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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 Mbp) 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 precisive 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

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CLINICAL PERSPECTIVE

What Is New?

- By genome-wide scan with polymorphic microsatellite markers and linkage analysis in a large family affected with autosomal-dominant atrial fibrillation (AF), a novel locus for AF was mapped on chromosome 1q24.2–q25.1, and by whole-exome sequencing and bioinformatics analyses, a new mutation in the *PRRX1* gene, NM_022716.4:c.319C>T;(p.Gln107*), was identified to cosegregate with AF in the family within the mapping region.
- 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 mutants had significantly diminished transactivation on the target genes *ISL1* and *SHOX2*, although with normal intracellular distribution, and the 2 mutations markedly decreased the ability of PRRX1 to bind the promoters of *ISL1* and *SHOX2*.

What Are the Clinical Implications?

- Identification of AF-causing genetic mutations implies potential implications for personalized prophylaxis and precise treatment of AF.
- The AF-related genetic data may be used for risk stratification and prognostic evaluation of patients with AF.

Nonstandard Abbreviations and Acronyms

WES	whole-exome sequencing
WT	wild type

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] Recently, 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 α 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 pronounced genetic heterogeneity of AF.[21,39–63] 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

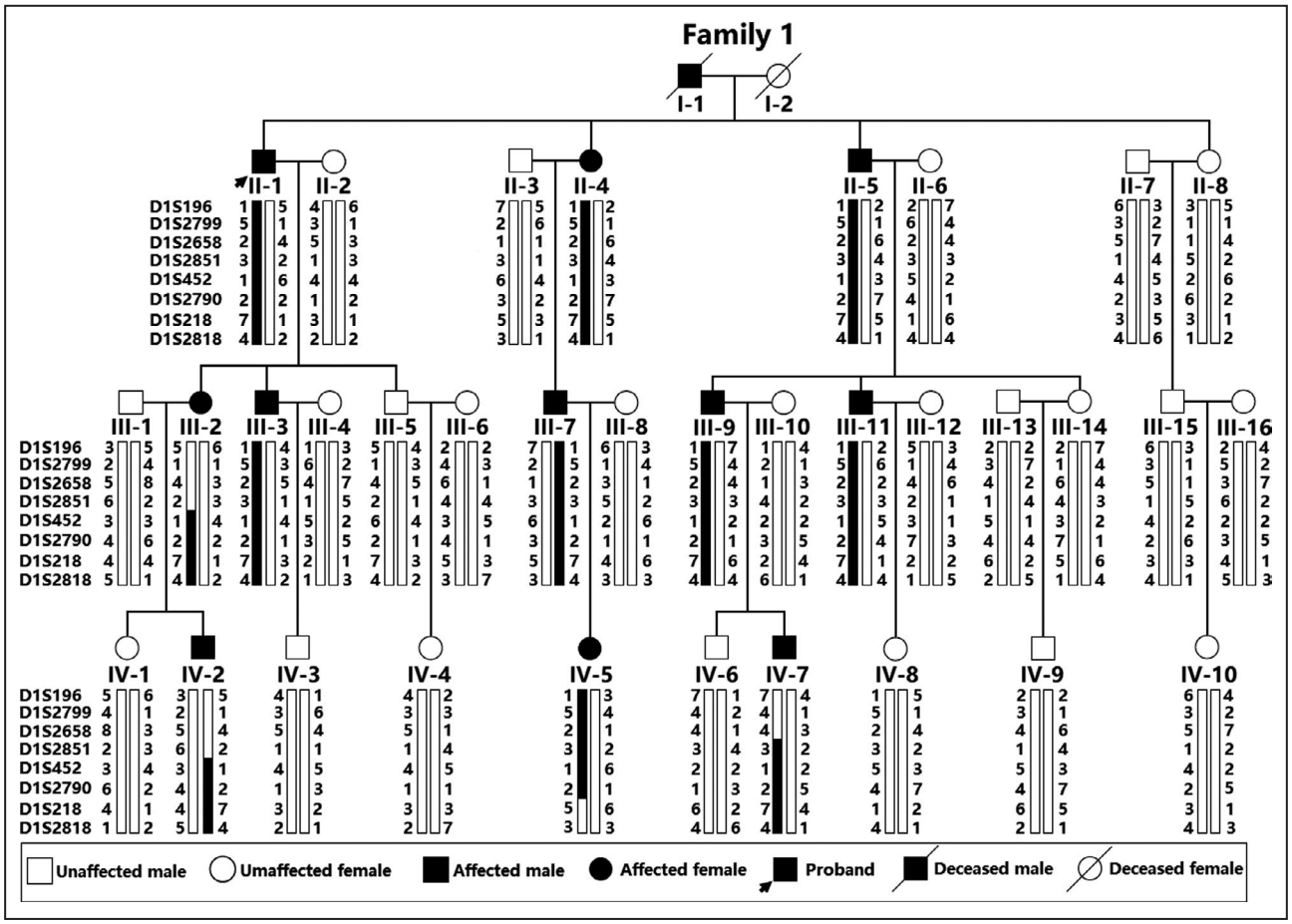

Figure 1. Pedigree structure and genetic mapping analysis of a 4-generation family with atrial fibrillation.
The 4-generation family with atrial fibrillation was arbitrarily designated as family 1. Family members are recognized by generations and numbers (Roman-Arabic numerals) given below pedigree symbols. A deceased family member is marked by a diagonal line through the symbol. A vertical bar beneath a family member means the chromosomal segment determined by genetic analysis. Microsatellite markers spanning the linkage region on chromosome 1q24.2–q25.1 are shown to the left of the pedigree, with each member's genotypes (represented by numbers) for each marker displayed next to the chromosome bars. The filled bars denote the chromosomal segment derived from the mutation-carrying chromosome of the affected ancestor (I-1).

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.[1,58] An individual with electrocardiographically documented AF was defined as "affected," whereas an individual without any proof of AF was classified as "unaffected."[58] Blood specimens were collected from all study participants. The present investigation adhered to the tenets outlined in the Declaration of Helsinki, and was approved by the local institutional ethics committee (approval number KS1101). 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.[58] 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

3130XL Genetic Analyzer (Applied Biosystems) following the manufacturer's manual. Linkage analysis was made using the software S.A.G.E. (Statistical Analysis for Genetic Epidemiology at http://darwin.cwru.edu/sage/), under an autosomal-dominant inheritance model with the allele frequency and penetrance value of AF set at 0.1% and 95%, respectively.[58] A 2-point logarithm of odds (LOD) score between each marker and the disease locus was calculated under a condition of evenly shared allele frequency, zero phenocopy rate, and no difference in male and female recombination rates. For fine mapping in the chromosomal region determined by the marker D1S452 with an initial supportive 2-point LOD score, 5 additional microsatellite markers (D1S196, D1S2799, D1S2658, D1S2851, and D1S2790) encompassing the chromosomal region were analyzed to validate the linkage and refine the recombination boundaries to delimit the critical chromosomal interval. These additional markers were selected from the Généthon human linkage maps, spanning the chromosomal region of ≈9.49-cM interval at a mean marker distance of 1.90 cM between 2 markers. Information on marker order as well as intermarker distances was derived from the linkage map of the Cooperative Human Linkage Center. Haplotypes of the family with AF were constructed using the software Cyrillic v2.1.3 (Cherwell Scientific, Oxford, UK) to ascertain the shared genomic regions among affected members in the family with AF and confine the recombination boundaries.

