A novel NKX2.5 loss-of-function mutation responsible for familial atrial fibrillation

RI-TAI HUANG[1], SONG XUE[1], YING-JIA XU[2], MIN ZHOU[1] and YI-QING YANG[3]

[1]Department of Cardiothoracic Surgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200127; Departments of [2]Cardiology and [3]Cardiovascular Research, Shanghai Chest Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200030, P.R. China

Received December 21, 2012; Accepted February 27, 2013

DOI: 10.3892/ijmm.2013.1316

Abstract. Atrial fibrillation (AF) represents the most common form of sustained cardiac arrhythmia and accounts for substantial morbidity and mortality. Increasing evidence demonstrates that abnormal cardiovascular development is involved in the pathogenesis of AF. In this study, the coding exons and splice sites of the *NKX2.5* gene, which encodes a homeodomain-containing transcription factor pivotal for normal cardiovascular morphogenesis, were sequenced in 110 unrelated index patients with familial AF. The available relatives of the mutation carrier and 200 unrelated ethnically-matched healthy individuals serving as controls were subsequently genotyped. The disease-causing potential of the identified *NKX2.5* variation was predicted by MutationTaster. The functional characteristics of the mutant NKX2.5 protein were analyzed using a dual-luciferase reporter assay system. As a result, a novel heterozygous NKX2.5 mutation, p.F145S, was identified in a family with AF transmitted as an autosomal dominant trait, which co-segregated with AF in the family with complete penetrance. The detected substitution, which altered the amino acid completely conserved evolutionarily across species, was absent in 400 control chromosomes and was automatically predicted to be causative. Functional analysis demonstrated that the NKX2.5 mutant was associated with significantly decreased transcriptional activity compared with its wild-type counterpart. To the best of our knowledge, this is the first report on the association of the NKX2.5 loss-of-function mutation with increased susceptibility to familial AF. The findings of the present study provide novel insights into the molecular mechanism underlying AF, suggesting the potential implications for the early prophylaxis and allele-specific therapy of AF.

Correspondence to: Professor Song Xue, Department of Cardiothoracic Surgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, P.R. China
E-mail: xuesong64@163.com

Dr Yi-Qing Yang, Department of Cardiovascular Research, Shanghai Chest Hospital, Shanghai Jiaotong University School of Medicine, 241 West Huaihai Road, Shanghai 200030, P.R. China
E-mail: yang99yang66@hotmail.com

Key words: arrhythmia, atrial fibrillation, genetics, transcription factor, NKX2.5, reporter gene analysis

Introduction

Atrial fibrillation (AF), typically characteristic of rapid and chaotic electrical activity in the atria with subsequent desynchronized atrial contractions, constitutes the most common type of cardiac arrhythmia in the setting of clinical practice, accounting for approximately one-third of hospitalizations for irregular heart rhythms (1). The prevalence of AF is approximately 1% in the general population, and increases markedly with advancing age, from approximately 0.5% of individuals in their fifties to nearly 10% of the octogenarian population (2). According to the Framingham Heart Study, the lifetime risk for development of AF is projected to be 25% for persons over the age of 40 (3). Currently, 2.5 million Americans are suffering from AF, but with the aging population and improved cardiovascular survival, that number is expected to exceed 16 million by the year 2050 (4). AF is responsible for substantially increased cardiovascular morbidity and mortality. Patients with AF have a 5-fold increased risk of stroke, and it is estimated that 15-20% of all strokes are attributable to AF (5). The risk of cerebrovascular thromboembolism ascribed to AF also increases strikingly with advancing age, rising from 1.5% at 50-59 years of age to 23.5% at 80-89 years of age (5). AF independently increases the risk of congestive heart failure and increases the risk of mortality 2-fold (6). Additionally, AF may contribute to complications such as adverse hemodynamics, reduced exercise tolerance, degraded quality of life, impaired cognition or dementia, and tachycardia-induced cardiomyopathy (7). Therefore, AF has become an immense and growing public health burden. Only in the United States, the costs attributable to the care of individuals with nonvalvular AF are in excess of $6.4 billion/year (8). Despite the high prevalence and clinical significance, the molecular mechanism underlying AF remains poorly understood.

Traditionally, AF has been perceived as a condition that occurs in the context of atrial electrical and structural remodeling that can result from miscellaneous cardiac and systemic disorders, including hypertension, coronary artery disease, rheumatic heart disease, congenital heart disease, chronic

pulmonary heart disease, cardiomyopathy, cardiac surgery, diabetes mellitus, hyperthyroidism and electrolyte imbalance (1). However, in 30-45% of AF patients, no obvious risk factors can be identified by routine medical examination, and such AF is termed 'idiopathic' or 'lone' (1).; up to 15% of these cases have a positive family history, and are thus defined as familial AF (9). Increasing evidence demonstrates the familial aggregation of AF and an enhanced vulnerability to AF in the close relatives of patients with AF, suggesting that genetic defects may be involved in the pathogenesis of AF in a subset of patients (10-16). Genome-wide genetic linkage analysis with highly polymorphic microsatellite markers mapped susceptibility loci for AF on human chromosomes 10q22, 6q14-16, 11p15.5, 5p13, 10p11-q21 and 5p15, of which AF-causing mutations in two genes, *KCNQ1* on chromosome 11p15.5 and *NUP155* on chromosome 5p13, were identified and functionally characterized (17-23). In addition, genetic screening of candidate genes revealed an increasing number of AF associated mutations in genes encoding potassium channel subunits (*KCNH2, KCNA5, KCNJ2, KCNJ8* and *KCNE1-5*), sodium channel subunits (*SCN5A* and *SCN1B-3B*), signaling peptide (NPPA), gap junctions (*GJA1* and *GJA5*), and others (24-44). Nevertheless, these causative mutations appear to be relatively rare causes of AF, and the genetic determinants for AF in an overwhelming majority of patients remain elusive.

