ORIGINAL RESEARCH

PRRX1 Loss-of-Function Mutations Underlying Familial Atrial Fibrillation

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BACKGROUND: Atrial fibrillation (AF) is the most common form of clinical cardiac dysrhythmia responsible for thromboembolic cerebral stroke, congestive heart failure, and death. Aggregating evidence highlights the strong genetic basis of AF. Nevertheless, AF is of pronounced genetic heterogeneity, and in an overwhelming majority of patients, the genetic determinants underpinning AF remain elusive.

METHODS AND RESULTS: By genome-wide screening with polymorphic microsatellite markers and linkage analysis in a 4-generation Chinese family affected with autosomal-dominant AF, a novel locus for AF was mapped to chromosome 1q24.2–q25.1, a 3.20-cM (≈4.19 Mb) interval between markers D1S2851 and D1S218, with the greatest 2-point logarithm of odds score of 4.8165 for the marker D1S452 at recombination fraction=0.00. Whole-exome sequencing and bioinformatics analyses showed that within the mapping region, only the mutation in the paired related homeobox 1 (PRRX1) gene, NM_022716.4:c.319C>T; (p.Gln107*), cosegregated with AF in the family. In addition, sequencing analyses of PRRX1 in another cohort of 225 unrelated patients with AF revealed a new mutation, NM_022716.4:c.437G>T; (p.Arg146Ile), in a patient. The 2 mutations were absent in 908 control subjects. Biological analyses in HeLa cells demonstrated that the 2 mutants had significantly diminished transactivation on the target genes ISL1 and SHOX2 and markedly decreased ability to bind the promoters of ISL1 and SHOX2 (2 genes causally linked to AF), although with normal intracellular distribution.

CONCLUSIONS: This study first indicates that PRRX1 loss-of-function mutations predispose to AF, which provides novel insight into the molecular pathogenesis underpinning AF, implying potential implications for precise prophylaxis and management of AF.

Key Words: atrial fibrillation ■ functional genomics ■ medical genetics ■ PRRX1 ■ reporter gene assay ■ translational regulation

Atrial fibrillation (AF), characterized by rapid and uncoordinated electrical activation of the atria and consequently ineffective atrial contraction, is the most prevalent form of clinical cardiac arrhythmia in humans, with an estimated prevalence of 0.5% in the total population worldwide, affecting >33 million individuals globally.1,2 The prevalence of AF exponentially increases with advancing age, increasing from ≈0.1% among adults aged <55 years to ≈10% in people aged ≥80 years.3 The lifetime risk for occurrence of AF was ≈25% in people aged ≥40 years, and this risk dramatically increased to ≈37% in those aged >55 years.4 Considering that AF is commonly silent and frequently undiagnosed (termed subclinical AF), the actual prevalence of AF could be substantially underestimated.5 As a supraventricular tachyarrhythmia, AF has been associated with degraded health-related quality of life, diminished exercise capacity, ischemic cerebral stroke or extracranial systemic thromboembolism, impaired cognition or dementia, chronic kidney disease, acute myocardial infarction, tachycardia-induced cardiomyopathy, congestive heart failure, sinoatrial node
dysfunction, ventricular arrhythmias, and sudden cardiac death.6–20 In fact, AF confers a 4- to 5-fold increased risk of ischemic stroke, accounting for 15% of all strokes, a 5-fold increased risk of heart failure, and a 2-fold increased risk of demise.20 Therefore, AF has become a major socioeconomic burden, given that existing treatment regimens are limited in efficacy and are seldom curative.20,21 Despite the increasing prevalence and important clinical significance of AF, its molecular pathogenesis remains incompletely defined. Previous epidemiological investigations have revealed that AF is frequently associated with various structural heart diseases and systemic comorbidities and other miscellaneous predisposing factors, encompassing congenital heart disease, coronary heart disease, rheumatic heart disease, cardiomyopathy, cardiac and noncardiac surgery, essential hypertension, hyperthyroidism, diabetes, chronic obstructive pulmonary disease, chronic kidney disease, inflammation, obstructive sleep apnea, imbalanced serum electrolytes, obesity, and unhealthy lifestyle.1,20,22–36 However, in up to 30% of AF cases, AF occurs in the absence of the above-mentioned, well-recognized cardiac conditions or modifiable risk precipitants (termed as idiopathic AF).2 Therefore, increasing studies have demonstrated substantial familial clustering of AF, with the heritability of AF being as high as 62%, which highlights a strong genetic component underlying AF.21 By genome-wide screening with polymorphic microsatellite markers and genotyping and linkage analysis in 3 families with AF, the first locus for AF was mapped on chromosome 10q22-24.37 By positional candidate gene analysis of a large family affected with autosomal-dominant AF, the first AF-causative gene, Ser140Gly-mutant KCNQ1, which encodes the \( \alpha \) subunit of the slowly repolarizing, delayed rectifying K\(^+\) channel, was identified on chromosome 11p15.5.38 To date, via genome-wide linkage analysis and association study, >160 chromosomal loci have been linked causally to AF, although for the vast majority of these genetic loci, the biological implications remain unclear.21,39–62 Moreover, in addition to chromosomal abnormalities (duplications/deletions), pathogenic mutations in >50 genes have been implicated with AF, of which most encode ion channels, gap junction channels, transcriptional factors, sarcomere proteins, and signaling molecules.21,39–62 Of note, loss-of-function mutations in TTN and MYL4 also cause AF.42,63 Nevertheless, these already well-established genetic defects only account for a minority of AF, because of a pronounced genetic heterogeneity of AF.21,39–62 Therefore, further research studies are warranted to better understand the complex genetic basis of AF.

## METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

## Recruitment of Study Subjects

In this investigation, a 36-member 4-generation Chinese family affected with AF was identified (for pedigree, see Figure 1). The family members available and another cohort of 200 unrelated patients with familial AF, who were matched with the affected family members for sex, age, and ethnicity, were enrolled as controls. In addition, another cohort of 225 unrelated patients experiencing AF as well as a total of 708 unrelated healthy subjects were recruited. All the study subjects underwent a comprehensive
review of clinical data, encompassing personal and medical histories (including symptoms such as palpitation and syncope, previously diagnosed cardiac conditions, and administration of medications), physical examination, transthoracic echocardiogram, standard 12-lead ECG, and routine laboratory tests. When indicated, a 72-hour ambulatory ECG and cardiac electrophysiological examination were conducted. For the family members who died, their medical records were examined. Clinical diagnosis and classification of AF was made as described previously. An individual with electrocardiographically documented AF was defined as “affected,” whereas an individual without any proof of AF was classified as “unaffected.” Blood specimens were collected from all study participants. Before study recruitment, written informed consent was obtained from each study participant.

**Genome-Wide Scan With Microsatellite Markers and Linkage Analysis**

Isolation of genomic DNA from blood leukocytes was conducted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). A genome-wide scan for genotyping in the family with AF was performed with the ABI PRISM Linkage Mapping Set version 2.5 (Applied Biosystems, Foster City, CA), comprising 376 polymorphic microsatellite markers spaced at an average resolution of 10 cm from chromosomes 1 to 22. Multiplex amplification of microsatellite markers was performed by polymerase chain reaction (PCR) with the AmpliTaq Gold DNA Polymerase (Applied Biosystems) on the Veriti 96-Well Thermal Cycler (Applied Biosystems). Genotyping was performed under the ABI PRISM
Whole-Exome Sequencing and Sequence Analysis of the Mapped Chromosomal Region