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.[58,64–67] 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), by using the HiSeq Sequencing Kit (Illumina) following the manufacturer's manual. Pipeline was applied to processing raw image data for calling bases and generating the set of sequence reads. Sequence reads were mapped to the human reference genome (hg19, GRCh37) via the Burrows-Wheeler Alignment software. The Genome Analysis Toolkit software was applied for calling variants (single-nucleotide polymorphisms, insertions, and deletions). On the basis of the possible inheritance modes 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.broadinstitute.org/) were retrieved to check its novelty.

Construction of Expression Plasmids and Site-Targeted Mutagenesis

Extraction of total RNA from human heart tissue specimens and generation of cDNA were conducted as described previously.[59] An 860-bp fragment from nucleotide 1 to 860 of the human paired related homeobox 1 (*PRRX1*) gene (GenBank accession No. NM_006902.4) containing the full-length open reading frame of *PRRX1* was amplified from cDNA by PCR using the PfuUltra High-Fidelity DNA Polymerase (Stratagene, Santa Clara, CA) and a specific pair of primers (forward primer: 5′-GCGGAATTCTGATTCGAGCGGGAAGAGGG-3′; reverse primer: 5′ -CGCCTCGAGTCCTCAGTTGACTGTTGGCA-3′). The yielded *PRRX1* cDNA was subjected to double digestion with restriction endonucleases *Eco*RI and *Xho*I (NEB, Ipswich, MA), and then inserted into the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) at the *Eco*RI-*Xho*I 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 QuikChange 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 −2007 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′-GTCAAGCTTGACGCTGCGCTTGAAGTCCC-3′), cut with *Kpn*I and *Hind*III (NEB), and inserted

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 *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′-GACGGTACCAAATGGAAGGGAAGACAGAT-3′; reverse primer: 5′-GTCGCTAGCGGCGCGGCTCGGG CACCTCT-3′), digested with *Kpn*I and *Nhe*I (NEB), and subcloned into pGL3 (Promega) to construct an *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 1×10^5 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 μg of empty pcDNA3.1 or 0.2 μg of WT PRRX1-pcDNA3.1 or 0.2 μg of mutant PRRX1-pcDNA3.1 or 0.1 μg of WT PRRX1-pcDNA3.1 plus 0.1 μg of empty pcDNA3.1 or 0.1 μg of WT PRRX1-pcDNA3.1 plus 0.1 μg of mutant PRRX1-pcDNA3.1, in combination with 1.0 μg of SHOX2-luciferase and 0.04 μg 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 μg for homozygous expression or 0.05 μg 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 μg) 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% nondenaturing 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

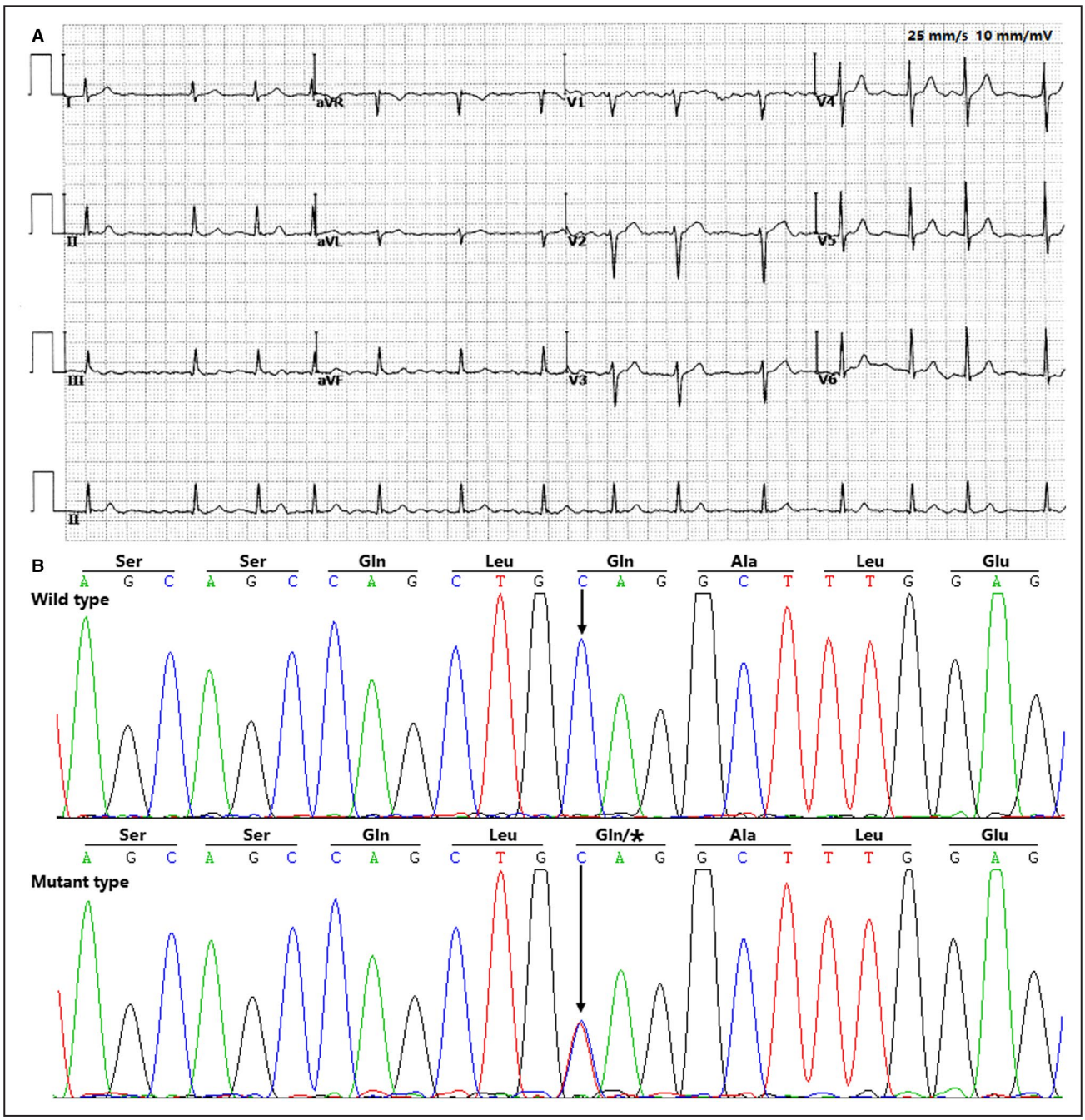

Figure 2. The PRRX1-Gln107* mutation causally linked to atrial fibrillation.
A, A representative standard 12-lead ECG from the proband affected with atrial fibrillation. **B**, Sequencing chromatograms of the affected proband and an unaffected control subject, exhibiting the heterozygous *PRRX1* mutation 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*).

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)	Sex	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
III-2	Female	52	36	Yes	Permanent	69	96	432	38	65
III-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.

