspirin	30 (20.0)
Digitalis	22 (14.7)
β-acceptor blocker	13 (8.7)
Calcium channel blocker	9 (6.0)

Continuous variables are expressed as the means ± standard deviation.

no significant difference between the patient and control groups in baseline characteristics including age, gender, body mass index, blood pressure, fasting blood glucose, serum lipid levels, left atrial dimension, left ventricular ejection fraction, heart rate at rest, as well as lifestyle (data not shown). The baseline clinical characteristics of the study subjects are summarized in Table II.

NKX2-6 mutation. By direct sequencing of the *NKX2-6* gene in the 150 unrelated patients with lone AF, a heterozygous mutation was identified in 1 patient, with a mutational prevalence of approximately 0.67%. Specifically, a substitution of cytosine for guanine in the third nucleotide of codon 175 (c.525G>C), predicting the transition of glutamine into histidine at amino acid position 175 (p.Q175H) was identified in the index patient from family 1. A representative 12-lead electrocardiogram of the proband with AF is shown in Fig. 1. The sequence chromatograms showing the detected heterozygous *NKX2-6* mutation of c.525G>C compared with its control sequence are shown in Fig. 2. The schematic diagrams of *NKX2-6* and NKX2-5 proteins showing the structural domains and location of the mutation identified in this study are presented in Fig. 3. The missense mutation was neither observed in the control population nor reported in the single nucleotide polymorphism (SNP) and HGM databases. Genetic screening of the family of the proband revealed that the mutation was present in all affected living family members, but absent in the unaffected family members examined. Analysis of the pedigree demonstrated that the mutation co-segregated with AF transmitted in an autosomal dominant pattern and with complete penetrance. The pedigree structure of the family is illustrated in Fig. 4. The phenotypic characteristics and status of the NKX2-6 mutation of the affected family members are listed in Table III.

Multiple alignments of NKX2-6 protein sequences. As shown in Fig. 5, a cross-species alignment of NKX2-6 protein sequences revealed that the altered amino acid p.Q175H was completely conserved evolutionarily, suggesting its functional importance.

Disease-causing potential of the NKX2-6 variation. The sequence variation of c.525G>C detected in the *NKX2-6* gene

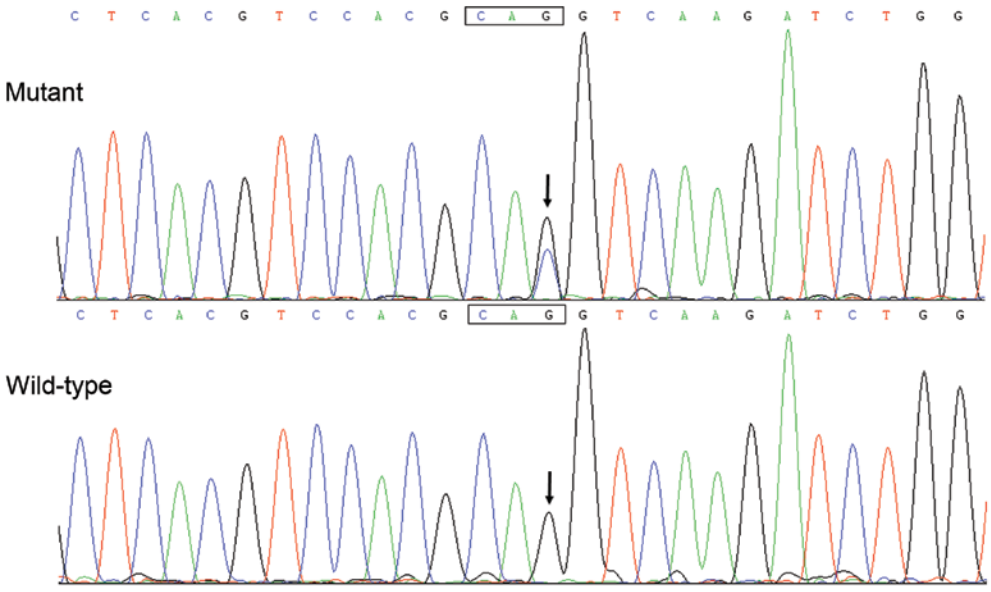

Figure 2. Sequence electropherograms showing the NKX2-6 variation compared with its control. The arrow indicates the heterozygous nucleotides of G/C in the proband from family 1 (mutant) or the homozygous nucleotides of G/G in the corresponding control individual (wild-type). The rectangle denotes the nucleotides comprising a codon of NKX2-6.