Emerging evidence indicates that abnormal embryological development of the cardiovascular system, particularly the pulmonary venous myocardium, is a major anatomic substrate for AF (45). Developmental biology studies substantiate the key role for several transcription factors, including NKX2.5, GATA4, GATA5 and GATA6, in the normal cardiovascular morphogenesis (46-48), and multiple mutations in GATA4, GATA5 and GATA6 have been causally associated with AF (49-57). NKX2.5 is a member of the NK2-family of transcription factors and its expression and functions overlap with those of the GATA family during cardiovascular development, particularly in synergistic regulation of target gene expressions cooperatively with GATA4 (58), which justifies *NKX2.5* as a prime candidate gene for AF.

To evaluate the prevalence of *NKX2.5* mutations in patients with familial AF and to explore the mechanism by which mutated *NKX2.5* results in or predisposes to AF, the coding exons and exon/intron boundaries of *NKX2.5* were sequenced in patients with familial AF in contrast to control individuals, and the functional characteristics of the mutant NKX2.5 were assessed in comparison with its wild-type counterpart using a dual-luciferase reporter assay system.

Materials and methods

Study subjects. A cohort of 110 unrelated index patients with familial AF was recruited from the Han Chinese population in China. The available relatives of the probands were enrolled. A total of 200 ethnically-matched unrelated healthy individuals were enlisted as controls. 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 (9,57). Briefly, AF was diagnosed by a standard 12-lead electrocardiogram demonstrating no P-waves and irregular R-R intervals irrespective 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 referred to as the presence of documented lone AF in additional two or more first- or second-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 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, regardless 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 more than seven days and requiring either pharmacologic therapy or electrical 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.

Genetic screening. Genomic DNA from all participants was isolated from blood lymphocytes with the Wizard Genomic DNA Extraction Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Initially, the whole coding region and splice junctions of the *NKX2.5* gene were sequenced in 110 unrelated index patients with familial AF. Subsequently, genotyping *NKX2.5* in the available relatives of the mutation carrier and 200 ethnically-matched unrelated healthy individuals who served as controls, was performed. The referential genomic DNA sequence of *NKX2.5* was derived from GenBank (accession no. NT_023133), a gene sequence database at the National Center for Biotechnical Information (NCBI; http://www.ncbi.nlm.nih.gov/). Using the online Primer 3 software (http://frodo.wi.mit.edu/), the primer pairs used to amplify the coding exons (exon 1-2) and intron-exon boundaries of *NKX2.5* by polymerase chain reaction (PCR) were designed as follows: primer 1, forward, 5'-CAC GAT GCA GGG AAG CTG-3' and reverse, 5'-AGT TTC TTG GGG ACG AAA GC-3' (the PCR product was 477 base pairs in size); primer 2, forward, 5'-CCT CCA CGA GGA TCC CTT AC-3' and reverse, 5'-CGA GTC CCC TAG GCA TGG-3' (the product was 463 base pairs); primer 3, forward, 5'-AGA ACC GGC GCT ACA AGT G-3' and reverse, 5'-GAG TCA GGG AGC TGT TGA GG-3' (the product was 473 base pairs). The PCR was performed using HotStar TaqDNA Polymerase (Qiagen, Hilden, Germany) on a Veriti 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 on an ABI-PRISM 3130xl DNA Analyzer (both from Applied Biosystems). The sequencing primers were the same as mentioned above for the specific region amplifications. DNA sequences were analyzed

with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was corroborated by re-sequencing of an independent PCR-generated amplicon from the subject and met the quality control threshold with a call rate exceeding 99%. For an identified sequence variant, the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) and NCBI's single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) online databases were queried to confirm its novelty.

Multiple alignments of NKX2.5 protein sequences across species. Conservation of the amino acid altered by missense mutation was appraised by aligning human NKX2.5 to chimpanzee, monkey, dog, cow, mouse, rat, chicken and zebrafish NKX2.5 using the HomoloGene and Show Multiple Alignment links on the NCBI's website (http://www.ncbi.nlm.nih.gov/homologene).

Prediction of the disease-causing potential of an NKX2.5 sequence variation. The disease-causing potential of an *NKX2.5* sequence variation was predicted by an online program, MutationTaster (http://www.mutationtaster.org), automatically giving a probability for an alteration to be either a pathogenic mutation or a benign polymorphism. Notably, the P-value given here is the probability of the prediction rather than the probability of error as used in t-test statistics, i.e., a value close to 1 indicates a high 'security' of the prediction.

Expression plasmids and site-directed mutagenesis. The recombinant expression plasmid NKX2.5-pEFSA and the atrial natriuretic factor (ANF)-luciferase reporter plasmid, which contains the 2600-bp 5'-flanking region of the *ANF* gene, i.e., ANF(-2600)-Luc, were kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chuo-ku, Chiba, Japan. The identified mutation was introduced into the wild-type *NKX2.5* using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

Luciferase reporter gene assays. COS-7 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 (hRluc/CMV; Promega) were used in transient transfection assays to examine the transcriptional activation function of the *NKX2.5* mutant. COS-7 cells were transfected with 0.4 μg of wild-type or mutant NKX2.5-pEFSA expression vector, 1.0 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 NKX2.5-pEFSA, 0.2 μg of mutant NKX2.5-pEFSA or empty vector pEFSA, 1.0 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. 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 NKX2.5.

Table I. Baseline demographics and clinical characteristics of the 110 unrelated probands with familial AF.

Parameters	Statistics
Baseline demographics	
Age at first diagnosis of AF (years)	42±6
Male (n, %)	71 (65)
Body mass index (kg/m^2)	23±2
Left ventricular ejection fraction (%)	64±5
Left atrial diameter (mm)	36±4
Personal history of AF (n, %)	
Type of AF at presentation	
Paroxysmal	63 (57)
Persistent	26 (24)
Permanent	21 (19)
History of cardioversion	86 (78)
Medical history (n, %)	
History of syncope	8 (7)
History of pacemaker	6 (5)
Thromboembolic complication	5 (5)
Hyperlipidemia	5 (5)
Arterial hypertension	4 (4)
Diabetes	2 (2)
Medications (n, %)	
Warfarin	70 (64)
Amiodarone	61 (55)
Digitalis	13 (12)
Aspirin	11 (10)
β-blocker	7 (6)
Calcium channel blocker	3 (3)

AF, atrial fibrillation.