Whole-exome sequencing (WES) and informatics analyses in 4 affected family members (II-1, III-2, III-3, and IV-2; Figure 1) and 2 unaffected family members (III-5 and IV-1; Figure 1) were fulfilled as described elsewhere. In short, for each family member to undergo WES, 3 μg of genomic DNA was fragmented randomly by an ultrasonicator (Covaris, Woburn, MA) to construct a whole-exome library, and captured with the SureSelect Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer’s protocol. The captured exome libraries were sequenced under the Illumina HiSeq 2000 Genome Analyzer (Illumina, San Diego, CA) and a specific pair of primers (forward primer: 5′-GCGGAATTCTGATTCGAGCGGGAAGAGGG-3′; reverse primer: 5′-TCGCTCGAGTCCTAGTTAGCTGTTGCA-3′). The yielded PRRX1 cDNA was subjected to double digestion with restriction endonucleases EcoRI and XhoI (NEB, Ipswich, MA), and then inserted into the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) at the EcoRI-XhoI sites to construct the eukaryotic gene expression plasmid PRRX1-pcDNA3.1. The mutant PRRX1-pcDNA3.1 plasmid was produced by PCR-based site-directed mutagenesis of wild-type (WT) PRRX1-pcDNA3.1 by using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers, and was validated by PCR sequencing. A 1750-bp DNA fragment (from −207 to −258, with the transcriptional start site numbered as +1) of the human SHOX2 gene (GenBank accession No. NC_000003.12), which harbors multiple consensus PRRX1-binding sites, (T/C)AATTA, was amplified from human genomic DNA by PCR with a specific pair of primers (forward primer: 5′-GACGGTACCTTTGAGAAACACTAATATAT-3′; reverse primer: 5′-GTCAAGCTTGGGAGCTGCCGTTGAAAGTCC-3′), cut with KpnI and HindIII (NEB), and inserted of AF in the family (Figure 1), the variants not matching any reasonable inheritance pattern of AF were filtered out. The variants passing the pedigree analysis were annotated with annotation of variance. When a potential AF-causing mutation was identified by WES analysis in the mapped genomic region, Sanger sequencing and segregation analysis in the whole family with AF were performed to further confirm it. Once a gene harboring an identified causative mutation for AF was identified, Sanger sequencing analysis of the gene was performed in another cohort of 225 unrelated patients with AF and 508 unrelated healthy individuals used as controls. For a confirmed deleterious genetic mutation, such population genetics databases as the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), the UK Biobank (https://www.ukbiobank.ac.uk/), the Single-Nucleotide Polymorphism database (https://www.ncbi.nlm.nih.gov/snp/), and the Genome Aggregation Database (http://gnomad.broad institute.org/) were retrieved to check its novelty.
into the basic vector pGL3 (Promega) to construct a SHOX2 promoter-driven firefly luciferase reporter plasmid (SHOX2-luciferase). Similarly, a 1680-bp sequence upstream of the translation start site (from \( -1 \) to \( -1680 \)) of the human \textit{ISL1} gene (GenBank accession No. NC_000005.10) was amplified from human genomic DNA by PCR with a specific pair of primers (forward primer: \( 5'\)-GACGGTAGAAATGGAGGAAAGCACAGAT-3'; reverse primer: \( 5'\)-GTCGCTAGCCGAGCTCCGGCACCTCT-3'), digested with KpnI and NheI (NEB), and subcloned into pGL3 (Promega) to construct an \textit{ISL1} promoter-driven firefly luciferase reporter plasmid (ISL1-luciferase).

