ORIGINAL RESEARCH

Variants in the *SCN5A* Promoter Associated With Various Arrhythmia Phenotypes

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***Background*—**Mutations in the coding sequence of *SCN5A*, which encodes the cardiac Na^+ channel α subunit, have been associated with inherited susceptibility to various arrhythmias. Variable expression of *SCN5A* is a possible mechanism responsible for this pleiotropic effect; however, it is unknown whether variants in the promoter and regulatory regions of *SCN5A* also modulate the risk of arrhythmias.

***Methods and Results*—**We resequenced the core promoter region of *SCN5A* and the regulatory regions of *SCN5A* transcription in 1298 patients with arrhythmia phenotypes (atrial fibrillation, n=444; sinus node dysfunction, n=49; conduction disease, n=133; Brugada syndrome, n=583; and idiopathic ventricular fibrillation, n=89). We identified 26 novel rare variants in the *SCN5A* promoter in 29 patients affected by various arrhythmias (atrial fibrillation, n=6; sinus node dysfunction, n=1; conduction disease, n=3; Brugada syndrome, n=14; idiopathic ventricular fibrillation, n=5). The frequency of rare variants was higher in patients with arrhythmias than in controls. In the alignment with chromatin immunoprecipitation sequencing data, the majority of variants were located at regions bound by transcription factors. Using a luciferase reporter assay, 6 variants (Brugada syndrome, n=3; idiopathic ventricular fibrillation, n=2; conduction disease, n=1) were functionally characterized, and each displayed decreased promoter activity compared with the wild-type sequences. We also identified rare variants in the regulatory region that were associated with atrial fibrillation, and the variant decreased promoter activity.

***Conclusions*—**Variants in the core promoter region and the transcription regulatory region of *SCN5A* were identified in multiple arrhythmia phenotypes, consistent with the idea that altered *SCN5A* transcription levels modulate susceptibility to arrhythmias.
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Key Words: arrhythmias • genetics • ion channels • sodium channels • transcription

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Voltage-gated sodium channels play a critical role in the generation and propagation of the cardiac action potential. Mutations in *SCN5A*, the gene encoding the major pore-forming sodium channel α subunit in the heart (Nav1.5), are associated with inherited susceptibility to a wide variety of arrhythmia syndromes, the "cardiac sodium channelopathies."[1–11] Gain-of-function mutations in *SCN5A* produce an enhanced sodium current during the action potential plateau and cause long QT syndrome (type 3),[1,2] whereas loss-of-function mutations produce a reduced sodium current and lead to various arrhythmias, including Brugada syndrome,[3] early repolarization syndrome,[4] idiopathic ventricular fibrillation,[5] cardiac conduction disease,[6] sinus node dysfunction,[7] atrial standstill,[8] and cardiomyopathy.[12,13] Both gain- and loss-of-function mutations are associated with atrial fibrillation.[9,10] Furthermore, sodium channel accessory subunit genes and sodium channel partner genes are also associated with arrhythmia syndromes.[14–19] Nevertheless, despite extensive efforts, most patients with arrhythmias suggestive of cardiac sodium channelopathies are genotype negative. Because reduced sodium channel expression is a major mechanism by which mutations in sodium channel genes alter sodium currents, leading to arrhythmia syndromes,[1–7] genetic variants in regulatory regions of *SCN5A* may cause arrhythmias.

Increasing evidence shows that the regions regulating *SCN5A* transcription play a critical role in the electrophysiology of the heart.[20–27] A region around the noncoding exon 1 was initially identified as the core *SCN5A* promoter.[21] Furthermore, it has been found that conserved noncoding sequences (CNSs) in intron 1 of *SCN5A* regulate promoter activity and cardiac conduction.[20] Single-nucleotide polymorphisms in the *SCN5A* core promoter region alter transcriptional activity, and a haplotype of the *SCN5A* promoter is associated with reduced transcriptional activity and the slowing of cardiac conduction.[22,23] A common haplotype of 2 single-nucleotide polymorphisms in the promoter region has recently been associated with the severity of arrhythmia phenotypes, including conduction disease and ventricular tachyarrhythmias, in a family affected by a loss-of-function mutation in the coding region of *SCN5A*.[24] Furthermore, evidence supporting the association of *SCN5A* transcriptional regulation and cardiac conduction includes the findings that genetic variation in a key cardiac transcription factor is associated with cardiac conduction in a genomewide association study and that the transcription factor regulates *SCN5A* expression.[25–27] We tested the hypothesis that variants in the *SCN5A* core promoter region influence susceptibility to cardiac electrical diseases.