Statistical analysis. Data are expressed as the 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 two-tailed P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the study subjects. A cohort of 110 unrelated index patients with familial AF and a total of 200 ethnically-matched unrelated healthy individuals used as controls were enrolled and clinically evaluated. None of the participants had apparent traditional risk factors for AF. There was no significant difference between patient and control groups in baseline characteristics including age, gender, body mass index, blood pressure, fasting blood glucose, serum lipid, left atrial dimension, left ventricular ejection fraction, heart rate at rest, as well as life style (data not shown). In the present study, four patients

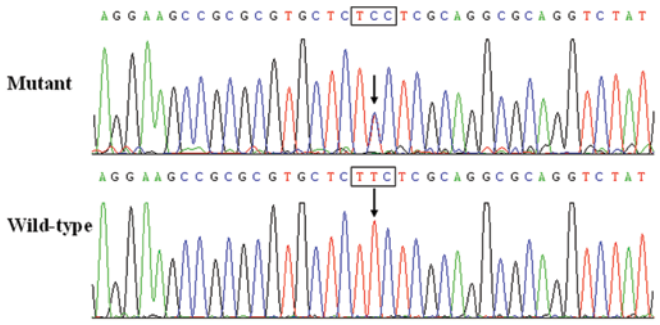

Figure 1. Sequence electropherograms showing the *NKX2.5* mutation and its corresponding control. The arrow indicates the heterozygous nucleotides of T/C in the index patient from Family 1 (mutant) or the homozygous nucleotides of T/T in the corresponding control individual (wild-type). The rectangle denotes the nucleotides comprising a codon of NKX2.5.

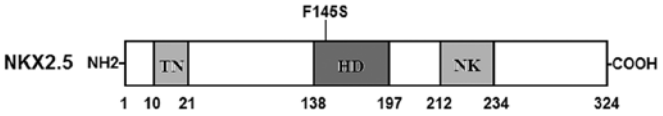

Figure 2. Schematic diagram of NKX2.5 protein structure depicting the location of a novel mutation. The mutation identified in patients with familial atrial fibrillation is noted above the diagram of the NKX2.5 protein. NH2, amino-terminus; TN, tinman domain; HD, homeodomain; NK, nucleotide kinase domain; and COOH, carboxyl-terminus.

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) is 140 or 90 mm Hg, respectively, but at the time of initial diagnosis of AF, their blood pressures were normal. The baseline clinical characteristics of the 110 index patients with familial AF are summarized in Table I.

NKX2.5 mutation. Direct sequencing of the coding exons and flanking introns of the *NKX2.5* gene was carried out following PCR amplification of genomic DNA from the 110 index patients with familial AF. A heterozygous *NKX2.5* mutation was identified in 1 out of 110 unrelated index patients, with a mutational prevalence of ~0.91% based on the patient cohort. In particular, a substitution of cytosine (C) for thymine (T) in the second nucleotide of codon 145 (c.434T>C), predicting the transition of phenylalanine (F) into serine (S) at amino acid 145 (p.F145S) was identified in an index patient from family 1. The sequence chromatograms showing the detected heterozygous *NKX2.5* mutation of c.434T>C compared with corresponding control sequence are shown in Fig. 1. A schematic diagram of NKX2.5 delineating the putative structural domains and location of the mutation identified in AF patients is presented in Fig. 2. The missense mutation was not found in the control population nor was it reported in the EVS's and NCBI's SNP databases. Genetic scan of the available family members of the mutation carrier showed that the mutation was present in all affected family members alive, but was absent in unaffected family members examined. Analysis of the pedigree showed that the mutation cosegregated with AF transmitted as an autosomal dominant trait in the family with complete penetrance. The pedigree structure of the family is presented in Fig. 3. The

Table II. Phenotypic characteristics and status of the NKX2.5 mutation of 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 (ms)	QT/QTc	LAD (mm)	LVEF (%)	NKX2.5 mutations
Family 1										F145S
I-1	M	72[a]	40	Permanent	73	116	432/475	42	58	N/A
II-2	F	54	32	Permanent	66	112	408/427	38	68	+/-
II-5	M	49	22	Persistent	79	94	360/412	36	62	+/-
III-1	M	28	28	Paroxysmal	75	96	394/439	33	64	+/-

AF, atrial fibrillation; F, female; M, male; QTc, corrected QT interval; N/A, not available or not applicable; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; +, indicates presence of mutation and -, denotes absence of mutation. [a]Age of death.

Table III. *NKX2.5* sequence variations identified in this study.

Exon	Nucleotide	Amino acid	Allele frequency	
			Patients	Controls
Exon 1	c.63A>G	p.E21E	43/220 (0.195)	82/400 (0.205)
Exon 2	c.434T>C	p.F145S	1/220 (0.005)	0/400 (0.000)
Exon 2	c.606C>G	p.L202L	4/220 (0.018)	6/400 (0.015)

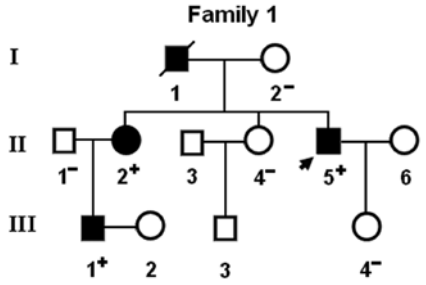

Figure 3. Pedigree structure of the family with familial AF, designated as family 1. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; closed symbols, affected members; open symbols, unaffected members; symbol with a slash, deceased member; arrow, proband; +, carriers of the heterozygous mutation; -, non-carriers.