**Cellular Transfection and Dual-Luciferase Assay**

HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS (Invitrogen) and seeded into a 24-well plate at a density of \( 1 \times 10^5 \) cells per well 24 hours before transfection. Cells were transiently transfected with various plasmids by using the Lipofectamine 3000 transfection reagent (Invitrogen). For analysis of the transactivation of SHOX2 by PRRX1, HeLa cells were transfected with 0.2 \( \mu \)g of empty pcDNA3.1 or 0.2 \( \mu \)g of WT PRRX1-pcDNA3.1 or 0.2 \( \mu \)g of mutant PRRX1-pcDNA3.1 or 0.1 \( \mu \)g of WT PRRX1-pcDNA3.1 plus 0.1 \( \mu \)g of empty pcDNA3.1 or 0.1 \( \mu \)g of WT PRRX1-pcDNA3.1 plus 0.1 \( \mu \)g of mutant PRRX1-pcDNA3.1, in combination with 1.0 \( \mu \)g of SHOX2-luciferase and 0.04 \( \mu \)g of pRL-TK (Promega). For analysis of the transactivation of ISL1 by PRRX1, the quantity of each expression plasmid (WT or mutant PRRX1-pcDNA3.1) was halved (namely, 0.1 \( \mu \)g for homozygous expression or 0.05 \( \mu \)g for heterozygous expression), whereas the plasmids ISL1-luciferase and pRL-TK (Promega) remained the same as the amount mentioned above. Notably, various amounts (ranging from 0.025 to 0.8 \( \mu \)g) of WT PRRX1-pcDNA3.1 were used to evaluate its dose-dependent activation of the promoters of SHOX2 and ISL2. Cells were collected and lysed 48 hours after transfection, and the luciferase activities of cellular lysates were measured on a GloMax 96 Microplate Luminometer (Promega), with the Dual-Glo Luciferase Assay System (Promega). The promoter activity was expressed as fold activation of Firefly luciferase relative to Renilla luciferase. At least 3 independent experiments were conducted in triplicate for each expression plasmid.

**Electrophoretic Mobility Shift Analysis**

Nuclear extracts were prepared from transfected HeLa cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer’s instructions. The DNA-binding ability of PRRX1 mutants was examined by electrophoretic mobility shift analysis using a 22-bp ISL1 promoter or SHOX2 promoter. The oligonucleotide with a PRRX1-binding site was synthesized, 5' end labeled with biotin, and incubated with the purified WT or mutant PRRX1 protein. The protein-DNA complexes were resolved by electrophoresis through 6% non-denaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at a constant voltage of 100 V, transferred to a positively charged nylon membrane (ThermoFisher Scientific, Waltham, MA), and cross-linked by UV exposure. The DNA-protein interaction was probed with streptavidin–horseradish peroxidase conjugates, and detected using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology), according to the manufacturer’s manual.

**Subcellular Distribution of the Mutant PRRX1 Proteins**

HeLa cells were grown on 14-mm glass coverslips in a 12-well plate. Forty-eight hours after transfection, cells were fixed using 4% paraformaldehyde for 10 minutes at room temperature and rinsed briefly in prechilled PBS. Fixed cells were then heated in antigen retrieval solution at 95 °C before being permeabilized with 0.5% Triton X-100 for 5 minutes. Subsequently, coverslips were blocked with 3% BSA for 30 minutes and incubated with rabbit anti-PRRX1 polyclonal antibody (Affinity Biologicals, Ancaster, ON, Canada) at a 1:200 dilution in a dark humidified chamber overnight at 4 °C. After being washed in PBS-Tween, cells were immunostained with goat anti-rabbit Alexa-Flour 594–conjugated secondary antibody at a 1:100 dilution for 1 hour at room temperature, keeping away from light. The nucleus was stained with 4',6-diamidino-2-phenylindole for 1 minute. Finally, coverslips were mounted with a drop of anti-fading mounting medium and sealed with clear nail polish to prevent drying and movement under the microscope. Images were acquired under a confocal laser-scanning fluorescence microscope (Leica Microsystems, Mannheim, Germany) using an oil objective (Leica Microsystems).

**Statistical Analysis**

Statistical analysis was performed using SPSS for Windows, Version 18.0 (SPSS, Chicago, IL). The quantitative data were represented as mean values with SD and compared by Student unpaired \( t \) test. A 2-tailed \( P<0.05 \) was considered as significantly different.