Methods

Study Participants

The study protocol was approved by the institutional review board of each institution. All participants provided written informed consent prior to the genetic and clinical investigations, in accordance with the standards of the Declaration of Helsinki and local ethics committees. This study included 1298 unrelated patients with arrhythmia phenotypes (atrial fibrillation, n=444; sinus node dysfunction, n=49; conduction disease, n=133; Brugada syndrome, n=583; idiopathic ventricular fibrillation, n=83; early repolarization syndrome, n=6) who did not have mutations in the coding sequences and flanking regions of *SCN5A*. Among the 1298 patients, 114 patients with Brugada syndrome and 376 patients with atrial fibrillation were white, and the remaining 808 patients were Japanese. We also resequenced the *SCN5A* promoter in 816 controls who were free from arrhythmias including 282 white and 534 Japanese participants. CNS23 and CNS28 in intron 1 of *SCN5A* regulate promoter activity,[20] and we resequenced CNS23 and CNS28 in 405 patients with atrial fibrillation, 65 patients with cardiac conduction disease, and 664 controls.

Genetic Analysis

A genetic analysis was performed using genomic DNA extracted from peripheral white blood cells with standard methods. The core promoter region of *SCN5A* and the CNSs were amplified by polymerase chain reaction using primers described elsewhere, and direct DNA sequencing was performed.[20] Variants found in controls, the 1000 Genomes Project data, the Tohoku Megabank Whole Genome data, or dbSNP (version 142) were excluded.[28]

Chromatin Immunoprecipitation Sequencing Analysis Using Adult Mouse Hearts

The association of the *SCN5A* promoter variants identified in this study with the key cardiac transcription factors was studied using data obtained from genomewide screens for binding sites of the key cardiac transcription factors in a previous study by our group (Figure 1).[25] FASTQ files from the GEO data sets for Tbx3 (GSE44821), Tbx5 (GSE21529), Nkx2-5 (GSE35151), and p300 and pol2 (GSE29184) as well as other markers of transcriptional activity were aligned to the mouse genome using the Galaxy server (http://galaxy.nbic.nl). Wiggle format data derived from these alignments were lifted over to the human genome (Hg18) for regions of interest and then uploaded as headed bedgraph track data to the University of California, Santa Cruz (UCSC) genome browser alongside positional data for the *SCN5A* promoter

