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Polymorphisms in the cardiac sodium channel promoter displaying variant *in vitro* expression activity

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Variable transcription of the cardiac sodium channel gene is a candidate mechanism determining arrhythmia susceptibility. We have previously cloned and characterized the core promoter and flanking region of *SCN5A*, encoding the cardiac sodium channel. Loss-of-function mutations in this gene have been reported in ~20% of patients with Brugada syndrome, an inherited cardiac electrical disorder associated with a high incidence of life-threatening arrhythmias. In this study, we identified DNA variants in the proximal 2.8 kb promoter region of *SCN5A* and determined their frequency in 1121 subjects. This population consisted of 88 Brugada syndrome patients with no *SCN5A* coding region mutation, and 1033 anonymized subjects from various ethnicities. Variant promoter activity was assayed in CHO cells and neonatal cardiomyocytes by transient transfection of promoter–reporter constructs. Single-nucleotide polymorphisms (SNPs) were identified at ~1/200 base pairs which are: 11 in the 5′-flanking region, 1 in exon 1, and 5 in intron 1. In addition, a haplotype consisting of two SNPs in complete linkage disequilibrium was identified. Minor allele frequencies were >5% in at least one ethnic panel at 5/19 polymorphic sites. *In vitro* functional analysis in cardiomyocytes identified four variants with significantly ($P < 0.05$) reduced reporter activity (up to 63% reduction). The largest changes were seen with c.–225–1790 G>A, which reduced reporter activity by 62.8% in CHO cells and 55% in cardiomyocytes. From these results, we can conclude that the *SCN5A* core promoter includes multiple DNA polymorphisms with altered *in vitro* activity, further supporting the concept of interindividual variability in transcription of this cardiac ion channel gene.

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Introduction

The *SCN5A* gene encodes the α -subunit of the predominant voltage-sensitive sodium channel expressed in cardiac muscle. The channel is an integral membrane protein that selectively allows sodium influx into the heart cell, propagates the initial upstroke of the action potential, and underlies fast conduction in human atrium and

ventricle.^{1,2} The human gene has been mapped to chromosome 3p21,³ and 'gain of function' mutations associated with defective fast inactivation cause type 3 of the congenital long QT syndrome.⁴ Loss-of-function mutations have also been described and linked to congenital arrhythmia syndromes, including conduction system failure,⁵ sick sinus syndrome,⁶ atrial fibrillation,⁷ and Brugada syndrome (BS),⁸ which predisposes to ventricular fibrillation (VF); in BS, *SCN5A*-coding region variants can be identified in ~20–30% of affected patients. Loss of sodium channel function has been associated with an increased risk for VF not only in BS, but also with acquired lesions, such as acute myocardial ischemia^{9,10} or therapy with sodium channel blocking drugs.^{9–11} Experimental evidence supports the idea that loss of sodium channel function can cause re-entrant excitation by slowing conduction¹² or by enhancing heterogeneity of repolarization.^{13–15} Taken together, therefore, these data suggest that loss of sodium channel function is a common mechanism predisposing to life-threatening cardiac arrhythmias. For that reason, variable *SCN5A* transcription could be a candidate modulator of arrhythmia risk, particularly in the presence of added factors such as myocardial ischemia, drugs, or mutations. However, the role of DNA variants within *SCN5A* transcriptional control has not been yet thoroughly investigated.^{16,17}

Cis-acting elements are usually conserved among species and functionally organized into modules in which each module integrates input from a specific set of transcription factors to direct a corresponding spatio-temporal expression pattern.^{18,19} While many regions of DNA may include regulatory functions, promoter elements are sites of assembly for transcription factors through a site-selection process that plays a central role in transcriptional regulation.^{20,21} One recent promoter screening and functional study indicated that around a third of promoter variants may alter gene expression to a functionally relevant extent.²² We have previously cloned and characterized the core promoter and flanking region of human and mouse *SCN5A* genes, and reported a single-nucleotide polymorphism (SNP), c.–225–92 C>A (numbering see Table 2), present in 6/142 normal alleles, which increased transcriptional activity by ~50% in cardiomyocytes.²³ In addition, we have reported a six-change haplotype variant within this region that is common in east Asian populations, which reduces transcriptional activity, and is associated with conduction slowing in human populations.²⁴ To further evaluate the functional effect of DNA variants in this region, we screened it for DNA variants in 88 patients with BS but no *SCN5A*-coding mutations, and 1033 control subjects of diverse ethnicities. We report here the frequency of these variants and their functional characterization, compared to wild-type, in promoter-reporter experiments.