**RESULTS**

**Baseline Clinical Characteristics of the Study Participants**

A 4-generation family with AF (Figure 1) was identified from the Chinese Han population, comprising 34
living family members (17 men and 17 women; aged from 19–73 years). The index patient of the family (II-1; Figure 1) was diagnosed with AF 39 years ago at the age of 34 years and received anti-arrhythmic pharmacologic therapy with amiodarone. He had no structural heart diseases, primary hypertension, or other systemic disorders. A representative 12-lead ECG recorded from the proband is exhibited in Figure 2A, showing the typical features of AF. The proband’s father (I-1; Figure 1) had medical history of AF, and died because of thromboembolic cerebral stroke at the age of 65 years. In the whole family, all the affected members had ECG-documented AF, whereas the unaffected family members had neither

![Figure 2. The PRRX1-Gln107* mutation causally linked to atrial fibrillation.](image)

**A.** A representative standard 12-lead ECG from the proband affected with atrial fibrillation. **B.** Sequencing chromatograms of the affected proband and an unaffected control subject, exhibiting the heterozygous PRRX1 mutation and its wild-type control. An arrow symbol points to the heterozygous nucleotides of C/T in the affected proband (mutant type) or the homozygous nucleotides of C/C in an unaffected subject (wild type). Sequencing analysis unveiled a heterozygous mutation of c.319C>T in PRRX1, resulting in a substitution of a premature stop codon for the glutamine (Gln)–encoding codon at amino acid 107 of PRRX1 (PRRX1-Gln107*).
history of AF nor symptoms of AF (palpitation, breathlessness, fatigue, dizziness, and chest discomfort), with normal ECGs. None had organic heart diseases or other systemic diseases predisposing to AF, such as coronary heart disease, essential hypertension, diabetes, hyperthyroidism, and obesity. Genetic analysis of the pedigree revealed that in the family AF is transmitted following an autosomal-dominant pattern of inheritance, with complete penetrance. The baseline clinical characteristics of the affected family members alive are summarized in Table 1. As shown in Table 1, 11 affected members of the family composed a group of familial AF (8 male patients and 3 female patients, with a mean age of 48 years, ranging from 21–73 years). Another cohort of 200 unrelated patients with familial AF (145 male patients and 55 female patients, with a mean age of 48 years, ranging from 21–73 years) were clinically investigated as controls, showing that they were matched with the affected family members for sex, age, and ethnicity. In addition, another cohort of 225 unrelated patients with AF was clinically assessed in contrast to a total of 708 unrelated healthy individuals without AF, and found that the patients (126 male cases and 99 female cases, with an average age of 49 years, ranging from 28–59 years) were matched with the healthy subjects (396 male subjects and 312 female subjects, with a mean age of 49 years, varying from 28–59 years) for ethnicity, sex, and age.

Mapping of a New Genetic Locus for AF on Chromosome 1q24.2–q25.1

A genome-wide screening was performed for 34 family members from the family affected with AF (Figure 1), with 376 polymorphic microsatellite markers spanning

Table 1. Clinical Characteristics of the Living Family Members Affected With AF, Carrying the Gln107* Mutation of PRRX1

| Subject information | Phenotype | ECG | Echocardiogram |
|---------------------|-----------|-----|---------------|
| Identity (family 1) | Age at study enrollment, y | Age at initial diagnosis of AF, y | Recurrent palpitation | AF (classification) | Heart rate, beats/min | QRS interval, ms | QTc, ms | LAD, mm | LVEF, % |
| II-1 Male 73 | 34 | Yes | Permanent | 84 | 93 | 403 | 39 | 60 |
| II-4 Female 71 | 38 | Yes | Permanent | 63 | 114 | 419 | 41 | 58 |
| II-5 Male 68 | 25 | Yes | Permanent | 83 | 78 | 408 | 45 | 62 |
| II-2 Female 52 | 36 | Yes | Permanent | 69 | 96 | 432 | 38 | 65 |
| II-3 Male 51 | 30 | Yes | Permanent | 106 | 90 | 422 | 42 | 64 |
| III-7 Male 50 | 29 | Yes | Permanent | 84 | 110 | 427 | 40 | 61 |
| III-9 Male 48 | 24 | Yes | Persistent | 89 | 96 | 472 | 36 | 66 |
| III-11 Male 45 | 23 | Yes | Persistent | 76 | 106 | 452 | 38 | 63 |
| IV-2 Male 26 | 25 | Yes | Paroxysmal | 96 | 84 | 457 | 34 | 64 |
| IV-5 Female 24 | 24 | Yes | Paroxysmal | 77 | 100 | 457 | 31 | 62 |
| IV-7 Male 21 | 21 | Yes | Paroxysmal | 100 | 86 | 436 | 29 | 68 |