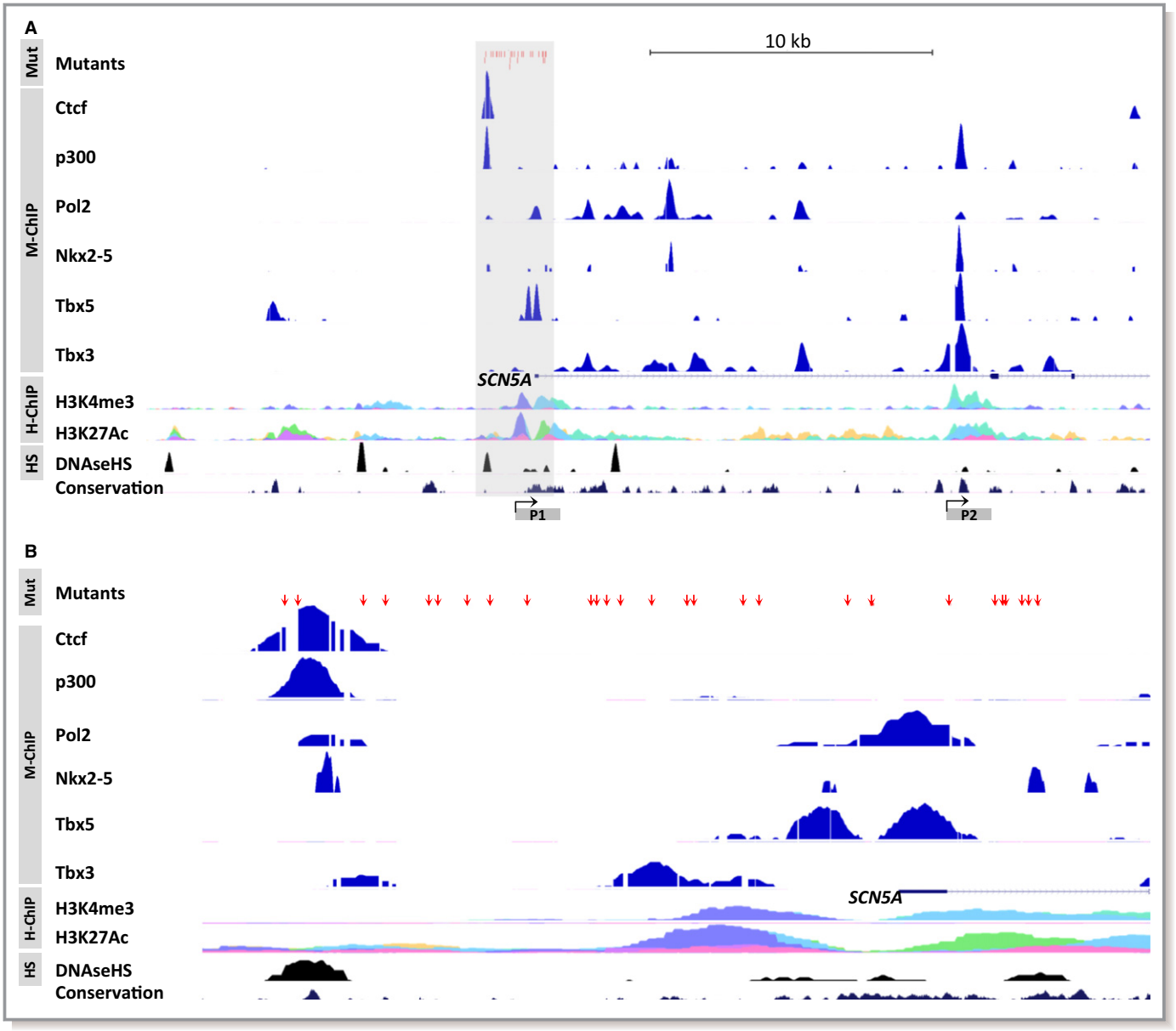

Figure 1. Human genomic promoter region of *SCN5A*. A, An overview of chromatin immunoprecipitation (ChIP) sequencing data sets including the *SCN5A* locus. B, A close-up of the core promoter region of *SCN5A* marked in panel A with a gray box. The genomic positions of the identified mutants are marked with red arrows showing their location with respect to various transcription factors (Ctcf, Pol2, p300, Nkx2-5, Tbx5 and Tbx3, lifted over to Hg18 from the mouse genome data sets) and other markers of transcriptional activity. The histone marks H3K4me3 and H3K27Ac and DNase hypersensitivity (HS) typically highlight regions of open active chromatin such as enhancers and promoters. Alternative promoters are marked P1 and P2. H-ChIP indicates human heart ChIP sequencing; Mut, locations of mutants; M-ChIP, mouse heart ChIP sequencing.

variants and other preselected tracks available for display on the UCSC browser.

Functional Analysis of Rare Variants

Transient transfection analyses were performed, as previously reported.[20–23] Briefly, the full-length human *SCN5A* promoter (haplotype A) was subcloned into the pGL3-Basic plasmid (Promega, Madison, WI).[21,23] Mutant constructs were prepared using a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. The pGL3-Basic plasmids carrying the wild-type or mutant *SCN5A* promoter (1 μg) were transfected into HEK293 cells, Chinese hamster ovary cells, and the mouse cardiomyocyte cell line HL-1 using Lipofectamine LTX (Invitrogen) or Fugene 6 transfection reagent (Roche Applied Science).[21–23] To study

the effects of variants in the CNSs on promoter activity, the pGL3-Basic plasmids carrying wild-type or mutant sequences with the SV40 promoter were also transfected into Chinese hamster ovary cells.[20] In each experiment, the pRL-TK plasmid (50 ng; Promega) encoding Renilla luciferase was cotransfected to normalize for experimental variability caused by differences in cell viability or transfection efficiency. Luminescence was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). The pGL3-Basic plasmid without promoter was tested in each experiment, and its activity level served as the baseline. Potential muscle-specific transcriptional regulatory modules in CNS28 were predicted using the M-SCAN algorithm (http://www.-cisreg.ca/cgi-bin/mscan/MSCAN).[20] To study the affinities of a human heart protein for the transcription factors that were predicted to bind to sequences within CNS28, an electrophoretic mobility shift assay was performed using the standard protocol. Briefly, double-stranded oligonucleotides containing the CNS28 sequence were incubated with a nuclear lysate from a human heart. A CNS28 unlabeled competitor probe was used as a control.