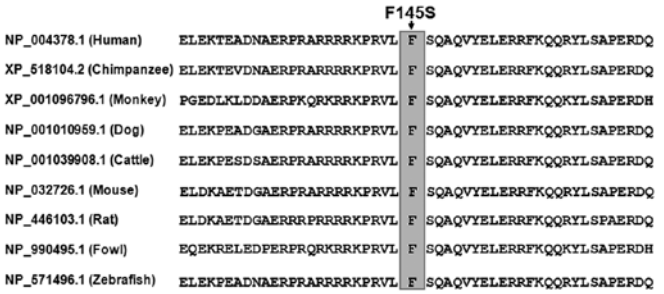

Figure 4. Multiple alignments of NKX2.5 protein sequences across various species. The altered amino acid p.F145 is completely conserved evolutionarily.

phenotypic characteristics and results of genetic screening of the affected family members are listed in Table II.

Notably, congenital atrial septal defect was confirmed by the early echocardiogram in patient I-1 and patient II-2 from family 1. In addition, two previously reported *NKX2.5* sequence polymorphisms, including c.63A>G and c.606C>G, were observed in both AF patients and control individuals. However, there was no significant difference in either of the two allele frequencies between the AF patient and healthy control groups. All the sequence variants and their allele frequencies are listed in Table III.

Alignment of multiple NKX2.5 protein sequences. A cross-species alignment of NKX2.5 protein sequences displayed that the altered amino acid was completely conserved evolutionarily, underscoring its functional importance (Fig. 4).

Disease-causing potential of the NKX2.5 variation. The sequence variation of c.434T>C detected in *NKX2.5* was automatically predicted to be a disease-causing mutation with a P-value of 0.999997. No SNP in the altered region was found in the MutationTaster database.

Transcriptional activity of the NKX2.5 mutant. The transcriptional activation function of the mutated NKX2.5 in COS-7 cells was characterized using a luciferase reporter, which was driven by the promoter of *ANP*, one of NKX2.5-directed cardiac target genes. The activity of the *ANP* promoter was expressed as fold activation of Firefly luciferase relative to Renilla luciferase. The same amounts of wild-type (0.4 μg) and F145S-mutant *NKX2.5* (0.4 μg) activated the ANP promoter by

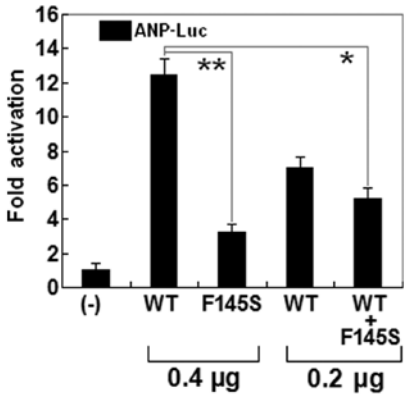

Figure 5. Functional defects associated with the NKX2.5 mutation. Activation of ANF-luciferase reporter in COS-7 cells by NKX2.5 wild-type (WT), or mutant, alone or in combination, demonstrated significantly reduced transactivational activity by mutant protein. Experiments were performed in triplicate and means ± standard deviations are shown. *P<0.0005 and **P<0.0001, when compared with wild-type NKX2.5.

~12- and ~3-fold, respectively. When the same amount of wild-type *NKX2.5* (0.2 μg) was cotransfected with F145S-mutant *NKX2.5* (0.2 μg), the induced activation of the ANP promoter was ~5-fold. These results suggest that the NKX2.5 mutation results in a significantly reduced transcriptional activation compared with its wild-type counterpart (Fig. 5).

Discussion

In the present study, a novel heterozygous NKX2.5 mutation, p.F145S, was identified in a family with familial AF. This missense mutation was present in all the affected family members examined but was absent in the unaffected family

members available and in the 400 normal chromosomes from a matched control population. A cross-species alignment of multiple NKX2.5 protein sequences exhibited that the altered amino acid was completely conserved evolutionarily. Functional analysis demonstrated that the mutant NKX2.5 was associated with a significantly decreased transcriptional activity. Therefore, it is highly likely that functionally impaired NKX2.5 is involved in the pathogenesis of AF in this family. To our knowledge, this is the first report on the relationship between NKX2.5 loss-of-function mutation and enhanced susceptibility to AF.

NKX2.5 was first discovered as a homologue of tinman, a *Drosophila* cardiac transcription factor. It shows high expression in early embryonic heart progenitor cells and persists through adulthood, indispensable for proper cardiovascular development and maturation (46). The human NKX2.5 gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids (46). The NKX2.5 protein contains a homeodomain (HD), an evolutionarily conserved domain that recognizes and binds to a consensus DNA motif, AAGTG. In addition to the HD, NKX2.5 contains N- and C-terminal regulatory domains. The HD is centrally located at amino acid positions 138-197 and is involved in nuclear translocation and interaction with other transcription factors as well as DNA binding (59). The NKX2.5 mutation of p.F145S identified in this study is located in HD, and may thus be expected to exert influence on the transcriptional activity of NKX2.5 by interfering with its nuclear distribution or DNA-binding ability.

Previous investigations revealed that NKX2.5 is an upstream regulator of several genes expressed during embryogenesis including the genes that encode ANF, brain natriuretic peptide and α-cardiac actin (60). As a well-known NKX2.5 downstream target molecule, ANF contains several NKX2.5 binding sites in its proximal promoter region, including -242 from the transcription start site, which has been confirmed as an *in vivo* binding site of NKX2.5 (61). Therefore, the functional characteristics of the NKX2.5 mutation can be explored by analysis of the transcriptional activity of the *ANF* promoter in cells transfected with NKX2.5 mutant in contrast to its wild-type counterpart. In this study, the functional role of the novel p.F145S mutation of NKX2.5 identified in familial AF patients was analyzed by transcriptional activity assays and the results demonstrated a significantly decreased transcriptional activity on a downstream gene. These findings indicate that NKX2.5 haploinsufficiency caused by mutation is potentially an alternative pathophysiological mechanism of AF.