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 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	(−∞)	−1.2490	0.5792	1.1302	1.2677	0.9418	0.4169
D1S2799	(−∞)	−1.9266	−0.0624	0.5361	0.7797	0.5818	0.2254
D1S2658	(−∞)	0.1533	1.3005	1.5739	1.4615	1.0175	0.4347
D1S2851	(−∞)	1.5556	2.0217	2.0176	1.6554	1.0932	0.4524
D1S452	4.8165	4.7379	4.4155	3.9929	3.0738	2.0432	0.9215
D1S2790	3.3113	3.2546	3.0217	2.7166	2.0536	1.3191	0.5696
D1S218	(−∞)	0.7466	1.8580	2.0844	1.8698	1.3098	0.5930
D1S2818	(−∞)	−0.3194	0.8463	1.1452	1.0955	0.7404	0.2950

θ Indicates recombination fraction; AF, atrial fibrillation; and LOD, logarithm of odds.

the whole human genome spaced by every 10 cM. 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

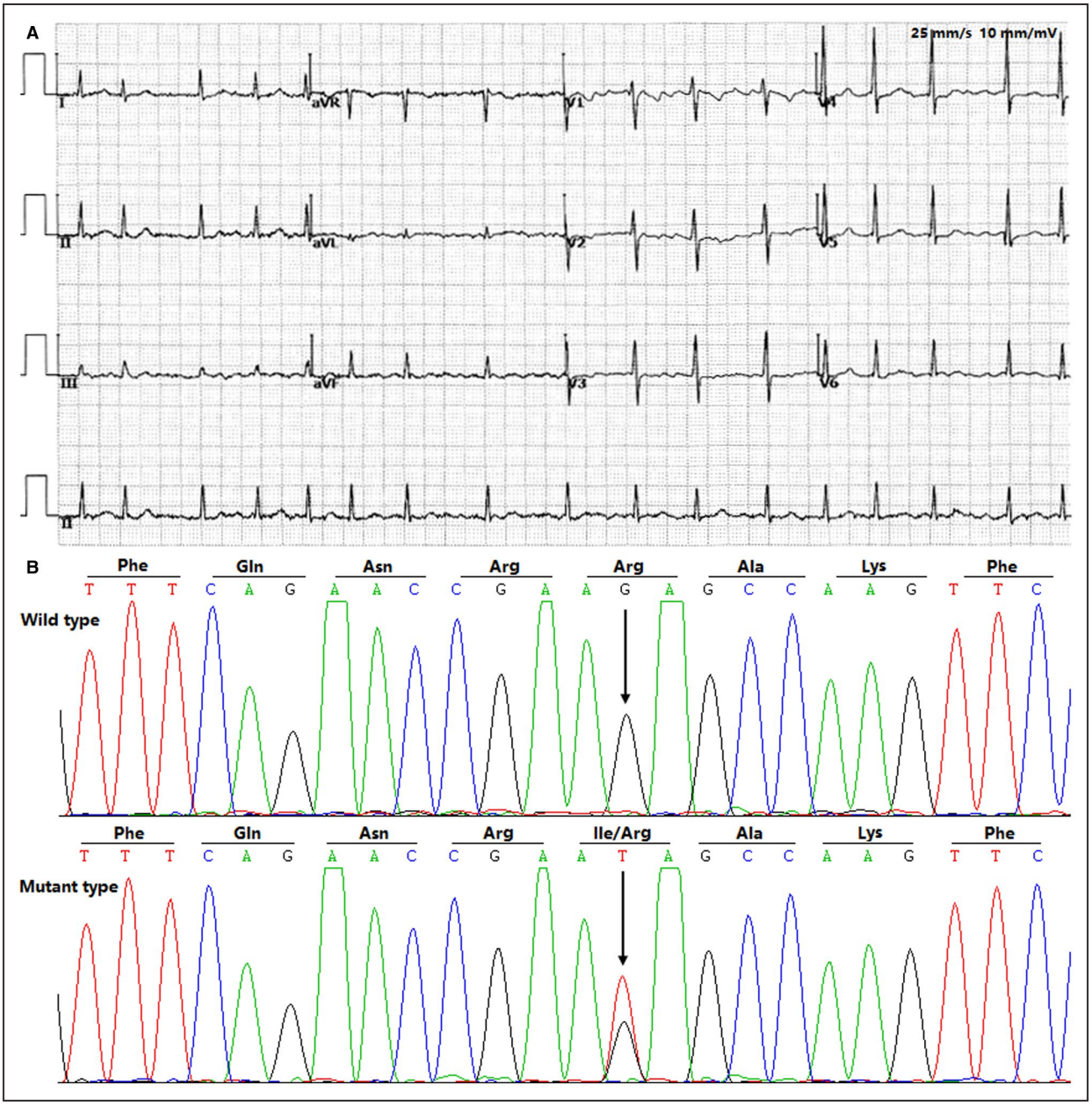

Figure 3. The PRRX1-Arg146Ile mutation responsible for atrial fibrillation.
A, A representative standard 12-lead ECG from the index patient experiencing atrial fibrillation. **B**, Sequencing electropherograms of the index patient and a healthy control individual, with the identified PRRX1-Arg146Ile 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 D1S2581 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 chr1:170688944C>T (GRCh37: NC_000001.10), equivalent to chr1:170719800C>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