Statistical Analysis

The Mann–Whitney *U* test or Fisher exact test was used to evaluate significant differences. All statistical analyses were performed using SPSS version 20 (IBM Corp). A 2-sided $P<0.05$ was considered statistically significant. Values are expressed as mean±SEM. The authors had full access to the data and take full responsibility for its integrity. All the authors have read and approved the paper as written.

Results

We identified 26 novel rare variants in the *SCN5A* promoter in 29 unrelated patients affected by various arrhythmias (atrial fibrillation, n=6; sinus node dysfunction, n=1; conduction disease, n=3; Brugada syndrome, n=14; idiopathic ventricular fibrillation, n=5) (Table). All variants were absent in controls and in the 1000 Genomes Project data.[28] The patients carrying a rare variant in the *SCN5A* promoter included 23 men (79%) and had a mean age of 44±20 years. One patient with atrial fibrillation had 2 variants. The same variants were identified in multiple unrelated patients (Table): c.-225-849insTG was identified in 3 patients with Brugada syndrome (patients 17–19), and c.-225-115G>T was identified in 1 patient with conduction disease (patient 4) and 1 patient with Brugada syndrome (patient 23). Among patients carrying a rare variant in the *SCN5A* promoter, conduction abnormalities were present in 18 patients (62%), and early repolarization or J-point elevation was present in 3 patients (12%). There was no abnormal QT interval in any patient. A family history of arrhythmias and/or sudden cardiac death was present in 11 patients (37%). Among patients with Brugada syndrome who were screened, rare variants in the *SCN5A* promoter were present in 14 of the 469 Japanese patients (3%) but were not present in any of the 114 white patients (0%).

In addition, 3 novel rare variants in the *SCN5A* promoter were identified in patients with arrhythmia phenotypes and in controls, but the frequency of the variants was not different. Similarly, the frequency of haplotype A/B, which has been associated with cardiac conduction,[23] did not differ between patients with arrhythmia phenotypes and controls (allelic frequency 0.22 for both groups).

Two promoters of *SCN5A* have been reported previously in mice, and the human *SCN5A* promoter can be mapped to the first promoter of the mouse.[29] The rare variants in the *SCN5A* promoter identified in patients affected by various arrhythmias were mapped to the mouse genome and aligned with data from genomewide screens for binding sites of the key cardiac transcription factors identified in a previous study from our group (Figure 1).[25] All *SCN5A* promoter variants were successfully mapped. The majority of the variants were located in regions bound by transcription factors that are important for both the development and maintenance of cardiac function, suggesting that the variants disrupt the associations of these factors that have been shown to affect an enhancer involved in *SCN5A* expression.[25] Using a luciferase reporter assay, 6 variants identified in patients with arrhythmias (Brugada syndrome, n=3; idiopathic ventricular fibrillation, n=2; conduction disease, n=1) were functionally characterized. Each mutant promoter consistently displayed decreased promoter activity compared with the wild-type sequences (Figure 2).

We identified a rare variant (c.-53+15025C>T) in CNS28, which is important for the transcriptional regulation of *SCN5A*,[20] in a patient with atrial fibrillation. This variant was absent in the controls and in the 1000 Genomes Project data. We also identified a variant (-53+15307G>A) in CNS28 in 7 of the 405 patients with atrial fibrillation and 1 of the 664 controls and found a higher incidence rate in patients with atrial fibrillation than in controls ($P=0.006$) (Figure 3). This variant is located within 1 of the 3 tandem binding sites for transcriptional enhancer factor 1 (TEF-1) in CNS28 and is predicted to disrupt the TEF-1 binding site, which is well conserved across species.[20] Electrophoretic mobility shift assays revealed a strong interaction between CNS28 and the nuclear protein isolated from the human heart, and the interaction was specifically blocked by anti-TEF antibodies, indicating that TEF-1 is associated with CNS28. Promoter reporter assays revealed that the variant was associated with decreased regulation of the promoter compared with the wild-type sequence.