The finding that functionally compromised NKX2.5 predisposes to AF may be partially due to the abnormally developed pulmonary vein myocardium. The pulmonary venous vessel is ensheathed by a layer of myocardium referred to as pulmonary myocardial sleeve, which has been verified to be responsible for the initiation and perpetuation of AF by several potential arrhythmogenic mechanisms including enhanced intrinsic pacemaker activity and liability to reentrance (62-64). Genetic-labeling lineage tracing studies have validated that NKX2.5 is expressed in the atria as well as in the pulmonary myocardium and is essential for the localized formation of the sinoatrial node during embryogenesis. NKX2.5 may functionally serve as a suppressor of the sinoatrial node lineage gene program, which limits pacemaker activity to the sinoatrial and atrioventricular nodes. When the level of NKX2.5 protein decreased in a hypomorphic model, the pulmonary cardiomyocytes shifted to connexin40-negative, HCN4-positive cells, a nodal-like phenotype with pacemaker activity (63). In *NKX2.5*-null mouse embryos, *HCN4* was activated along the entire embryonic heart tube, whereas *connexin40* expression was inhibited, ectopic pacemaker cells were observed throughout the heart tube (64). Hence, NKX2.5 loss-of-function mutation presumably contributes to formation of the pulmonary myocardium sleeve and switch of the pulmonary myocardium to a sinoatrial node-like phenotype, creating an atrial electrophysiological matrix in favor of AF.

There are some downstream genes transactivated by NKX2.5, and mutations in several target genes have been linked to AF, including the genes encoding ANF and gap junction protein connexin40 (38,39,41-43). Therefore, it is probable that mutated NKX2.5 confers susceptibility to AF by decreasing expressions of target genes.

It is of note that congenital atrial septal defect was documented in 2 AF patients carrying p.F145S mutation of NKX2.5. Similarly, congenital cardiovascular malformations were previously confirmed in AF patients carrying GATA4, GATA5 or GATA6 mutations (49-57). Markedly, a long list of mutations in these genes has been implicated in a wide variety of congenital cardiovascular anomalies (65-75). These observational results indicate that AF may share a common genetic origin with congenital heart disease.

In conclusion, the findings of the present study provide novel insights into the molecular mechanism of AF, suggesting potential implications for early prophylaxis and allele-specific therapy of this common arrhythmia.

Acknowledgements

The authors are grateful to the participants for their dedication to the study. This study was supported by grants from the National Natural Science Fund of China (81270161, 81070153 and 30570768), the National Basic Research Program of China (2010CB912604), the Personnel Development Foundation of Shanghai, China (2010019), and the Key Program of Basic Research of Shanghai, China (10JC1414000, 10JC1414001 and 10JC1414002).

References

1. Fuster V, Rydén LE, Cannom DS, Crijns HJ, Curtis AB, Ellenbogen KA, Halperin JL, Kay GN, Le Huezey JY, Lowe JE, Olsson SB, Prystowsky EN, Tamargo JL, Wann LS, Smith SC Jr, Priori SG, Estes NA III, Ezekowitz MD, Jackman WM, January CT, Lowe JE, Page RL, Slotwiner DJ, Stevenson WG, Tracy CM, Jacobs AK, Anderson JL, Albert N, Buller CE, Creager MA, Ettinger SM, Guyton RA, Halperin JL, Hochman JS, Kushner FG, Ohman EM, Stevenson WG, Tarkington LG and Yancy CW; American College of Cardiology Foundation/American Heart Association Task Force: 2011 ACCF/AHA/HRS focused updates incorporated into the ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. Circulation 123: e269-e367, 2011.
2. Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV and Singer DE: Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. JAMA 285: 2370-2375, 2001.