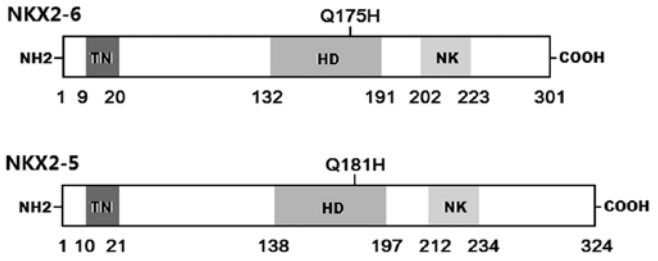

Figure 3. Schematic diagrams of NKX2-6 and NKX2-5 protein structures with the identified mutation indicated. The mutation identified in patients with familial atrial fibrillation is shown above the structural domains. NH2, amino-terminus; TN, tinman domain; HD, homeodomain; NK, nucleotide kinase domain; COOH, carboxyl-terminus.

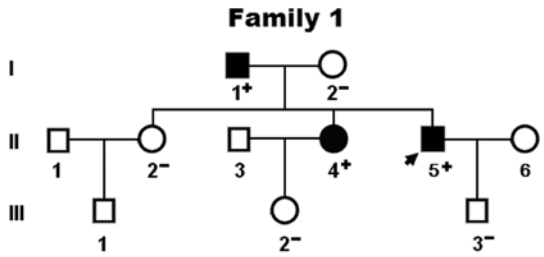

Figure 4. Pedigree structure of the family with atrial fibrillation. Family members are identified by generations and numbers. Square, male family member; circle, female member; closed symbol, affected member; open symbol, unaffected member; arrow, proband; +, carrier of the heterozygous missense mutation; -, non-carrier.

was predicted by MutationTaster to be a disease-causing mutation with a P-value of 0.998. No SNPs in the altered region were reported in the MutationTaster database. Accordingly, the amino acid substitution of Q175H in NKX2-6 was also predicted by PolyPhen-2 to be possibly damaging, with a score of 1.000 (sensitivity, 0.00; specificity, 1.00).

Reduced transcriptional activity of Q181H-mutant NKX2-5. Due to unknown target genes of NKX2-6, Q181H-mutant NKX2-5 was used as a surrogate to assess the functional sequelae of Q175H-mutant NKX2-6. As shown in Fig. 6, the same amount (0.4 μg) of wild-type and mutant *NKX2-5* activated the ANF-luciferase (*ANF*) promoter by approximately 11- and 4-fold, respectively. When the same amount of wild-type NKX2-5 (0.2 μg) was co-transfected with mutant NKX2-5 (0.2 μg), the induced activation of the ANF promoter was approximately 6-fold. These results demonstrate that Q181H-mutant NKX2-5 is associated with a significantly diminished transcriptional activity compared with its wild-type counterpart, suggesting that the homeobox substitution Q175H results in functional defects of NKX2-5

Discussion

In this study, a novel heterozygous mutation of p.Q175H in the *NKX2-6* gene was identified in a family with familial AF. The missense mutation co-segregated with AF in the family and was absent in the 400 reference chromosomes from an ethnically-matched control population. A cross-species alignment of multiple NKX2-6 protein sequences revealed that the altered amino acid was completely conserved evolutionarily. Functional analyses *in vitro* and *in silico* indicated that the homeobox substitution resulted in functional impairment. Therefore, it is highly likely that mutant NKX2-6 predisposes these mutation carriers to AF.

NKX2-6 is a vertebrate homolog of *Drosophila* homeobox-containing protein termed 'tinman'. It is expressed in early embryonic heart progenitor cells, playing an important role in proper cardiovascular development (102-105). The human *NKX2-6* gene maps to chromosome 8p21.2 and consists of 2 exons encoding a protein of 301 amino acids (104). The NKX2-6 protein contains an evolutionarily conserved homeodomain that recognizes and binds to a consensus DNA motif,

Table III. Phenotypic characteristics and status of NKX2-6 mutation in the affected pedigree members.

Subject information				Phenotype	Electrocardiogram			Echocardiogram		Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of AF (years)	AF (classification)	Heart rate (beats/min)	QRS interval (msec)	QTc	LAD (mm)	LVEF (%)	NKX2-6 mutation
Family 1										Q175H
I-1	M	65	40	Permanent	87	84	425	40	65	+/-
II-4	F	40	35	Persistent	82	86	498	35	60	+/-
II-5	M	38	37	Paroxysmal	84	90	437	38	67	+/-

AF, atrial fibrillation; F, female; M, male; QTc, corrected QT interval; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; +, present; -, absent.

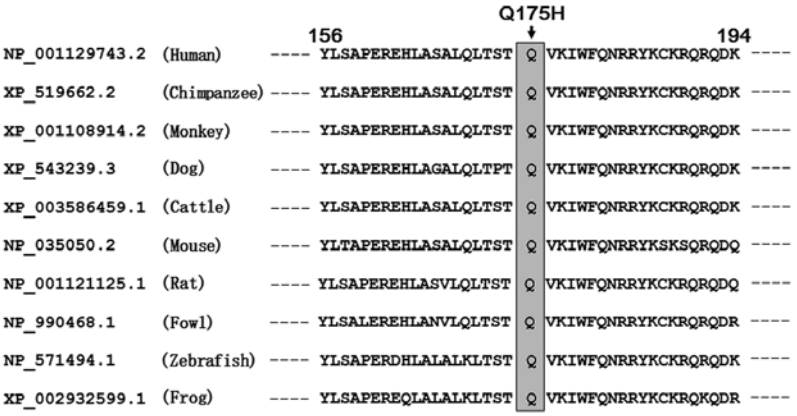

Figure 5. Multiple alignments of NKX2-6 protein sequences among species. The altered amino acid of p.Q175H is completely conserved evolutionarily across species.