Table. Clinical Characteristics of Patients Carrying a Rare Variant in *SCN5A* Promoter

Patient No.	Sex	Age at Onset, y	Disease	Promoter Variant	ECG Abnormalities	Family History of Arrhythmias	Family History of Sudden Death
1	F	62	SND	c.-225-820T>C	No	No	No
2	M	71	Conduction disease	c.-53+147delG	Prolonged PR interval	No	No
3	F	82	Conduction disease	c.-53+265_+269GGGTT	Prolonged PR interval	No	No
4	F	9	Conduction disease	c.-225-115G>T	Left bundle branch block	Conduction disease	No
5	M	39	AF	c.-225-1340G>T	Prolonged PR interval	SVT	No
6	F	28	AF	c.-225-1315G>T	No	AF	No
7	F	47	AF	c.-225-1161A>G, c.-53+241C>A	No	No	No
8	M	17	AF	c.-53+167G>T	No	AF	No
9	M	64	AF, SND	c.-53+175delA	No	AF, SND	Yes
10	M	58	AF	c.-53+222G>A	RBBB, prolonged PR interval	No	No
11	M	21	Brugada syndrome	c.-225-1763T>C	RBBB	No	No
12	M	75	Brugada syndrome	c.-225-1723C>T	RBBB, AF	No	No
13	M	44	Brugada syndrome	c.-225-1531C>T	No	No	Yes
14	F	54	Brugada syndrome	c.-225-1467G>A	No	No	No
15	M	67	Brugada syndrome	c.-225-782_779delGTTT	RBBB	No	No
16	M	32	Brugada syndrome	c.-225-866insTA	No	No	No
17	M	67	Brugada syndrome	c.-225-849insTG	RBBB, left anterior hemiblock	No	Yes
18	M	54	Brugada syndrome	c.-225-849insTG	Intraventricular block, inferior early repolarization	No	No
19	M	34	Brugada syndrome	c.-225-849insTG	RBBB	No	Yes
20	M	22	Brugada syndrome	c.-225-688T>C	No	No	No
21	M	43	Brugada syndrome	c.-225-587_-584 del CAGT	Prolonged PR interval	No	Yes
22	M	38	Brugada syndrome	c.-225-565T>C	Inferior early repolarization	No	No
23	M	57	Brugada syndrome	c.-225-115G>T	No	No	No
24	M	39	Brugada syndrome	c.-53+11G>A	RBBB	No	Yes
25	M	13	IVF	c.-225-1228A>G	RBBB	No	No
26	M	26	IVF	c.-225-1052G>A	No	No	No
27	M	61	IVF	c.-225-420G>C	Prolonged PR interval	No	No
28	M	15	IVF	c.-225-374 G>T	J-point elevation	ERS	Yes
29	M	41	IVF	c.-225-51_-42del CCGACCCCGC	RBBB	No	No
	Male, n=23 (79%)	44±20			Conduction abnormalities, n=18 (62%)	n=7 (24%)	n=7 (24%)

AF indicates atrial fibrillation; ERS, early repolarization syndrome; IVF, idiopathic ventricular fibrillation; RBBB, right bundle branch block; SND, sinus node dysfunction; SVT, supraventricular tachycardia.

Discussion

We identified rare variants in the core promoter region of *SCN5A* in patients with multiple arrhythmia syndromes. Chromatin immunoprecipitation sequencing analysis revealed that the majority of the promoter variants were localized to regions bound by transcription factors and other factors that are important for transcriptional activity. In the functional analysis, the variants in the *SCN5A* promoter displayed decreased activity compared with the wild-type sequences. We also identified a rare variant in the regulatory region of *SCN5A* transcription that was associated with decreased promoter activity. Our findings suggest that variability in *SCN5A* transcription affects susceptibility to a wide variety of arrhythmias.