3. Lloyd-Jones DM, Wang TJ, Leip EP, Larson MG, Levy D, Vasan RS, D'Agostino RB, Massaro JM, Beiser A, Wolf PA and Benjamin EJ: Lifetime risk for development of atrial fibrillation: the Framingham Heart Study. Circulation 110: 1042-1046, 2004.
4. Miyasaka Y, Barnes ME, Gersh BJ, Cha SS, Bailey KR, Abhayaratna WP, Seward JB and Tsang TS: Secular trends in incidence of atrial fibrillation in Olmsted County, Minnesota, 1980 to 2000, and implications on the projections for future prevalence. Circulation 114: 119-125, 2006.
5. Wolf PA, Abbott RD and Kannel WB: Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. Stroke 22: 983-988, 1991.
6. Benjamin EJ, Wolf PA, D'Agostino RB, Silbershatz H, Kannel WB and Levy D: Impact of atrial fibrillation on the risk of death: the Framingham Heart Study. Circulation 98: 946-952, 1998.
7. Magnani JW, Rienstra M, Lin H, Sinner MF, Lubitz SA, McManus DD, Dupuis J, Ellinor PT and Benjamin EJ: Atrial fibrillation: current knowledge and future directions in epidemiology and genomics. Circulation 124: 1982-1993, 2011.
8. Coyne KS, Paramore C, Grandy S, Mercader M, Reynolds M and Zimetbaum P: Assessing the direct costs of treating nonvalvular atrial fibrillation in the United States. Value Health 9: 348-356, 2006.
9. Darbar D, Herron KJ, Ballew JD, Jahangir A, Gersh BJ, Shen WK, Hammill SC, Packer DL and Olson TM: Familial atrial fibrillation is a genetically heterogeneous disorder. J Am Coll Cardiol 41: 2185-2192, 2003.
10. Ellinor PT, Yoerger DM, Ruskin JN and MacRae CA: Familial aggregation in lone atrial fibrillation. Hum Genet 118: 179-184, 2005.
11. Arnar DO, Thorvaldsson S, Manolio TA, Thorgeirsson G, Kristjansson K, Hakonarson H and Stefansson K: Familial aggregation of atrial fibrillation in Iceland. Eur Heart J 27: 708-712, 2006.
12. Junttila MJ, Raatikainen MJ, Perkiömäki JS, Hong K, Brugada R and Huikuri HV: Familial clustering of lone atrial fibrillation in patients with saddleback-type ST-segment elevation in right precordial leads. Eur Heart J 28: 463-468, 2007.
13. Christophersen IE, Ravn LS, Budtz-Joergensen E, Skytthe A, Haunsoe S, Svendsen JH and Christensen K: Familial aggregation of atrial fibrillation: a study in Danish twins. Circ Arrhythm Electrophysiol 2: 378-383, 2009.
14. Yang YQ, Zhang XL, Wang XH, Tan HW, Shi HF, Fang WY and Liu X: Familial aggregation of lone atrial fibrillation in the Chinese population. Intern Med 49: 2385-2391, 2010.
15. Lubitz SA, Yin X, Fontes JD, Magnani JW, Rienstra M, Pai M, Villalon ML, Vasan RS, Pencina MJ, Levy D, Larson MG, Ellinor PT and Benjamin EJ: Association between familial atrial fibrillation and risk of new-onset atrial fibrillation. JAMA 304: 2263-2269, 2010.
16. Fox CS, Parise H, D'Agostino RB Sr, Lloyd-Jones DM, Vasan RS, Wang TJ, Levy D, Wolf PA and Benjamin EJ: Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring. JAMA 291: 2851-2855, 2004.
17. Brugada R, Tapscott T, Czernuszewicz GZ, Marian AJ, Iglesias A, Mont L, Brugada J, Girona J, Domingo A, Bachinski LL and Roberts R: Identification of a genetic locus for familial atrial fibrillation. N Engl J Med 336: 905-911, 1997.
18. Ellinor PT, Shin JT, Moore RK, Yoerger DM and MacRae CA: Locus for atrial fibrillation maps to chromosome 6q14-16. Circulation 107: 2880-2883, 2003.
19. Chen YH, Xu SJ, Bendahhou S, Wang XL, Wang Y, Xu WY, Jin HW, Sun H, Su XY, Zhuang QN, Yang YQ, Li YB, Liu Y, Xu HJ, Li XF, Ma N, Mou CP, Chen Z, Barhanin J and Huang W: KCNQ1 gain-of-function mutation in familial atrial fibrillation. Science 299: 251-254, 2003.
20. Oberti C, Wang L, Li L, Dong J, Rao S, Du W and Wang Q: Genome-wide linkage scan identifies a novel genetic locus on chromosome 5p13 for neonatal atrial fibrillation associated with sudden death and variable cardiomyopathy. Circulation 110: 3753-3759, 2004.
21. Zhang X, Chen S, Yoo S, Chakrabarti S, Zhang T, Ke T, Oberti C, Yong SL, Fang F, Li L, de la Fuente R, Wang L, Chen Q and Wang QK: Mutation in nuclear pore component NUP155 leads to atrial fibrillation and early sudden cardiac death. Cell 135: 1017-1027, 2008.
22. Volders PG, Zhu Q, Timmermans C, Eurlings PM, Su X, Arens YH, Li L, Jongbloed RJ, Xia M, Rodriguez LM and Chen YH: Mapping a novel locus for familial atrial fibrillation on chromosome 10p11-q21. Heart Rhythm 4: 469-475, 2007.