AF indicates atrial fibrillation; LAD, left atrial diameter; LVEF, left ventricular ejection fraction; and QTc, corrected QT interval.

Table 2. Two-Point LOD Scores for the Markers on Chromosome 1q24.2-q25.1 at Different θ Values in the Family Affected With AF

| Marker | LOD scores at θ |
|--------|-----------------|
|        | 0.00 | 0.01 | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| D1S196 | 4.8165 | 4.7379 | 4.4155 | 3.9929 | 3.0738 | 2.0432 | 0.9215 |
| D1S2799 | 3.3113 | 3.2546 | 3.0217 | 2.7166 | 2.0536 | 1.3911 | 0.5696 |
| D1S2658 | 0.7466 | 1.8580 | 2.0844 | 1.8698 | 1.3098 | 0.5930 |
| D1S2818 | 0.7466 | 1.8580 | 2.0844 | 1.8698 | 1.3098 | 0.5930 |

θ Indicates recombination fraction; AF, atrial fibrillation; and LOD, logarithm of odds.
Genetic linkage analysis indicated significant linkage of AF to 1 marker, D1S452, with a maximum 2-point LOD score of 4.7379 at recombination fraction=0.00 (Table 2 and Figure 1). Two-point LOD scores for D1S452 remained >3 irrespective of variations in the phenocopy prevalence from 0% to 5% and variations in the penetrance from 60% to 99%. Analysis of the pairwise LOD scores showed no significant linkage to markers on other chromosomal regions (Table 2). To confine the AF-linked chromosomal region, 5 additional markers (D1S196, D1S2799, D1S2658, D1S2851, and D1S2790) were genotyped, and by haplotype analysis (Figure 1 and Table 2), the disease locus was ultimately mapped to chromosome 1q24.2–q25.1 (GRCh38, chr1:170 347 964–174 533 955), a 3.20-cM

Figure 3. The PRRX1-Arg146Ile mutation responsible for atrial fibrillation.
A, A representative standard 12-lead ECG from the index patient experiencing atrial fibrillation. B, Sequencing electropherograms of the index patient and a healthy control individual, with the identified PRRX1-Arg146Ile mutation indicated by an arrow. Sequence analysis revealed a heterozygous mutation of c.437G>T in PRRX1, leading to a transversion of arginine (Arg) into isoleucine (Ile) at amino acid 146 of PRRX1 (PRRX1-Arg146Ile).
(≈4.19 Mbp) interval between markers D1S2851 and D1S218. The chromosomal segment between markers D1S2851 and D1S218, where 95 genes (including 31 protein-encoding genes, 34 RNA-encoding genes, and 30 pseudo genes) were defined, was heterozygous for all the affected family members with AF (Figure 1).