The sodium channel plays an important role in normal cardiac function. The generation and propagation of electrical impulses throughout the atria, the ventricles, and the Purkinje network are critically dependent on normal sodium channel function; reductions in the sodium current slow the heart rate and cardiac conduction, as has been shown in heterozygous *SCN5A* knockout mice and during therapy with sodium channel blockers.[30] Recent genomewide association studies have shown that the heritability of cardiac conduction is ≈30% to 40%, and these studies have consistently identified an association of the *SCN5A* and *SCN10A* loci with variation in cardiac conduction as one of the most significant association signals.[27,31–34] Furthermore, loss-of-function mutations in *SCN5A* cause isolated conduction disease, and decreased sodium channel expression is a mechanism underlying such disease.[6,35,36] In the present study, rare variants in the core promoter region of *SCN5A* were identified in patients with isolated conduction disease. Furthermore, 62% of patients carrying a rare variant in the *SCN5A* promoter had conduction abnormalities, further supporting the hypothesis that the sodium channel expression level is important for cardiac conduction. Similarly, the sodium channel controls cardiac excitability, and rare variants in the *SCN5A* promoter were identified in patients with sinus node dysfunction, which can be caused by mutations in the coding region of *SCN5A*.[7,8]

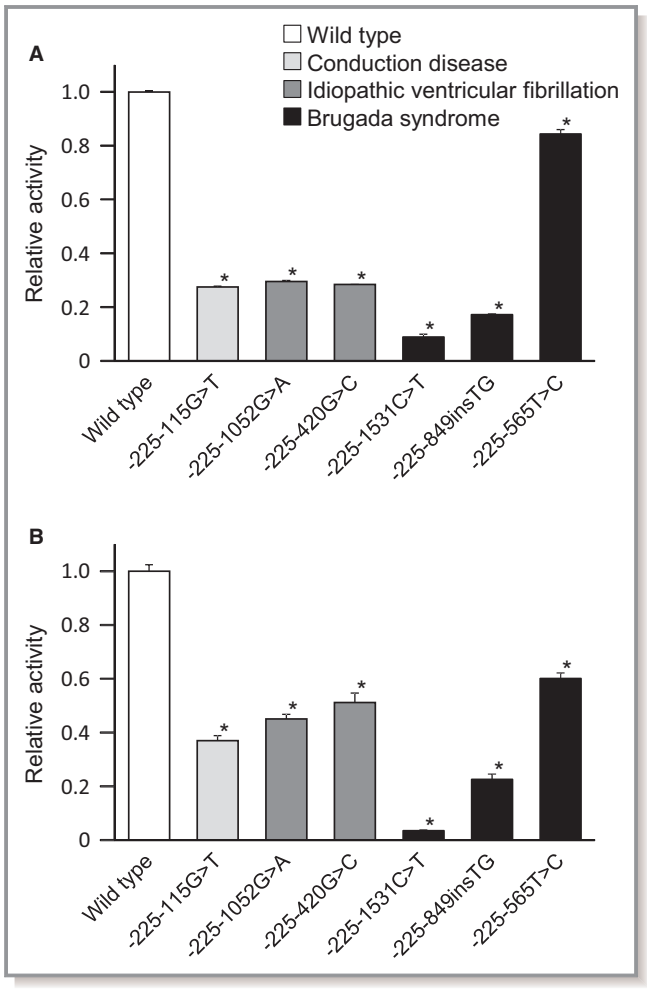

Figure 2. Promoter activity of rare variants in the *SCN5A* promoter region. In both HEK293 cells (A) and HL-1 cardiomyocytes (B), each mutant promoter (n=4) displayed decreased activity compared with the wild-type promoter (n=8). **P*<0.01 vs the wild-type promoter.

To date, mutations in 20 different genes have been associated with Brugada syndrome, and sodium channel dysfunction is an important inherited mechanism of this disease.[37–43] The causative genes of Brugada syndrome include sodium channel genes (α-subunit gene *SCN5A* and β-subunit genes) and sodium channel partner genes (*GPD1L*, *MOG1*, *SLMAP*, and *PKP2*).[38–40,42] Mutations in *TRPM4* that can affect the resting membrane potential and thus reduce sodium channel availability have been identified recently in patients with Brugada syndrome.[41] The present study added variants in the *SCN5A* promoter to the genetic cause of Brugada syndrome resulting in decreased sodium current. Evidence that common variants in 3 genes including *SCN5A*, *SCN10A*, and *HEY2*, all of which affect cardiac conduction, are associated with Brugada syndrome in a recent genomewide association study further supports this hypothesis.[44] We screened for rare variants in the *SCN5A* promoter in Japanese and European patients with Brugada syndrome, but we identified unique variants only in the Japanese patients. The prevalence of Brugada syndrome is high in Southeast Asia compared with other areas, and our findings may explain, at least in part, this difference in prevalence.[39,45] Loss-of-function mutations in *SCN5A* are also associated with other forms of idiopathic ventricular fibrillation, including early repolarization syndrome characterized by J-point elevation in the inferolateral leads and idiopathic ventricular fibrillation without J-point elevation.[4,5] We identified rare variants in the *SCN5A* promoter in patients with these forms of idiopathic ventricular fibrillation. Furthermore, right bundle-branch block has recently been associated with idiopathic ventricular