23. Darbar D, Hardy A, Haines JL and Roden DM: Prolonged signal-averaged P-wave duration as an intermediate phenotype for familial atrial fibrillation. J Am Coll Cardiol 51: 1083-1089, 2008.
24. Olesen MS, Bentzen BH, Nielsen JB, Steffensen AB, David JP, Jabbari J, Jensen HK, Haunsø S, Svendsen JH and Schmitt N: Mutations in the potassium channel subunit KCNE1 are associated with early-onset familial atrial fibrillation. BMC Med Genet 13: 24, 2012.
25. Yang Y, Xia M, Jin Q, Bendahhou S, Shi J, Chen Y, Liang B, Lin J, Liu Y, Liu B, Zhou Q, Zhang D, Wang R, Ma N, Su X, Niu K, Pei Y, Xu W, Chen Z, Wan H, Cui J, Barhanin J and Chen Y: Identification of a KCNE2 gain-of-function mutation in patients with familial atrial fibrillation. Am J Hum Genet 75: 899-905, 2004.
26. Lundby A, Ravn LS, Svendsen JH, Hauns S, Olesen SP and Schmitt N: KCNE3 mutation V17M identified in a patient with lone atrial fibrillation. Cell Physiol Biochem 21: 47-54, 2008.
27. Zeng Z, Tan C, Teng S, Chen J, Su S, Zhou X, Wang F, Zhang S, Gu D, Makielski JC and Pu J: The single nucleotide polymorphisms of I(Ks) potassium channel genes and their association with atrial fibrillation in a Chinese population. Cardiology 108: 97-103, 2007.
28. Ravn LS, Aizawa Y, Pollevick GD, Hofman-Bang J, Cordeiro JM, Dixen U, Jensen G, Wu Y, Burashnikov E, Haunso S, Guerchicoff A, Hu D, Svendsen JH, Christiansen M and Antzelevitch C: Gain of function in IKs secondary to a mutation in KCNE5 associated with atrial fibrillation. Heart Rhythm 5: 427-435, 2008.
29. Hong K, Bjerregaard P, Gussak I and Brugada R: Short QT syndrome and atrial fibrillation caused by mutation in KCNH2. J Cardiovasc Electrophysiol 16: 394-396, 2005.
30. Xia M, Jin Q, Bendahhou S, He Y, Larroque MM, Chen Y, Zhou Q, Yang Y, Liu Y, Liu B, Zhu Q, Zhou Y, Lin J, Liang B, Li L, Dong X, Pan Z, Wang R, Wan H, Qiu W, Xu W, Eurlings P, Barhanin J and Chen Y: A Kir2.1 gain-of-function mutation underlies familial atrial fibrillation. Biochem Biophys Res Commun 332: 1012-1019, 2005.
31. Delaney JT, Muhammad R, Blair MA, Kor K, Fish FA, Roden DM and Darbar D: A KCNJ8 mutation associated with early repolarization and atrial fibrillation. Europace 14: 1428-1432, 2012.
32. Olson TM, Alekseev AE, Liu XK, Park S, Zingman LV, Bienengraeber M, Sattiraju S, Ballew JD, Jahangir A and Terzic A: Kv1.5 channelopathy due to KCNA5 loss-of-function mutation causes human atrial fibrillation. Hum Mol Genet 15: 2185-2191, 2006.
33. Yang Y, Li J, Lin X, Yang Y, Hong K, Wang L, Liu J, Li L, Yan D, Liang D, Xiao J, Jin H, Wu J, Zhang Y and Chen YH: Novel KCNA5 loss-of-function mutations responsible for atrial fibrillation. J Hum Genet 54: 277-283, 2009.
34. Olson TM, Michels VV, Ballew JD, Reyna SP, Karst ML, Herron KJ, Horton SC, Rodeheffer RJ and Anderson JL: Sodium channel mutations and susceptibility to heart failure and atrial fibrillation. JAMA 293: 447-454, 2005.
35. Watanabe H, Darbar D, Kaiser DW, Jiramongkolchai K, Chopra S, Donahue BS, Kannankeril PJ and Roden DM: Mutations in sodium channel beta1- and beta2-subunits associated with atrial fibrillation. Circ Arrhythm Electrophysiol 2: 268-275, 2009.
36. Wang P, Yang Q, Wu X, Yang Y, Shi L, Wang C, Wu G, Xia Y, Yang B, Zhang R, Xu C, Cheng X, Li S, Zhao Y, Fu F, Liao Y, Fang F, Chen Q, Tu X and Wang QK: Functional dominant-negative mutation of sodium channel subunit gene SCN3B associated with atrial fibrillation in a Chinese GeneID population. Biochem Biophys Res Commun 398: 98-104, 2010.
37. Olesen MS, Jespersen T, Nielsen JB, Liang B, Møller DV, Hedley P, Christiansen M, Varró A, Olesen SP, Haunsø S, Schmitt N and Svendsen JH: Mutations in sodium channel β-subunit SCN3B are associated with early-onset lone atrial fibrillation. Cardiovasc Res 89: 786-793, 2011.
38. Hodgson-Zingman DM, Karst ML, Zingman LV, Heublein DM, Darbar D, Herron KJ, Ballew JD, de Andrade M, Burnett JC Jr and Olson TM: Atrial natriuretic peptide frameshift mutation in familial atrial fibrillation. N Engl J Med 359: 158-165, 2008.
39. Ren X, Xu C, Zhan C, Yang Y, Shi L, Wang F, Wang C, Xia Y, Yang B, Wu G, Wang P, Li X, Wang D, Xiong X, Liu J, Liu Y, Liu M, Liu J, Tu X and Wang QK: Identification of NPPA variants associated with atrial fibrillation in a Chinese GeneID population. Clin Chim Acta 411: 481-485, 2010.