Identification of Pathogenic Mutations in PRRX1

WES was performed in 4 affected (II-1, III-2, III-3, and IV-2; Figure 1) and 2 unaffected family members (III-5 and IV-1; Figure 1). Analysis of the sequenced DNA sequences within the mapping chromosomal region from marker D1S2581 to D1S218, only the genetic variant chr:1:17068944C>T (GRCh37: NC_000001.10), equivalent to chr:1:170719800C>T (GRCh38: NC_000001.11) or NM_022716.4:c.319C>T;(p. Gln107*) in the PRRX1 gene, encoding a key cardiac transcription factor, was validated by Sanger sequencing and cosegregated with AF in the family. The sequence chromatograms of the heterozygous c.319C>T mutation in PRRX1 and its WT control are displayed in Figure 2B. This variation was not observed in another cohort of 200 unrelated patients with familial AF. In addition, sequencing analysis of the coding exons and splicing donors/acceptors of PRRX1 in another cohort of 225 unrelated patients with idiopathic AF revealed a new mutation, NM_022716.4:c.437G>T; (p. Arg146Ile), in a male patient, who was aged 32 years with a positive family history of AF. Sequencing analysis of the missense mutation carrier’s parents revealed that the mutation was present in his 57-year-old father affected with AF and absent in his 55-year-old healthy mother. A standard 12-lead ECG recorded from the missense mutation carrier is shown in Figure 3A. The sequence electropherograms of the heterozygous c.437G>T mutation in PRRX1 and its WT control are given in Figure 3B. The 2 heterozygous PRRX1 mutations were neither detected

![Figure 4](image-url)
Diminished Transcriptional Activity of the Mutant PRRX1 Proteins

As shown in Figure 4, WT PRRX1 showed a dose-dependent transactivation of the SHOX2 promoter (Figure 4A) or the ISL1 promoter (Figure 4C). However, compared with their WT counterparts, both Gln107*-mutant PRRX1 (Q107*) and Arg146Ile-mutant PRRX1 (R146I), in either the homozygous or heterozygous state, showed a significantly diminished transcriptional activity on the SHOX2 promoter (Figure 4B) or the ISL1 promoter (Figure 4D). Specifically, as shown in Figure 4B, homozygous WT activated the SHOX2 promoter by ≈42-fold, whereas homozygous Q107* and R146I activated the SHOX2 promoter by ≈5- and ≈7-fold, respectively (WT versus Q107*: t=45.8808, P<0.00001; WT versus R146I: t=45.4836, P<0.00001); heterozygous Q107* and R146I activated the SHOX2 promoter by ≈14- and ≈13-fold, respectively (WT versus WT+Q107*: t=22.9480, P=0.00002; WT versus WT+R146I: t=18.3957, P=0.00005). Similarly, as shown in Figure 4D, homozygous WT activated the ISL1 promoter by ≈78-fold, whereas homozygous Q107* and R146I activated the ISL1 promoter by ≈25- and ≈27-fold, respectively (WT versus Q107*: t=44.5860, P<0.00001; WT versus R146I: t=42.0624, P<0.00001); heterozygous Q107* and R146I activated the ISL1 promoter by ≈39- and ≈44-fold, respectively (WT versus WT+Q107*: t=31.5338, P<0.00001; WT versus WT+R146I: t=13.2953, P=0.00019).

Reduced DNA-Binding Ability of the Mutant PRRX1 Proteins

As shown in Figure 5, the nuclear extracts of HeLa cells were able to interact with the biotinylated ISL1 or SHOX2 probe to yield complexes. As shown in Figure 5A, the ability of Q107* or R146I to bind to the ISL1 promoter DNA significantly reduced compared with that of WT. Similarly, as shown in Figure 5B, the ability of Q107* or R146I to bind to the SHOX2 promoter DNA markedly decreased to an undetectable level.

Normal Nuclear Distribution of the Mutant PRRX1 Proteins

As shown in Figure 6, in transfected cells, WT normally localized to the nucleus. Similar with WT, both Q107* and R146I had a normal intracellular localization.

Figure 5. Decreased DNA-binding ability of PRRX1 resulted from Gln107* or Arg146Ile mutation.