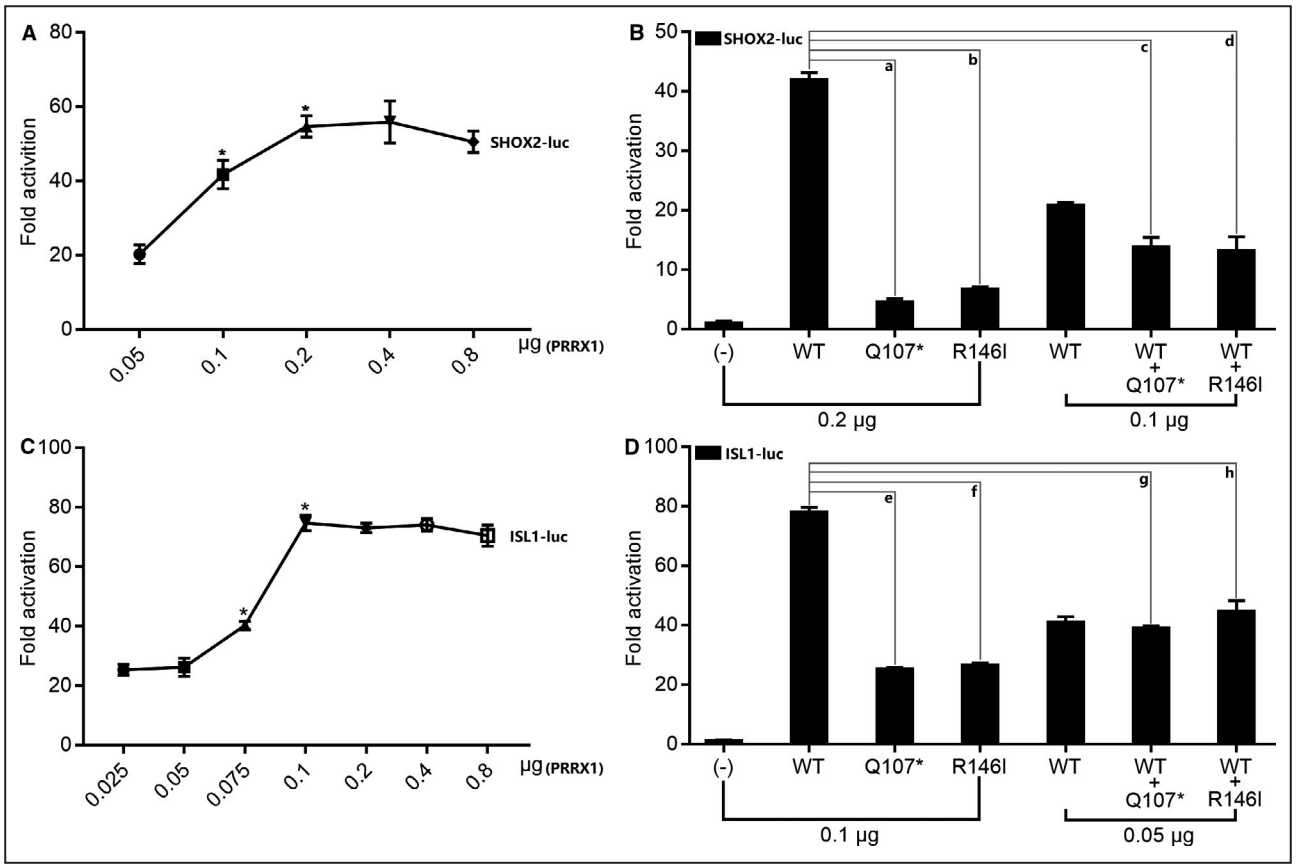

Figure 4. Diminished transcriptional activity of PRRX1 caused by Gln107* or Arg146Ile mutation.
A, In cultured HeLa cells, PRRX1 transcriptionally activated the *SHOX2* promoter in a dose-dependent manner. **B**, Transactivation of the *SHOX2* promoter-driven luciferase by wild-type PRRX1 (WT) or Gln107*-mutant PRRX1 (Q107*) or R146I-mutant PRRX1 (R146I), alone or in combination, showed that Gln107* or R146I had a significantly reduced transcriptional activity. **C**, In cultured HeLa cells, PRRX1 transcriptionally activated the *ISL1* promoter in a dose-dependent mode. **D**, Transactivation of the *ISL1* promoter-driven luciferase by WT or Q107* or R146I, alone or in combination, showed that Gln107* or R146I had a significantly decreased transcriptional activity. Transfection experiments were performed in triplicate, and the results are expressed as means±SD. All the letters from a to h indicate $P<0.001$, when compared with the same amounts of WT.

in 708 unrelated control individuals nor retrieved in such population genetics databases as Human Gene Mutation Database, UK Biobank, Single-Nucleotide Polymorphism database, and Genome Aggregation Database, indicating novel mutations.

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.

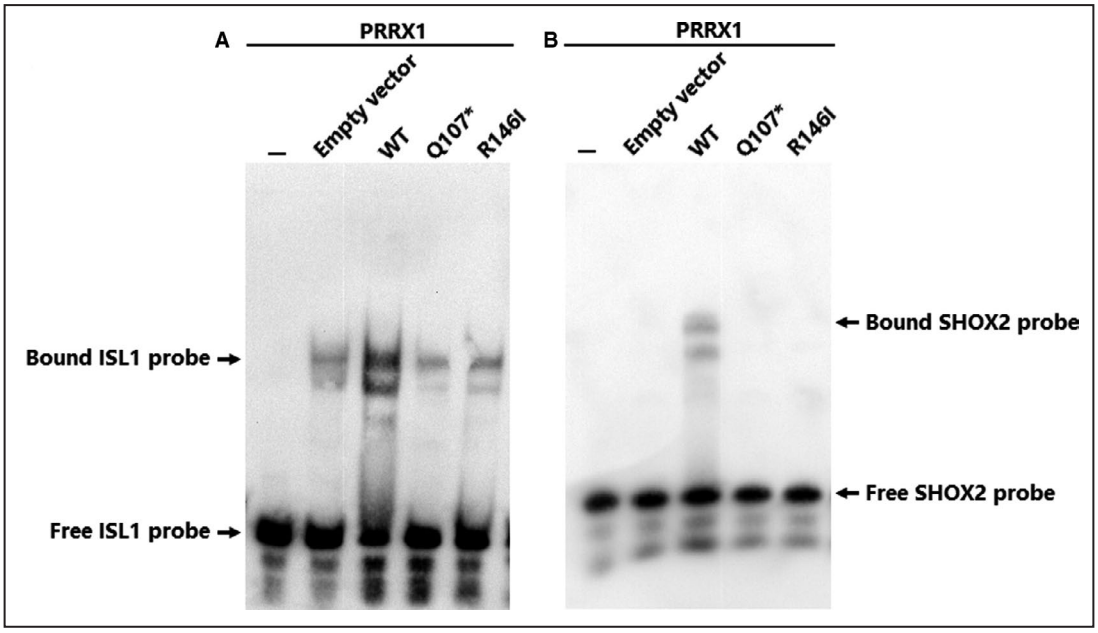

Figure 5. Decreased DNA-binding ability of PRRX1 resulted from Gln107* or Arg146Ile mutation.
A, The ability of wild-type PRRX1 (WT), Gln107*-mutant PRRX1 (Q107*), or R146I-mutant PRRX1 (R146I) to bind the *ISL1* promoter DNA. **B**, The ability of WT, Q107*, or R146I to bind the *SHOX2* promoter DNA. 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.