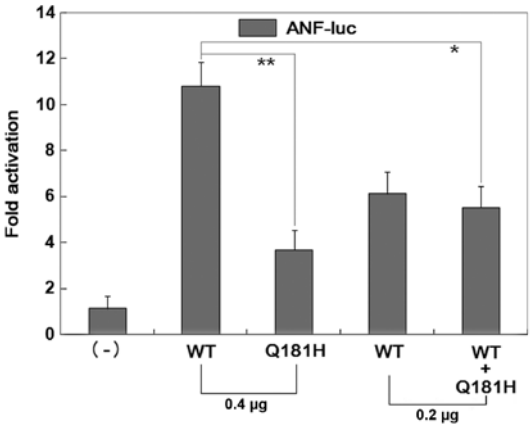

Figure 6. Q181H-mutant NKX2-5 shows impaired transcriptional activation. Activation of atrial natriuretic factor promoter driven luciferase reporter in COS-7 cells by wild-type (WT) or Q181H-mutant NKX2-5, alone or together, showed significantly reduced transcriptional activation by the mutant protein. Experiments were performed in triplicate, and mean and standard deviations are shown. **P<0.001 and *P<0.005, respectively, when compared with wild-type NKX2-5. WT, wild-type.

AAGTG. The homeodomain is centrally located at amino acid positions 132-191 and is predominantly involved in target DNA binding, nuclear translocation, as well as interaction with other transcription factors (104,106). The NKX2-6 mutation of p.Q175H identified in this study is located in the homeodomain, and thus may exert influence on the transcriptional activity of NKX2-6 by interfering with its nuclear distribution or DNA-binding ability.

In order to assess the functional characteristics of the Q175H homeodomain substitution in NKX2-6, NKX2-5 was selected as a surrogate based on the following reasons: firstly, no downstream genes or a binding site recognition sequence for NKX2-6 have been substantiated. Secondly, NKX2-5 and NKX2-6 are 2 members of the NK2 family of transcription factors, and they share a highly conserved structural motif, particularly for the homeodomain. Thirdly, biochemical assays of NKX2-5 have been well characterized. Finally, there are *in vivo* studies corroborating that NKX2-5 may compensate for the lack of NKX2-6 during embryogenesis, therefore suggesting that some DNA binding and transcriptional regulatory activities are shared (102-104). Consequently, functional analysis of Q181H-mutant NKX2-5 instead of Q175H NKX2-6 revealed that the homeodomain substitution significantly diminished transcriptional activity on a target gene, *ANF*. These findings suggest that the haploinsufficiency or dominant-negative effects caused by the *NKX2-6* mutation are potentially

alternative molecular pathological mechanisms responsible for the development of AF.

The findings that genetically compromised *NKX2-6* enhances the vulnerability to AF may be partially attributed to the abnormal cardiovascular development (65). During murine embryogenesis, NKX2-6 is expressed at opposite poles of the developing heart: from E8-8.5 in posterior myocardial progenitors, then the sinus venosa and dorsal pericardium, and from E9.5 in the outflow tract myocardium (102). Although the targeted disruption of *NKX2-6* has been shown to result in normal mouse embryos with no obvious heart malformations, *NKX2-5* mRNA expression expanded to the regions of *NKX2-6,* indicative of functional compensation for loss of *NKX2-6* in these embryos. Furthermore, overlapping functions for *NKX2-6* and *NKX2-5* had been verified in double knockout mouse embryos, in which the development of the atria was less advanced, indicating that these 2 genes are essential for cardiac development (103). Given the high degree of homology between NKX2-6 and NKX2-5 over the homeodomain (90% identity) and overlapping cardiac expression patterns, these data suggest the possibility that NKX2-6 and NKX2-5 synergistically regulate some target genes (104). In humans, mutations in both NKX2-5 and NKX2-6 have been associated with various congenital cardiovascular defects (66-68,104,105), and moreover, multiple NKX2-5 mutations have been causally implicated in AF (88-90), which suggest that mutant NKX2-6 may presumably contribute to the development of AF through a similar transcriptional mechanism.

In conclusion, to the best of our knowledge, the present study associates mutant NKX2-6 with enhanced susceptibility to AF for the first time, providing novel insight into the molecular pathogenesis of AF and suggesting potential strategies for the antenatal prophylaxis and personalized treatment of AF.

Acknowledgements

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