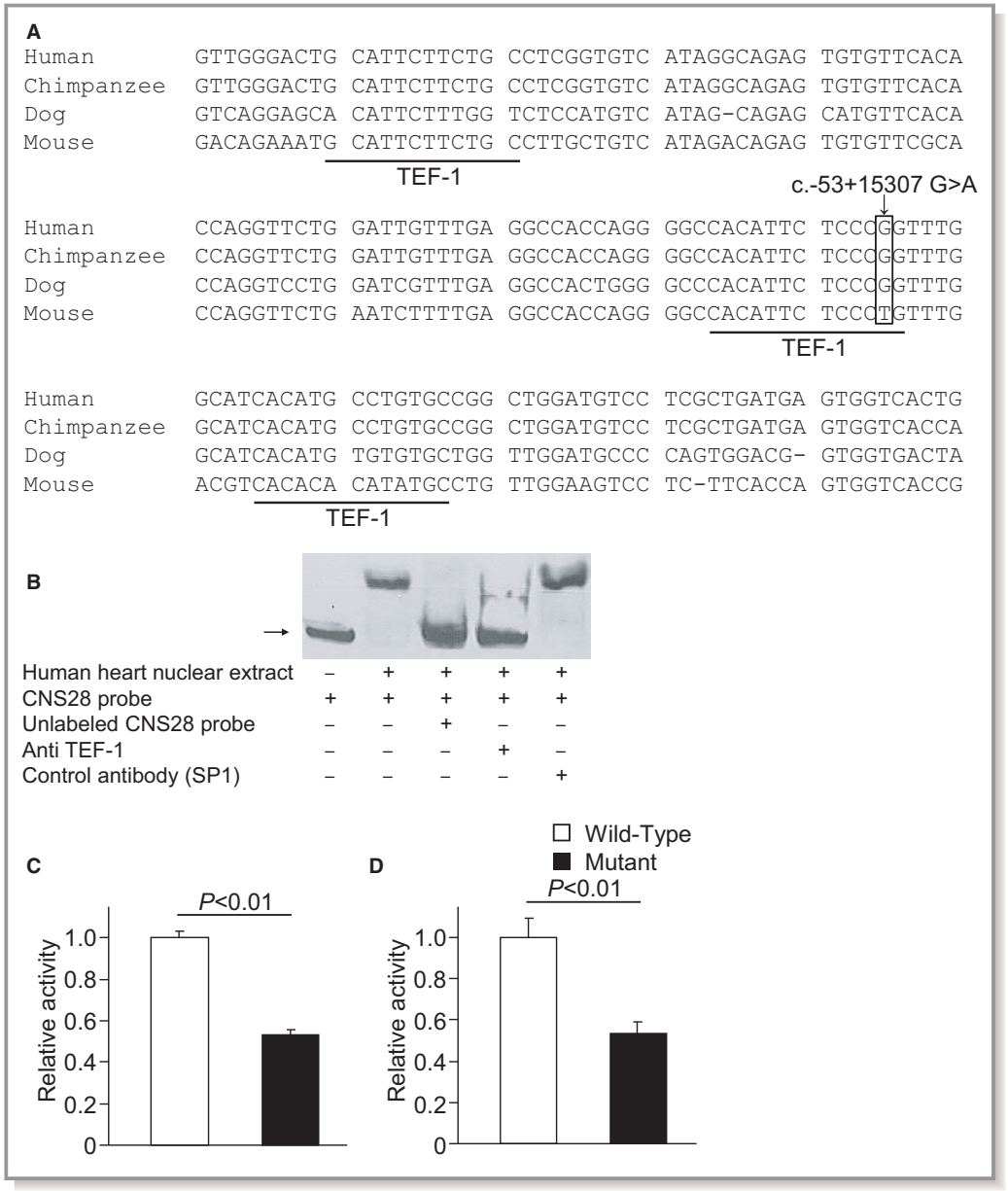

Figure 3. A variant in the conserved noncoding sequence 28 (CNS28) in intron 1 of *SCN5A* that regulates *SCN5A* transcription associated with atrial fibrillation. A, CNS28 includes 3 tandem binding sites for the transcriptional enhancer factor 1 (TEF-1). A variant in CNS28 is predicted to disrupt the second binding site for TEF-1. B, Electrophoretic mobility shift assays revealed a strong interaction between CNS28 and the nuclear protein isolated from the human heart. The interaction was specifically blocked by anti–TEF antibody. In both Chinese hamster ovary cells (C) and HL-1 cardiomyocytes (D), the variant (n=4) decreased regulation of the promoter compared with the wild-type sequence (n=4).

fibrillation, and we also identified a rare variant in the *SCN5A* promoter in a patient with this disorder.[46]

Atrial fibrillation is the most common arrhythmia, with a high lifetime risk of 1 in 4 to 6.[47] A number of studies have demonstrated a genetic contribution to atrial fibrillation susceptibility, and sodium channel dysfunction is an important pathogenic mechanism of atrial fibrillation.[9,10,48–50] Mutations in *SCN5A* that result in either increases or decreases in sodium currents account for up to 6% of patients with atrial fibrillation, and we identified rare variants in the *SCN5A* promoter region in patients with atrial fibrillation.[9,10,48,49] Furthermore, a recent genomewide association study with a large cohort showed that polymorphisms in *SCN5A* are associated with a risk of atrial fibrillation.[50] These polymorphisms in *SCN5A* have been associated with cardiac conduction in previous genomewide association studies, suggesting that the polymorphisms result in decreased sodium currents.[27,31] Our findings that a variant in the

regulatory region of *SCN5A* transcription reduced promoter activity and was associated with atrial fibrillation are in accordance with previous reports.

Genetic screening is usually performed for nonsynonymous mutations in the coding exons and flanking intronic sequences, but genetic causes have not been sufficiently identified in patients with arrhythmia syndromes, especially those with arrhythmias that may result from loss-of-function mutations in sodium channel genes.[51] In a recent study, 80% of the genome was shown to have biochemical functions, particularly portions outside of the protein-coding regions, and increasing evidence suggests the importance of noncoding regions in cardiac electrophysiology.