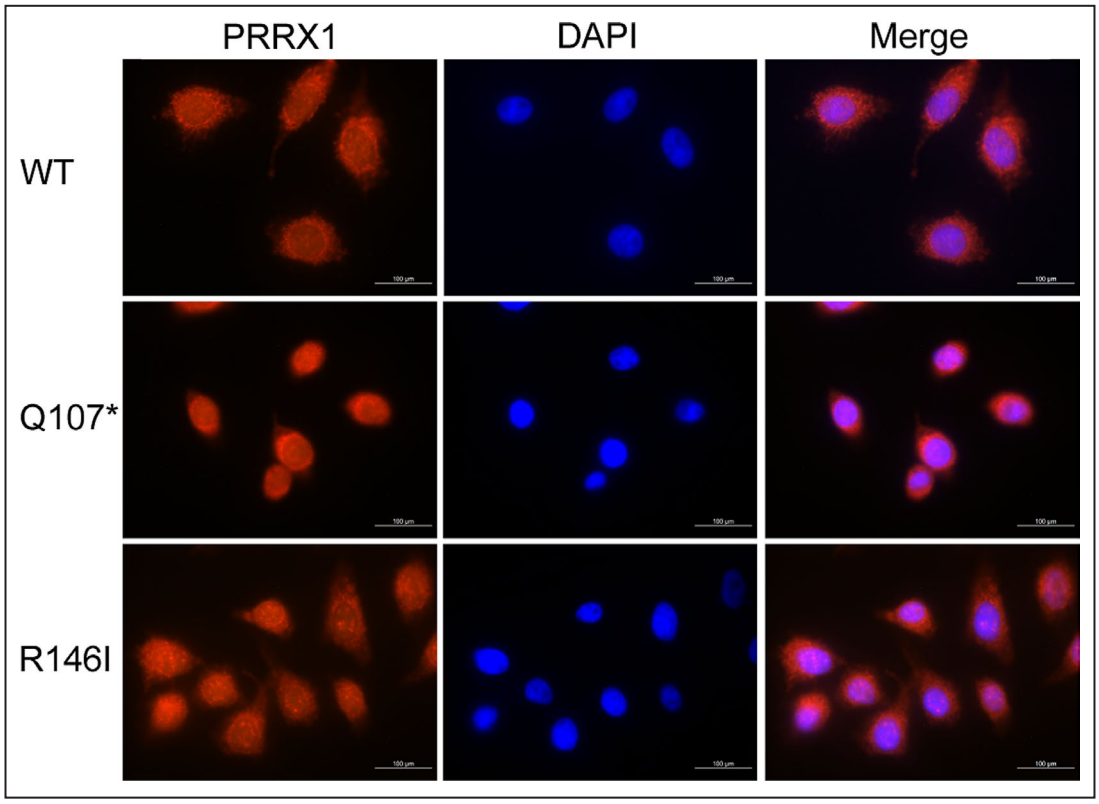

Figure 6. Normal subcellular distribution of wild-type and mutant PRRX1 proteins.
Subcellular localizations of wild-type PRRX1 (WT), Gln107*-mutant PRRX1 (Q107*), and R146I-mutant PRRX1 (R146I) were determined by immunofluorescence in HeLa cells. HeLa cells, transfected with WT, Q107*, or R146I, were stained with anti-FLAG (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). Enlarged images of FLAG and DAPI staining and merged images of HeLa cells were presented. WT, Q107*, or R146I was localized exclusively to the nuclei with normal subcellular distribution. Bar=100 μm.

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.[49,56,68,69] 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.[70] 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,[71–73] a major source of ectopic beats triggering AF in most patients.[74] PRRX1 has been validated to mediate the epithelial-to-mesenchymal transition, a key feature of human heart development.[75] Moreover, in *Prrx1*-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 *Prrx1* in the development of vascular and perivascular matrix.[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,[77–82] and loss-of-function mutations in both *SHOX2* and *ISL1* have been involved in the pathogenesis of AF.[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*) ≈63 kb upstream of the *PRRX1* gene, with significantly increased risk of AF in both Europeans and Asians.[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.[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.[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.

REFERENCES

1. January CT, Wann LS, Alpert JS, Calkins H, Cigarroa JE, Cleveland JC, Conti JB, Ellinor PT, Ezekowitz MD, Field ME, et al. 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines and the Heart Rhythm Society. *Circulation*. 2014;130:e199–e267. doi: 10.1161/CIR.0000000000000041
2. Chugh SS, Havmoeller R, Narayanan K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, Kim YH, McAnulty JH, Zheng ZJ, et al. Worldwide epidemiology of atrial fibrillation: a global burden of disease 2010 study. *Circulation*. 2014;129:837–847. doi: 10.1161/CIRCULATIONAHA.113.005119
3. Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV, 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*. 2001;285:2370–2375. doi: 10.1001/jama.285.18.2370
4. Weng LC, Preis SR, Hulme OL, Larson MG, Choi SH, Wang B, Trinquart L, McManus DD, Staerk L, Lin H, et al. Genetic predisposition, clinical risk factor burden, and lifetime risk of atrial fibrillation. *Circulation*. 2018;137:1027–1038. doi: 10.1161/CIRCULATIONAHA.117.031431
5. Noseworthy PA, Kaufman ES, Chen LY, Chung MK, Elkind MSV, Joglar JA, Leal MA, McCabe PJ, Pokorney SD, Yao X, et al. Subclinical and device-detected atrial fibrillation: pondering the knowledge gap: a scientific statement from the American Heart Association. *Circulation*. 2019;140:e944–e963. doi: 10.1161/CIR.0000000000000740
6. Pan Y, Wang Y, Wang Y. Investigation of causal effect of atrial fibrillation on Alzheimer disease: a Mendelian randomization study. *J Am Heart Assoc*. 2020;9:e014889. doi: 10.1161/JAHA.119.014889
7. Bunch TJ. Atrial fibrillation and dementia. *Circulation*. 2020;142:618–620. doi: 10.1161/CIRCULATIONAHA.120.045866
8. Kim D, Yang PS, Sung JH, Jang E, Yu HT, Kim TH, Uhm JS, Kim JY, Pak HN, Lee MH, et al. Less dementia after catheter ablation for atrial fibrillation: a nationwide cohort study. *Eur Heart J*. 2020;41:4483–4493. doi: 10.1093/eurheartj/ehaa726
9. Sanchez JM, Jolly SE, Dewland TA, Tseng ZH, Nah G, Vittinghoff E, Marcus GM. Incident strokes among American Indian individuals with atrial fibrillation. *J Am Heart Assoc*. 2021;10:e019581. doi: 10.1161/JAHA.120.019581
10. Martinez C, Wallenhorst C, Rietbrock S, Freedman B. Ischemic stroke and transient ischemic attack risk following vitamin K antagonist cessation in newly diagnosed atrial fibrillation: a cohort study. *J Am Heart Assoc*. 2020;9:e014376. doi: 10.1161/JAHA.119.014376
11. Maeda T, Nishi T, Funakoshi S, Tada K, Tsuji M, Satoh A, Kawazoe M, Yoshimura C, Arima H. Risks of bleeding and stroke based on CHA2DS2-VASc scores in Japanese patients with atrial fibrillation: a large-scale observational study using real-world data. *J Am Heart Assoc*. 2020;9:e014574. doi: 10.1161/JAHA.119.014574
12. Shi M, Chen LY, Bekwelem W, Norby FL, Soliman EZ, Alam AB, Alonso A. Association of atrial fibrillation with incidence of extracranial systemic embolic events: the ARIC Study. *J Am Heart Assoc*. 2020;9:e016724. doi: 10.1161/JAHA.120.016724
13. Belkouche A, Yao H, Putot A, Chagué F, Rochette L, Danchin N, Fauchier L, Zeller M, Cottin Y. The multifaceted interplay between atrial fibrillation and myocardial infarction: a review. *J Clin Med*. 2021;10:198. doi: 10.3390/jcm10020198
14. Kim IC. Atrial fibrillation and heart failure with preserved ejection fraction: two chronic troublemakers. *Heart Fail Clin*. 2021;17:377–386. doi: 10.1016/j.hfc.2021.03.001
15. Hickey KT, Colombo PC, Naka Y, Garan AR, Yuzefpolskaya M, Garan H, Wan EY, Sciacca RR, Goldenthal I, Biviano AB. Atrial fibrillation is associated with recurrent ventricular arrhythmias after LVAD implant:

incidence and impact in a consecutive series. *J Cardiovasc Transl Res*. 2020;13:199–203. doi: 10.1007/s12265-019-09914-0
16. Singh SM, Abdel-Qadir H, Pang A, Fang J, Koh M, Dorian P, Wijeysundera HC, Ko DT. Population trends in all-cause mortality and cause specific-death with incident atrial fibrillation. *J Am Heart Assoc*. 2020;9:e016810. doi: 10.1161/JAHA.120.016810
17. Tanaka Y, Shah NS, Passman R, Greenland P, Lloyd-Jones DM, Khan SS. Trends in cardiovascular mortality related to atrial fibrillation in the United States, 2011 to 2018. *J Am Heart Assoc*. 2021;10:e020163. doi: 10.1161/JAHA.120.020163
18. Buckley BJR, Harrison SL, Fazio-Eynullayeva E, Underhill P, Lane DA, Thijssen DHJ, Lip GYH. Exercise-based cardiac rehabilitation and all-cause mortality among patients with atrial fibrillation. *J Am Heart Assoc*. 2021;10:e020804. doi: 10.1161/JAHA.121.020804
19. Yang PS, Sung JH, Jang E, Yu HT, Kim TH, Uhm JS, Kim JY, Pak HN, Lee MH, Joung B. Catheter ablation improves mortality and other outcomes in real-world patients with atrial fibrillation. *J Am Heart Assoc*. 2020;9:e015740. doi: 10.1161/JAHA.119.015740
20. Virani SS, Alonso A, Aparicio HJ, Benjamin EJ, Bittencourt MS, Callaway CW, Carson AP, Chamberlain AM, Cheng S, Delling FN, et al. Heart disease and stroke statistics–2021 update: a report from the American Heart Association. *Circulation*. 2021;143:e254–e743. doi: 10.1161/CIR.0000000000000950
21. Roselli C, Rienstra M, Ellinor PT. Genetics of atrial fibrillation in 2020: GWAS, genome sequencing, polygenic risk, and beyond. *Circ Res*. 2020;127:21–33. doi: 10.1161/CIRCRESAHA.120.316575
22. Waldmann V, Khairy P. Pathophysiology, risk factors, and management of atrial fibrillation in adult congenital heart disease. *Card Electrophysiol Clin*. 2021;13:191–199. doi: 10.1016/j.ccep.2020.10.007
23. Rowin EJ, Sridharan A. Thinking outside the heart to treat atrial fibrillation in hypertrophic cardiomyopathy. *J Am Heart Assoc*. 2020;9:e016260. doi: 10.1161/JAHA.120.016260
24. Garg L, Gupta M, Sabzwari SRA, Agrawal S, Agarwal M, Nazir T, Gordon J, Bozorgnia B, Martinez MW. Atrial fibrillation in hypertrophic cardiomyopathy: prevalence, clinical impact, and management. *Heart Fail Rev*. 2019;24:189–197. doi: 10.1007/s10741-018-9752-6
25. London B. FISHing for answers in postoperative atrial fibrillation. *J Am Heart Assoc*. 2012;1:e002931. doi: 10.1161/JAHA.112.002931
26. Conen D, Wang MK, Devereaux PJ, Whitlock R, McIntyre WF, Healey JS, Yuan F, Yusuf S, Lamy A. New-onset perioperative atrial fibrillation after coronary artery bypass grafting and long-term risk of adverse events: an analysis from the CORONARY trial. *J Am Heart Assoc*. 2021;10:e020426. doi: 10.1161/JAHA.120.020426
27. Taha A, Nielsen SJ, Bergfeldt L, Ahlsson A, Friberg L, Björck S, Franzén S, Jeppsson A. New-onset atrial fibrillation after coronary artery bypass grafting and long-term outcome: a population-based nationwide study from the SWEDEHEART registry. *J Am Heart Assoc*. 2021;10:e017966. doi: 10.1161/JAHA.120.017966
28. Hyun J, Cho MS, Nam GB, Kim M, Do U, Kim J, Choi KJ, Kim YH. Natural course of new-onset postoperative atrial fibrillation after non-cardiac surgery. *J Am Heart Assoc*. 2021;10:e018548. doi: 10.1161/JAHA.120.018548
29. Elbadawi A, Elgendy IY, Mahmoud AH, Ogunbayo GO, Saad M, Megaly M, Alotaki E, Mentias A, Barakat AF, London B. Outcomes of surgical ablation in patients with atrial fibrillation undergoing cardiac surgeries. *Ann Thorac Surg*. 2019;107:1395–1400. doi: 10.1016/j.athoracsur.2018.10.040
30. Higa S, Maesato A, Ishigaki S, Suenari K, Chen YJ, Chen SA. Diabetes and endocrine disorders (hyperthyroidism/hypothyroidism) as risk factors for atrial fibrillation. *Card Electrophysiol Clin*. 2021;13:63–75. doi: 10.1016/j.ccep.2020.11.005
31. Vyas V, Hunter RJ, Longhi MP, Finlay MC. Inflammation and adiposity: new frontiers in atrial fibrillation. *Europace*. 2020;22:1609–1618. doi: 10.1093/europace/euaa214
32. Packer M. Characterization, pathogenesis, and clinical implications of inflammation-related atrial myopathy as an important cause of atrial fibrillation. *J Am Heart Assoc*. 2020;9:e015343. doi: 10.1161/JAHA.119.015343
33. Linz D, Nattel S, Kalman JM, Sanders P. Sleep apnea and atrial fibrillation. *Card Electrophysiol Clin*. 2021;13:87–94. doi: 10.1016/j.ccep.2020.10.003
34. Bertomeu-Gonzalez V, Moreno-Arribas J, Esteve-Pastor MA, Roldán-Rabadán I, Muñiz J, Raña-Míguez P, Ruiz-Ortiz M, Cequier Á, Bertomeu-Martínez V, Badimón L, et al. Association of body mass index with clinical outcomes in patients with atrial fibrillation: a report from the FANTASIIA registry. *J Am Heart Assoc*. 2020;9:e013789. doi: 10.1161/JAHA.119.013789