Methods

Study populations

Patients The BS was diagnosed in 88 patients following standard published criteria.^{25,26} Cases are from multiple medical centers that include the Vanderbilt University Medical Center ($n=13$), the Academic Medical Center, University of Amsterdam ($n=38$), the University Hospital Münster ($n=27$), and the LMU University Clinics Großhadern, Munich ($n=10$). A blood sample was obtained from each patient for extraction of DNA from lymphocytes. DNA was then harvested and archived. The investigation conforms with the principles outlined in the Declaration of Helsinki and informed consent was obtained from all individuals. All patients had been previously screened for *SCN5A*-coding region mutations and no mutation had been identified.

Reference populations

Four reference populations were studied:

1. A set of randomly selected and unrelated Dutch Caucasian individuals ($n=98$).
2. Randomly selected and unrelated individuals from the general population living in Southern Germany, surveyed during 2002 and 2003 ($n=702$, from the KORA S4 survey, a population-based sub-survey originating from the WHO MONICA project).²⁷
3. A set of randomly selected, unrelated individuals with ethnicities representing those of the middle Tennessee area ($n=71$; 48 white and 23 black).
4. A subset of ethnicity-defined but otherwise anonymized individuals ($n=162$, Coriell Polymorphism Discovery Resource multi-ethnicity panels that is available from the National Human Genome Research Institute).²⁸ The Coriell samples used in this study included four different ethnicity panels, consisting of unrelated and apparently healthy individuals. The panels screened in this study were as follows: (a) the Caucasian Panel (self-declared Caucasians), $n=47$; (b) the African American Panel (individuals of self-declared African ancestry or 3/4 grandparents with African ancestry), $n=45$; (c) Han Chinese from Los Angeles Panel (individuals with all four grandparents born in Taiwan, China, or Hong Kong), $n=26$; and (d) the Mexican–American Community of Los Angeles Panel (individuals with three or four grandparents born in Mexico), $n=44$.

Thus, a total of 2066 control alleles and 176 alleles in patients with BS were studied.

Screening for variants

Polymorphism identification Polymorphism identification was carried out in the BS patient group ($n=88$) and the Coriell group ($n=162$). The screened segment included 2.1 kb of 5' upstream sequence of exon 1, exon 1 (which is

173 bp and non-coding), and the proximal intron 1 regions that are relatively GC-rich (60.6% GC content) and highly conserved compared to mouse and rat. Proximal intron 1 of 439 bp was screened in the patient group, while in the Coriell group, the screening was extended to 708 bp of proximal intron 1. A 2.8 kb PCR fragment encompassing these regions was amplified with primer pair 1F/1R (Table 1), using the LA-PCR method (LA PCR kit, TaKaRa). Thermal cycling conditions were 94°C for 1 min, followed by 40 cycles of 94°C for 30 s, 64°C for 30 s, and 68°C for 3 min. The intron 1 fragment was amplified with primer pair 9F/9R (Table 1), using AmpliTaq Gold (Roche). Thermal cycling conditions were 95°C for 10 min, followed by 36 cycles of 95°C for 1 min, 64°C for 40 s, 72°C for 40 s, followed by 72°C for 10 min. After purification (QiaQuick PCR purification kit, Qiagen), the PCR product was sequenced using primers 1F-8R and 9R and the ABI BigDye Terminator Sequencing kit (Applied Biosystems), according to standard protocols.