40. Thibodeau IL, Xu J, Li Q, Liu G, Lam K, Veinot JP, Birnie DH, Jones DL, Krahn AD, Lemery R, Nicholson BJ and Gollob MH: Paradigm of genetic mosaicism and lone atrial fibrillation: physiological characterization of a connexin 43-deletion mutant identified from atrial tissue. Circulation 122: 236-244, 2010.
41. Gollob MH, Jones DL, Krahn AD, Danis L, Gong XQ, Shao Q, Liu X, Veinot JP, Tang AS, Stewart AF, Tesson F, Klein GJ, Yee R, Skanes AC, Guiraudon GM, Ebihara L and Bai D: Somatic mutations in the connexin 40 gene (GJA5) in atrial fibrillation. N Engl J Med 354: 2677-2688, 2006.
42. Yang YQ, Zhang XL, Wang XH, Tan HW, Shi HF, Jiang WF, Fang WY and Liu X: Connexin40 nonsense mutation in familial atrial fibrillation. Int J Mol Med 26: 605-610, 2010.
43. Yang YQ, Liu X, Zhang XL, Wang XH, Tan HW, Shi HF, Jiang WF and Fang WY: Novel connexin40 missense mutations in patients with familial atrial fibrillation. Europace 12: 1421-1427, 2010.
44. Mahida S, Lubitz SA, Rienstra M, Milan DJ and Ellinor PT: Monogenic atrial fibrillation as pathophysiological paradigms. Cardiovasc Res 89: 692-700, 2011.
45. Mommersteeg MT, Christoffels VM, Anderson RH and Moorman AF: Atrial fibrillation: a developmental point of view. Heart Rhythm 6: 1818-1824, 2009.
46. Akazawa H and Komuro I: Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases. Pharmacol Ther 107: 252-268, 2005.
47. Pikkarainen S, Tokola H, Kerkelä R, and Ruskoaho H: GATA transcription factors in the developing and adult heart. Cardiovasc Res 63: 196-207, 2004.
48. Peterkin T, Gibson A, Loose M and Patient R: The roles of GATA-4, -5 and -6 in vertebrate heart development. Semin Cell Dev Biol 16: 83-94, 2005.
49. Posch MG, Boldt LH, Polotzki M, Richter S, Rolf S, Perrot A, Dietz R, Ozcelik C and Haverkamp W: Mutations in the cardiac transcription factor GATA4 in patients with lone atrial fibrillation. Eur J Med Genet 53: 201-203, 2010.
50. Yang YQ, Wang MY, Zhang XL, Tan HW, Shi HF, Jiang WF, Wang XH, Fang WY and Liu X: GATA4 loss-of-function mutations in familial atrial fibrillation. Clin Chim Acta 412: 1825-1830, 2011.
51. Jiang JQ, Shen FF, Fang WY, Liu X and Yang YQ: Novel GATA4 mutations in lone atrial fibrillation. Int J Mol Med 28: 1025-1032, 2011.
52. Wang J, Sun YM and Yang YQ: Mutation spectrum of the GATA4 gene in patients with idiopathic atrial fibrillation. Mol Biol Rep 39: 8127-8135, 2012.
53. Yang YQ, Wang J, Wang XH, Wang Q, Tan HW, Zhang M, Shen FF, Jiang JQ, Fang WY and Liu X: Mutational spectrum of the GATA5 gene associated with familial atrial fibrillation. Int J Cardiol 157: 305-307, 2012.
54. Wang XH, Huang CX, Wang Q, Li RG, Xu YJ, Liu X, Fang WY and Yang YQ: A novel GATA5 loss-of-function mutation underlies lone atrial fibrillation. Int J Mol Med 31: 43-50, 2013.
55. Yang YQ, Wang XH, Tan HW, Jiang WF, Fang WY and Liu X: Prevalence and spectrum of GATA6 mutations associated with familial atrial fibrillation. Int J Cardiol 155: 494-496, 2012.
56. Yang YQ, Li L, Wang J, Zhang XL, Li RG, Xu YJ, Tan HW, Wang XH, Jiang JQ, Fang WY and Liu X: GATA6 loss-of-function mutation in atrial fibrillation. Eur J Med Genet 55: 520-526, 2012.
57. Li J, Liu WD, Yang ZL and Yang YQ: Novel GATA6 loss-of-function mutation responsible for familial atrial fibrillation. Int J Mol Med 30: 783-790, 2012.
58. Zhang Y, Rath N, Hannenhalli S, Wang Z, Cappola T, Kimura S, Atochina-Vasserman E, Lu MM, Beers MF and Morrisey EE: GATA and Nkx factors synergistically regulate tissue-specific gene expression and development in vivo. Development 134: 189-198, 2007.
59. Pradhan L, Genis C, Scone P, Weinberg EO, Kasahara H and Nam HJ: Crystal structure of the human NKX2.5 homeodomain in complex with DNA target. Biochemistry 51: 6312-6319, 2012.
60. Tanaka M, Chen Z, Bartunkova S, Yamasaki N and Izumo S: The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development 126: 1269-1280, 1999.
61. Warren SA, Terada R, Briggs LE, Cole-Jeffrey CT, Chien WM, Seki T, Weinberg EO, Yang TP, Chin MT, Bungert J and Kasahara H: Differential role of Nkx2-5 in activation of the atrial natriuretic factor gene in the developing versus failing heart. Mol Cell Biol 31: 4633-4645, 2011.
62. Haïssaguerre M, Jaïs P, Shah DC, Takahashi A, Hocini M, Quiniou G, Garrigue S, Le Mouroux A, Le Métayer P and Clémenty J: Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. N Engl J Med 339: 659-666, 1998.
63. Mommersteeg MT, Brown NA, Prall OW, de Gier-de Vries C, Harvey RP, Moorman AF and Christoffels VM: Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. Circ Res 101: 902-909, 2007.
64. Mommersteeg MT, Hoogaars WM, Prall OW, de Gier-de Vries C, Wiese C, Clout DE, Papaioannou VE, Brown NA, Harvey RP, Moorman AF and Christoffels VM: Molecular pathway for the localized formation of the sinoatrial node. Circ Res 100: 354-362, 2007.
65. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE and Seidman JG: Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281: 108-111, 1998.
66. Wang J, Xin YF, Liu XY, Liu ZM, Wang XZ and Yang YQ: A novel NKX2-5 mutation in familial ventricular septal defect. Int J Mol Med 27: 369-375, 2011.
67. Liu XY, Wang J, Yang YQ, Zhang YY, Chen XZ, Zhang W, Wang XZ, Zheng JH and Chen YH: Novel NKX2-5 mutations in patients with familial atrial septal defects. Pediatr Cardiol 32: 193-201, 2011.
68. Wang J, Liu XY and Yang YQ: Novel NKX2-5 mutations responsible for congenital heart disease. Genet Mol Res 10: 2905-2915, 2011.
69. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, Rothrock CR, Eapen RS, Hirayama-Yamada K, Joo K, Matsuoka R, Cohen JC and Srivastava D: GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424: 443-447, 2003.
70. Wang J, Fang M, Liu XY, Xin YF, Liu ZM, Chen XZ, Wang XZ, Fang WY, Liu X and Yang YQ: A novel GATA4 mutation responsible for congenital ventricular septal defects. Int J Mol Med 28: 557-564, 2011.
71. Liu XY, Wang J, Zheng JH, Bai K, Liu ZM, Wang XZ, Liu X, Fang WY and Yang YQ: Involvement of a novel GATA4 mutation in atrial septal defects. Int J Mol Med 28: 17-23, 2011.
72. Wang J, Luo XJ, Xin YF, Liu Y, Liu ZM, Wang Q, Li RG, Fang WY, Wang XZ and Yang YQ: Novel GATA6 mutations associated with congenital ventricular septal defect or tetralogy of fallot. DNA Cell Biol 31: 1610-1617, 2012.
73. Yang YQ, Li L, Wang J, Liu XY, Chen XZ, Zhang W, Wang XZ, Jiang JQ, Liu X and Fang WY: A novel GATA4 loss-of-function mutation associated with congenital ventricular septal defect. Pediatr Cardiol 33: 539-546, 2012.
74. Zheng GF, Wei D, Zhao H, Zhou N, Yang YQ and Liu XY: A novel GATA6 mutation associated with congenital ventricular septal defect. Int J Mol Med 29: 1065-1071, 2012.
75. McCulley DJ and Black BL: Transcription factor pathways and congenital heart disease. Curr Top Dev Biol 100: 253-277, 2012.