35. Poorthuis MHF, Sherliker P, de Borst GJ, Carter JL, Lam KBH, Jones NR, Halliday A, Lewington S, Bulbulia R. Joint associations between body mass index and waist circumference with atrial fibrillation in men and women. *J Am Heart Assoc*. 2021;10:e019025. doi: 10.1161/JAHA.120.019025
36. Chung MK, Eckhardt LL, Chen LY, Ahmed HM, Gopinathannair R, Joglar JA, Noseworthy PA, Pack QR, Sanders P, Trulock KM, et al. Lifestyle and risk factor modification for reduction of atrial fibrillation: a scientific statement from the American Heart Association. *Circulation*. 2020;141:e750–e772. doi: 10.1161/CIR.0000000000000748
37. Brugada R, Tapscott T, Czernuszewicz GZ, Marian AJ, Iglesias A, Mont L, Brugada J, Girona J, Domingo A, Bachinski LL, et al. Identification of a genetic locus for familial atrial fibrillation. *N Engl J Med*. 1997;336:905–911. doi: 10.1056/NEJM199703273361302
38. Chen YH, Xu SJ, Bendahhou S, Wang XL, Wang Y, Xu WY, Jin HW, Sun H, Su XY, Zhuang QN, et al. KCNQ1 gain-of-function mutation in familial atrial fibrillation. *Science*. 2003;299:251–254. doi: 10.1126/science.1077771
39. Roselli C, Chaffin MD, Weng LC, Aeschbacher S, Ahlberg G, Albert CM, Almgren P, Alonso A, Anderson CD, Aragam KG, et al. Multi-ethnic genome-wide association study for atrial fibrillation. *Nat Genet*. 2018;50:1225–1233. doi: 10.1038/s41588-018-0133-9
40. Nielsen JB, Thorolfsdottir RB, Fritsche LG, Zhou W, Skov MW, Graham SE, Herron TJ, McCarthy S, Schmidt EM, Sveinbjornsson G, et al. Biobank-driven genomic discovery yields new insight into atrial fibrillation biology. *Nat Genet*. 2018;50:1234–1239. doi: 10.1038/s41588-018-0171-3
41. Choi SH, Weng LC, Roselli C, Lin H, Haggerty CM, Shoemaker MB, Barnard J, Arking DE, Chasman DI, Albert CM, et al. Association between Titin loss-of-function variants and early-onset atrial fibrillation. *JAMA*. 2018;320:2354–2364. doi: 10.1001/jama.2018.18179
42. Ahlberg G, Refsgaard L, Lundegaard PR, Andreasen L, Ranthe MF, Linscheid N, Nielsen JB, Melbye M, Haunsø S, Sajadieh A, et al. Rare truncating variants in the sarcomeric protein titin associate with familial and early-onset atrial fibrillation. *Nat Commun*. 2018;9:4316. doi: 10.1038/s41467-018-06618-y
43. Thorolfsdottir RB, Sveinbjornsson G, Sulem P, Nielsen JB, Jonsson S, Halldorsson GH, Melsted P, Ivarsdottir EV, Davidsson OB, Kristjansson RP, et al. Coding variants in RPL3L and MYZAP increase risk of atrial fibrillation. *Commun Biol*. 2018;1:68. doi: 10.1038/s42003-018-0068-9
44. Drabkin M, Zilberberg N, Menahem S, Mulla W, Halperin D, Yogev Y, Wormser O, Perez Y, Kadir R, Etzion Y, et al. Nocturnal atrial fibrillation caused by mutation in KCND2, encoding pore-forming (α) subunit of the cardiac Kv4.2 potassium channel. *Circ Genom Precis Med*. 2018;11:e002293. doi: 10.1161/CIRCGEN.118.002293
45. Yamada N, Asano Y, Fujita M, Yamazaki S, Inanobe A, Matsuura N, Kobayashi H, Ohno S, Ebana Y, Tsukamoto O, et al. Mutant KCNJ3 and KCNJ5 potassium channels as novel molecular targets in bradyarrhythmias and atrial fibrillation. *Circulation*. 2019;139:2157–2169. doi: 10.1161/CIRCULATIONAHA.118.036761
46. Menon A, Hong L, Savio-Galimberti E, Sridhar A, Youn SW, Zhang M, Kor K, Blair M, Kupershmidt S, Darbar D. Electrophysiologic and molecular mechanisms of a frameshift NPPA mutation linked with familial atrial fibrillation. *J Mol Cell Cardiol*. 2019;132:24–35. doi: 10.1016/j.yjmcc.2019.05.004
47. Han M, Zhao M, Cheng C, Huang Y, Han S, Li W, Tu X, Luo X, Yu X, Liu Y, et al. Lamin A mutation impairs interaction with nucleoporin NUP155 and disrupts nucleocytoplasmic transport in atrial fibrillation. *Hum Mutat*. 2019;40:310–325. doi: 10.1002/humu.23691
48. Goodyer WR, Dunn K, Caleshu C, Jackson M, Wylie J, Moscarello T, Platt J, Reuter C, Smith A, Trela A, et al. Broad genetic testing in a clinical setting uncovers a high prevalence of titin loss-of-function variants in very early onset atrial fibrillation. *Circ Genom Precis Med*. 2019;12:e002713. doi: 10.1161/CIRCGEN.119.002713
49. Hoffmann S, Paone C, Sumer SA, Diebold S, Weiss B, Roeth R, Clauss S, Klier I, Kääb S, Schulz A, et al. Functional characterization of rare variants in the SHOX2 gene identified in sinus node dysfunction and atrial fibrillation. *Front Genet*. 2019;10:648. doi: 10.3389/fgene.2019.00648
50. Ghazizadeh Z, Kiviniemi T, Olafsson S, Plotnick D, Beerens ME, Zhang K, Gillon L, Steinbaugh MJ, Barrera V, Sui SH, et al. Metastable atrial state underlies the primary genetic substrate for MYL4 mutation-associated