[23,24,52] A haplotype of the *SCN5A* promoter that is commonly found in Asian persons affects transcriptional activity and modulates cardiac conduction in healthy persons, indicating that genetically determined variable transcription of the sodium channel occurs in the human heart.[23] This haplotype has also modulated the extent to which sodium channel blockers slow conduction in patients with Brugada syndrome. Furthermore, another common haplotype of the *SCN5A* promoter is associated with the severity of phenotypes caused by a nonsynonymous mutation in *SCN5A* in a family affected with conduction disease and Brugada syndrome.[24] In the present study, variants in the *SCN5A* promoter were associated with a wide variety of arrhythmia syndromes. Although variants in the *SCN5A* promoter may not be monogenic causes of arrhythmias, they may increase susceptibility to arrhythmias. This hypothesis is supported by evidence from a recent study that arrhythmia syndrome can be caused by multiple genetic factors.[44] Future studies may elucidate an additional role of noncoding sequences in the pathogenesis of arrhythmia syndromes.

This study has several limitations. The frequency of variants was low, and thus the variants may not be responsible in a large number of patients. Although we used multiple control sets including direct sequencing data in control participants who were free from arrhythmias and in public data sets, the number of controls for white patients was relatively small. This study included only white and Japanese patients, and the results of this study may not be applied to other ethnicities. Linkage or segregation analysis was not conducted because DNA was not available in family members of probands included in this study. The same variants (c.-225-849insTG and c.-225-115G>T) were identified in multiple unrelated probands from different families. Although we did not check the relatedness of the probands, these 2 variants were absent in controls and in public databases and resulted in decreased promoter activity, suggesting that these 2 variants are associated with arrhythmia susceptibility. Although a relatively large number of rare variants in the *SCN5A* promoter region were identified, the number of variants that were functionally tested was limited. The functional analyses of variants were performed using HEK293 cells, Chinese hamster ovary cells, and mouse atrial myocytes (HL-1) and the in vitro characteristics were consistent with the phenotype in the patients, but the environment was different from that in the native human cardiomyocyte, especially from that in ventricular myocytes.

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Disclosures

None.

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