Polymorphism genotyping Polymorphism genotypes for the KORA population ($n = 702$) were determined using PCR followed by primer extension reactions and MALDI-TOF mass spectrometric analysis (Sequenom, San Diego, CA, USA) using the Homogenous MassEXTEND[®] Assay (Sequenom). PCR was performed using primers flanking the relevant SNPs. Prior to the primer extension reaction, unincorporated primers and dNTPs were removed from the PCR product by treatment with 0.2 U Shrimp Alkaline Phosphatase (20 min at 37°C, 10 min at 85°C). Primer extension was performed by addition of a mastermix containing extension primer, dNTP/ddNTPs, and Thermo-sequenase (Amersham) to 7 μ l dephosphorylated PCR product, followed by a thermal cycling protocol as follows: 94°C for 2 min, 55 cycles of 94°C for 5 s, 52°C for 5 s, and 72°C for 10 s. Samples were desalted by adding Spectro-Clean Resin. Fifteen nanoliters of sample were then spotted onto the pad of the 384-SpectroCHIP[™] bioarray and subsequently subjected to mass spectrometric analysis.

Table 1 Sequence of oligonucleotide primers

Primer number	Sequence
1F	5'-TAGGAAGTGCCTGTCTCCAGACACCTGTTG-3'
1R	5'-CGCTCTCTGGAACACATTCATGGCG-3'
2F	5'-GACTCTGGGAAGCGGCTG-3'
3F	5'-AGAAGTGTGCCCCATAGGTCC-3'
4R	5'-ATGGTCACAGGCACACACACCGG-3'
5F	5'-CTAAGTCATGTGGGTGTCTAAG-3'
6R	5'-CCATAGGCATACCTGCACTGACA-3'
7F	5'-GTTTGTTAATGTGACCCTGTC-3'
8R	5'-TGCTGCTTGGCGCGGACTCG-3'
9F	5'-GCCTGCCGCCCCCAACTTTCCTC-3'
9R	5'-CCCCCATCCTCATCACCACCTAACAG-3'

Abbreviations: F, forward primer; R, reverse primer.

Polymorphism genotyping in the remaining control populations ($n = 169$) was performed by direct sequencing or restriction enzyme analysis according to standard protocols.

Plasmid construction To investigate the functional consequences of DNA variants in the screened region, 0.5 μ g of human genomic DNA was used as a template to amplify a fragment that contained the -2190 to +613 segment, using primer pairs 1F and 1R (Table 1). This PCR product was designated as P1 as in our previous report.²³ After cloning the P1 fragment into the pGEM-T Easy vector, the plasmid was digested with *Nco*I and *Sac*I and the insert was subcloned into the pGL3-Basic vector (Promega), which contains the firefly luciferase-coding sequence to generate a wild-type *SCN5A* promoter-luciferase fusion construct designated as pGL-P1 WT. All constructs containing mutations in the 5' upstream region were subsequently generated using pGL-P1 WT as template. To extend the functional characterization on those DNA variants identified in exon 1 and flanking intron 1 regions, a construct encompassing the -261 to +613 segment (designated P+613 WT in our previous report²³) was generated and subsequently served as template to generate mutated constructs containing variants in exon 1 and flanking intron 1.

Variants were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) using pGL-P1 WT or pGL-P+613 WT as template, following the manufacturer's instructions. All constructs were verified by direct sequencing.

Transient transfection assays Transient transfection analyses were performed as previously reported.²³ In brief, reporter activity was assayed in neonatal mouse cardiomyocytes and in CHO cells. One-day-old B6D2 mice were anesthetized and killed. Neonatal hearts were removed and placed in 1 \times PBS solution. Ventricular segments were digested by Trypsin-Versene (Biofluids Inc.) and cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% (v/v) NuSerum (Beckton Dickinson), 2.5 mM thymidine, and penicillin-streptomycin (10 U/ml and 10 mg/ml respectively) in a humidified 5% CO₂ atmosphere at 37°C. Cells were allowed to attach for 48 h before being used. Chinese hamster ovary K1 (CHO-K1) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described.²³

The *SCN5A* promoter-luciferase fusion constructs (1 μ g DNA) were transfected into the neonatal mouse cardiac cells using Fugene 6 (Roche), and into CHO cells using lipofectamine reagent (Invitrogen). In each experiment, the pRL-TK plasmid (0.05 μ g; Promega), encoding Renilla luciferase, was co-transfected to normalize for experimental variability caused by differences in cell viability or

transfection efficiency. Luminescence was measured 48 h after transfection by using the Dual-Luciferase Reporter Assay System (Promega). The pGL3-Basic (promoterless) plasmid was tested in each experiment and its activity level served as the baseline.