A, The ability of wild-type PRRX1 (WT), Gln107*-mutant PRRX1 (Q107*), or R146I-mutant PRRX1 (R146I) to bind the ISL1 promoter DNA. B, The ability of WT, Q107*, or R146I to bind the SHOX2 promoter DNA. Electrophoretic mobility shift assay showed that WT normally bound the ISL1 promoter DNA (A) or the SHOX2 promoter DNA (B), but either Q107* or R146I had a significantly decreased DNA-binding affinity for the ISL1 promoter DNA (A) or the SHOX2 promoter DNA (B), when compared with WT.
DISCUSSION

In the present investigation, by genome-wide scan with polymorphic microsatellite markers and linkage analysis in a 4-generation family with AF, a new genetic locus for AF was mapped to chromosome 1q24.2–q25.1. WES and bioinformatics analyses as well as Sanger sequencing analyses revealed that within the mapping chromosomal region, only the mutation in the \( PRRX1 \) gene, NM_022716.4:c.319C>T;p. Gln107*, cosegregated with AF in the whole family with complete penetrance. In addition, by Sanger sequencing analysis of \( PRRX1 \) in another cohort of 225 unrelated patients with AF, a novel mutation, NM_022716.4:c.437G>T; p. Arg146Ile, was identified in 1 patient. Sequencing analysis of the missense mutation carrier’s parents unveiled that the mutation was present in his father with AF and absent in his mother without AF. The 2 heterozygous mutations were neither observed in 1816 control chromosomes nor found in such population genetics databases as Human Gene Mutation Database, UK Biobank, Single-Nucleotide Polymorphism database, and Genome Aggregation Database. Functional research studies demonstrated that the 2 mutants had significantly diminished transactivation on the target genes \( ISL1 \) and \( SHOX2 \), and remarkably reduced the ability to bind the promoters of \( ISL1 \) and \( SHOX2 \), 2 genes that have been causally linked to AF. Therefore, it is likely that genetically compromised \( PRRX1 \) contributes to AF in these mutation carriers.

In humans, \( PRRX1 \) maps on chromosome 1q24.2, coding for a member of the paired family of homeobox-containing transcription factor proteins localized to the nucleus. This DNA-associated protein is amply expressed in the developing cardiovascular system throughout embryogenesis, predominantly in mesenchymal tissues, encompassing the heart, great arteries, and pulmonary veins, a major source of ectopic beats triggering AF in most patients. \( PRRX1 \) has been validated to mediate the epithelial-to-mesenchymal transition, a key feature of human heart development. Moreover, in \( Prx1 \)-null mice, vascular malformations occurred, including abnormal positioning and awkward curvature of the aortic arch, a misdirected and elongated ductus arteriosus, and an anomalous retroesophageal
right subclavian artery, indicating a pivotal role for Prx1 in the development of vascular and perivascular matrix.\textsuperscript{76}

In the current study, PRRX1 was verified to physically interact with and transcriptionally regulate SHOX2 and ISL1, 2 critical downstream genes essential for the development of cardiac pacing and conducting system.\textsuperscript{77–82}

and loss-of-function mutations in both SHOX2 and ISL1 have been involved in the pathogenesis of AF.\textsuperscript{49,56,68,69}

Hence, PRRX1 mutations may predispose to AF by reducing expression of such important target genes as SHOX2 and ISL1.

Recently, multiple genome-wide association studies consistently associated a top genetic variant at the AF locus on chromosome 1q24, a single-nucleotide polymorphism (rs3903239)\textsuperscript{63} kb upstream of the PRRX1 gene, with significantly increased risk of AF in both Europeans and Asians.\textsuperscript{83}

Further studies demonstrated that this AF-associated variant reduced the activity of an enhancer upstream of PRRX1, leading to diminished expression of PRRX1 in human left atrial tissue.\textsuperscript{83}

Moreover, loss of PRRX1 expression was shown to result in shortening of the atrial action potential duration in human cardiomyocytes and embryonic zebrafish myocardium, creating a matrix susceptible to AF.\textsuperscript{83}

Collectively, these findings indicate PRRX1 as the AF-causative gene at this locus and unveil the electrophysiological mechanism by which the genetic association at the 1q24 locus contributes to AF.

**CONCLUSIONS**

In conclusion, this study first reports PRRX1 loss-of-function mutations underlying AF, which provides new insight into the molecular pathogenesis of AF, suggesting potential implications for precision medicine of AF in a subset of patients.

**ARTICLE INFORMATION**

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**Disclosures**

None.

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