atrial fibrillation. *Circulation*. 2020;141:301–312. doi: 10.1161/CIRCULATIONAHA.119.044268
51. van Ouwerkerk AF, Bosada FM, Liu J, Zhang J, van Duijvenboden K, Chaffin M, Tucker NR, Pijnappels D, Ellinor PT, Barnett P, et al. Identification of functional variant enhancers associated with atrial fibrillation. *Circ Res*. 2020;127:229–243. doi: 10.1161/CIRCRESAHA.119.316006
52. Wang B, Lunetta KL, Dupuis J, Lubitz SA, Trinquart L, Yao L, Ellinor PT, Benjamin EJ, Lin H. Integrative omics approach to identifying genes associated with atrial fibrillation. *Circ Res*. 2020;126:350–360. doi: 10.1161/CIRCRESAHA.119.315179
53. Choi SH, Jurgens SJ, Weng L-C, Pirruccello JP, Roselli C, Chaffin M, Lee C-Y, Hall AW, Khera AV, Lunetta KL, et al. Monogenic and polygenic contributions to atrial fibrillation risk: results from a national biobank. *Circ Res*. 2020;126:200–209. doi: 10.1161/CIRCRESAHA.119.315686
54. Vad OB, Paludan-Müller C, Ahlberg G, Kalstø SM, Ghouse J, Andreasen L, Haunsø S, Tveit A, Sajadieh A, Christophersen IE, et al. Loss-of-function variants in cytoskeletal genes are associated with early-onset atrial fibrillation. *J Clin Med*. 2020;9:372. doi: 10.3390/jcm9020372
55. Hansen TH, Yan Y, Ahlberg G, Vad OB, Refsgaard L, dos Santos JL, Mutsaers N, Svendsen JH, Olesen MS, Bentzen BH, et al. A novel loss-of-function variant in the chloride ion channel gene Clcn2 associates with atrial fibrillation. *Sci Rep*. 2020;10:1453. doi: 10.1038/s41598-020-58475-9
56. Wu SH, Wang XH, Xu YJ, Gu JN, Yang CX, Qiao Q, Guo XJ, Guo YH, Qiu XB, Jiang WF, et al. ISL1 loss-of-function variation causes familial atrial fibrillation. *Eur J Med Genet*. 2020;63:104029. doi: 10.1016/j.ejmg.2020.104029
57. Chalazan B, Mol D, Darbar FA, Ornelas-Loredo A, Al-Azzam B, Chen Y, Tofovic D, Sridhar A, Alzahrani Z, Ellinor P, et al. Association of rare genetic variants and early-onset atrial fibrillation in ethnic minority individuals. *JAMA Cardiol*. 2021;6:811–819. doi: 10.1001/jamacardio.2021.0994
58. Li RG, Xu YJ, Ye WG, Li YJ, Chen H, Qiu XB, Yang YQ, Bai D. Connexin45 (GJC1) loss-of-function mutation contributes to familial atrial fibrillation and conduction disease. *Heart Rhythm*. 2021;18:684–693. doi: 10.1016/j.hrthm.2020.12.033
59. Li N, Xu YJ, Shi HY, Yang CX, Guo YH, Li RG, Qiu XB, Yang YQ, Zhang M. KLF15 loss-of-function mutation underlying atrial fibrillation as well as ventricular arrhythmias and cardiomyopathy. *Genes (Basel)*. 2021;12:408. doi: 10.3390/genes12030408
60. Feghaly J, Zakka P, London B, MacRae CA, Refaat MM. Genetics of atrial fibrillation. *J Am Heart Assoc*. 2018;7:e009884. doi: 10.1161/JAHA.118.009884
61. Victorino J, Alvarez-Franco A, Manzanares M. Functional genomics and epigenomics of atrial fibrillation. *J Mol Cell Cardiol*. 2021;157:45–55. doi: 10.1016/j.yjmcc.2021.04.003
62. Kim JA, Chelu MG, Li N. Genetics of atrial fibrillation. *Curr Opin Cardiol*. 2021;36:281–287. doi: 10.1097/HCO.0000000000000840
63. Orr N, Arnaout R, Gula LJ, Spears DA, Leong-Sit P, Li Q, Tarhuni W, Reischauer S, Chauhan VS, Borkovich M, et al. A mutation in the atrial-specific myosin light chain gene (MYL4) causes familial atrial fibrillation. *Nat Commun*. 2016;7:11303. doi: 10.1038/ncomms11303
64. Yaoita N, Satoh K, Satoh T, Shimizu T, Saito S, Sugimura K, Tatebe S, Yamamoto S, Aoki T, Kikuchi N, et al. Identification of the novel variants in patients with chronic thromboembolic pulmonary hypertension. *J Am Heart Assoc*. 2020;9:e015902. doi: 10.1161/JAHA.120.015902
65. Jiang X, Shao Y, Araj FG, Amin AA, Greenberg BM, Drazner MH, Xing C, Mammen PPA. Heterozygous cystic fibrosis transmembrane regulator gene missense variants are associated with worse cardiac function in patients with Duchenne muscular dystrophy. *J Am Heart Assoc*. 2020;9:e016799. doi: 10.1161/JAHA.120.016799
66. Ware SM, Wilkinson JD, Tariq M, Schubert JA, Sridhar A, Colan SD, Shi L, Canter CE, Hsu DT, Webber SA, et al. Genetic causes of cardiomyopathy in children: first results from the pediatric cardiomyopathy genes study. *J Am Heart Assoc*. 2021;10:e017731. doi: 10.1161/JAHA.120.017731
67. Ramchand J, Wallis M, Macciocca I, Lynch E, Farouque O, Martyn M, Phelan D, Chong B, Lockwood S, Weintraub R, et al. Prospective evaluation of the utility of whole exome sequencing in dilated cardiomyopathy. *J Am Heart Assoc*. 2020;9:e013346. doi: 10.1161/JAHA.119.013346
68. Hoffmann S, Clauss S, Berger IM, Weiß B, Montalbano A, Röth R, Bucher M, Klier I, Wakili R, Seitz H, et al. Coding and non-coding variants in the SHOX2 gene in patients with early-onset atrial fibrillation. *Basic Res Cardiol*. 2016;111:36. doi: 10.1007/s00395-016-0557-2
69. Li N, Wang ZS, Wang XH, Xu YJ, Qiao Q, Li XM, Di RM, Guo XJ, Li RG, Zhang M, et al. A SHOX2 loss-of-function mutation underlying familial atrial fibrillation. *Int J Med Sci*. 2018;15:1564–1572. doi: 10.7150/ijms.27424
70. Grueneberg DA, Natesan S, Alexandre C, Gilman MZ. Human and Drosophila homeodomain proteins that enhance the DNA-binding activity of serum response factor. *Science*. 1992;257:1089–1095. doi: 10.1126/science.257.5073.1089
71. Leussink B, Brouwer A, el Khattabi M, Poelmann RE, Gittenberger-de Groot AC, Meijlink F. Expression patterns of the paired-related homeobox genes MHox/Prx1 and S8/Prx2 suggest roles in development of the heart and the forebrain. *Mech Dev*. 1995;52:51–64. doi: 10.1016/0925-4773(95)00389-i
72. Bergwerff M, Gittenberger-de Groot AC, DeRuiter MC, van Iperen L, Meijlink F, Poelmann RE. Patterns of paired-related homeobox genes PRX1 and PRX2 suggest involvement in matrix modulation in the developing chick vascular system. *Dev Dyn*. 1998;213:59–70. doi: 10.1002/(SICI)1097-0177(199809)213:1<59:AID-AJA6>3.0.CO;2-X
73. Libório TN, Acquafreda T, Matizonkas-Antonio LF, Silva-Valenzuela MG, Ferraz AR, Nunes FD. In situ hybridization detection of homeobox genes reveals distinct expression patterns in oral squamous cell carcinomas. *Histopathology*. 2011;58:225–233. doi: 10.1111/j.1365-2559.2011.03751.x
74. Haïssaguerre M, Jaïs P, Shah DC, Takahashi A, Hocini M, Quiniou G, Garrigue S, Le Mouroux A, Le Métayer P, Clémenty J. Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *N Engl J Med*. 1998;339:659–666. doi: 10.1056/NEJM199809033391003
75. Ocaña OH, Córcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S, Barrallo-Gimeno A, Cano A, Nieto MA. Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. *Cancer Cell*. 2012;22:709–724. doi: 10.1016/j.ccr.2012.10.012
76. Bergwerff M, Gittenberger-de Groot AC, Wisse LJ, DeRuiter MC, Wessels A, Martin JF, Olson EN, Kern MJ. Loss of function of the Prx1 and Prx2 homeobox genes alters architecture of the great elastic arteries and ductus arteriosus. *Virchows Arch*. 2000;436:12–19. doi: 10.1007/pl00008193
77. Blaschke RJ, Hahurij ND, Kuijper S, Just S, Wisse LJ, Deissler K, Maxelon T, Anastassiadis K, Spitzer J, Hardt SE, et al. Targeted mutation reveals essential functions of the homeodomain transcription factor Shox2 in sinoatrial and pacemaking development. *Circulation*. 2007;115:1830–1838. doi: 10.1161/CIRCULATIONAHA.106.637819
78. Espinoza-Lewis RA, Yu L, He F, Liu H, Tang R, Shi J, Sun X, Martin JF, Wang D, Yang J, et al. Shox2 is essential for the differentiation of cardiac pacemaker cells by repressing Nkx2-5. *Dev Biol*. 2009;32:376–385. doi: 10.1016/j.ydbio.2008.12.028
79. Hoffmann S, Schmitteckert S, Raedecke K, Rheinert D, Diebold S, Roeth R, Weiss B, Granzow M, Niesler B, Griesbeck A, et al. Network-driven discovery yields new insight into Shox2-dependent cardiac rhythm control. *Biochim Biophys Acta Gene Regul Mech*. 2021;1864:194702. doi: 10.1016/j.bbagrm.2021.194702
80. Liang X, Zhang Q, Cattaneo P, Zhuang S, Gong X, Spann NJ, Jiang C, Cao X, Zhao X, Zhang X, et al. Transcription factor ISL1 is essential for pacemaker development and function. *J Clin Invest*. 2015;125:3256–3268. doi: 10.1172/JCI68257
81. Vedantham V, Galang G, Evangelista M, Deo RC, Srivastava D. RNA sequencing of mouse sinoatrial node reveals an upstream regulatory role for Islet-1 in cardiac pacemaker cells. *Circ Res*. 2015;116:797–803. doi: 10.1161/CIRCRESAHA.116.305913
82. Galang G, Mandla R, Ruan H, Jung C, Sinha T, Stone NR, Wu RS, Mannion BJ, Allu PKR, Chang K, et al. ATAC-Seq reveals an Isl1 enhancer that regulates sinoatrial node development and function. *Circ Res*. 2020;127:1502–1518. doi: 10.1161/CIRCRESAHA.120.317145
83. Tucker NR, Dolmatova EV, Lin H, Cooper RR, Ye J, Hucker WJ, Jameson HS, Parsons VA, Weng LC, Mills RW, et al. Diminished PRRX1 expression is associated with increased risk of atrial fibrillation and shortening of the cardiac action potential. *Circ Cardiovasc Genet*. 2017;10:e001902. doi: 10.1161/CIRCGENETICS.117.001902