Statistical analysis

Allele frequencies between the (Caucasian) BS patient population and the (Caucasian) KORA control population were compared using χ^2 statistical analysis. Expression activity of mutant construct was divided by that of wild type that was obtained from the same transfection experiment to generate activity relative to wild type. For each construct analyzed, data obtained was based on at least six separate transient transfection experiments. Data are presented as mean \pm SE. Reporter gene-expression activities were compared between wild-type and mutant sequences. Statistical significance level was examined with Student's *t*-test. Throughout, a value of $P < 0.05$ was considered as being statistically significant. All statistical analyses were conducted with SPSS software (version: SPSS 13.0).

Results

Polymorphism identification

Seventeen SNPs and a haplotype consisting of two SNPs (c. [-225-1823 C>T; -225-834 T>C]) in complete linkage disequilibrium were identified in the 2.8 kb screened region (Table 2). Seven SNPs (c.-225-1790 G>A, c.-225-1657 A>G, c.-225-1294 T>G, c.-225-988 G>T, c.-225-667 A>G, c.-53+167 G>T, c.-53+274 G>A) were only identified in the control population and at low minor allele frequency (<2%). Six SNPs and the 2-SNP haplotype were identified in both case and control populations (Table 2). Among all variants identified, the most common were c.-225-2038 G>T, c.-53+114 C>T, c.-53+708 G>A, and the 2-SNP haplotype, with minor allele frequencies of >5% in at least one studied population (Table 2). Rarer alleles were often represented in a single ethnic group. For example, c.-225-1790 G>A, c.-225-1657 A>G, and the intronic variant c.-53+649 G>T were only identified in the Coriell African American control panel with minor allele frequencies of ~ 1 , ~ 1 , and $\sim 3\%$ respectively. In the 5' upstream region, 8 out of the 11 polymorphisms were identified in the -1 to -2 kb region while only three occurred within the region from the transcription start site through -1 kb. The overall frequency of the 19 variants described here and those previously reported^{24,23} within the screened region was $\sim 1/200$ base pairs. The nucleotide changes of the novel variants and variants described before were 50% transition (pyrimidine to pyrimidine or purine to purine), 46.2% transversion (purine to pyrimidine or vice versa), and 3.8% insertion/deletions. This is in agreement with previous reports indicating that transitions are more common than transversions.²⁹

No differences in allele frequencies were detected between the (Caucasian) BS patient population and the (Caucasian) KORA control population.

In vitro functional assays

Table 3 and Figure 1 show the effects of 12 of the 17 SNPs and the haplotype identified in this study on *SCN5A* promoter activities. Two rare variants, c.-225-1790 G>A, and the intron 1 variant c.-53+167 G>T reduced expression activities significantly in both cardiac myocytes and non-cardiac (CHO) cells. Compared with wild type, promoter activity in c.-225-1790 G>A was 0.45 ± 0.08 in cardiomyocytes and 0.37 ± 0.06 in CHO cells; promoter activity of c.-53+167 G>T was 0.47 ± 0.09 in cardiomyocytes and 0.58 ± 0.09 in CHO cells. The c.-225-1790 G>A variant was only identified in the Coriell African American sample, and c.-53+167 G>T was identified in the Middle Tennessee control population (Table 2).

Promoter activity in c.-225-775 T>A, identified in 1/176 alleles in the BS population and in Dutch and KORA controls, was significantly reduced in cardiomyocytes (0.76 ± 0.04 relative to wild type). Promoter activity of this variant was slightly but not statistically significantly reduced in CHO cells (0.82 ± 0.04 relative to wild type). In a similar cardiac-specific pattern, promoter activity in c.-225-1315 G>T identified in 1/176 alleles in the BS population and in KORA controls was significantly reduced in cardiomyocytes (0.55 ± 0.07 relative to wild type). The c.-206 G>A variant, also identified in 1/176 alleles in the BS population and in Dutch and KORA controls, only reduced promoter activity in CHO cells (0.78 ± 0.03).

About half of the characterized variants (7/12 SNPs and the haplotype) displayed activity similar to wild type in the functional assay (Table 3, Figure 1). Figure 2 summarizes the results of this and previous studies.^{24,23} *In vitro* functional analysis of variants in this study identified five novel variants with reporter activity reduced by >20% ($P < 0.05$) compared to wild type. The largest changes were seen with c.-225-1790 G>A. In addition, the previously reported²³ variant in the core promoter region (c.-225-92 C>A) showed increased activity by >20% ($P < 0.05$) and the six-change haplotype produced approximately 62% reduction of reporter activity in cardiomyocytes.²⁴

Discussion

This study systematically analyzed DNA variants within the *SCN5A* promoter in patients with BS and a large data set of control populations. We demonstrate that DNA variants are common within the transcription regulatory regions, and that five novel variants have a significant impact on sodium channel expression *in vitro*.

Transcription regulatory sequences constitute only a small fraction of the roughly 95% of the human genome that does not encode proteins, but determine the level,

Table 2 DNA variants identified in the transcriptional regulatory regions of *SCN5A* gene

DNA variants	Allele frequency in patients and controls								Reference
	Brugada syndrome patients (n = 176)	Dutch controls (n = 196)	KORA controls (n = 1404)	Coriell Caucasian controls (n = 94)	Coriell African American controls (n = 90)	Coriell hispanic controls (n = 88)	Coriell LA-Han controls (n = 52)	Middle TN controls (n = 142)	
<i>5' upstream region</i>									
c.-225-2038 G>T	0.13	N/A	0.13	0.18	0.096	0.27	0.14	N/A	This study
c.-225-1790 G>A	0	N/A	0	0	0.011	0	0	N/A	This study
c.-225-1744 G>C	0.028	N/A	0.019	0.011	0	0.011	0	N/A	This study
c.-225-1657 A>G	0	N/A	0	0	0.011	0	0	N/A	This study
c.-225-1315 G>T	0.006	N/A	0.004	0	0	0	0	N/A	This study
c.-225-1294 T>G	0	0.005	0	0	0	0	0	N/A	This study
c.-225-1223 C>T	0.006	N/A	0	N/A	N/A	N/A	N/A	N/A	This study
c.-225-1131 G>C	0.006	N/A	0.006	N/A	N/A	N/A	N/A	N/A	This study
c.-225-988 G>T	0	0.005	0	0	0	0	0	N/A	This study
c.-225-775 T>A	0.006	0.01	0.01	0	0	0	0	N/A	This study
c.-225-667 A>G	0	N/A	0.001	N/A	N/A	N/A	N/A	N/A	This study
<i>Core promoter</i>									
c.-225-92 C>A	0	0	0	0	0	0	0	0.035	Yang et al ²³
<i>Exon 1</i>									
c.-206 G>A	0.006	0.03	0.006	0	0	0	0	0	This study
<i>Intron 1</i>									
c.-53+114 C>T	0	N/A	0	0.013	0.011	0.011	0.27	0.01	This study
c.-53+167 G>T	0	N/A	0	0	0	0	0	0.007	This study
c.-53+274 G>A	0	N/A	0	0	0	0	0	0.007	This study
c.-53+649 G>T	N/A	N/A	0	0	0.033	0	0	N/A	This study
c.-53+708 G>A	N/A	N/A	0.001	0	0.16	0.011	0	N/A	This study
<i>Haplotype</i>									
c.[-225-1823 C>T;-225-834 T>C]	0.22	N/A	0.17	N/A	0.02	N/A	N/A	N/A	This study

Numbering based on *SCN5A* promoter RefSeq: AY_148488 and cDNA RefSeq: NM_000335 with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Abbreviations: *n*, allele number; N/A, not available.

Table 3 Functional consequences of DNA variants and potential effects on *cis-trans* interaction

DNA variants	Activity relative to wild type		Generation	Putative binding sites for trans-acting protein
	CHO cells	Cardiomyocytes		
<i>5' upstream region</i>				
c.-225-2038 G>T	0.97 ± 0.16	1.05 ± 0.17	—	—
c.-225-1790 G>A	0.37 ± 0.06*	0.45 ± 0.08*	GATA1(GATA-binding factor 1)	ZIC2 (Zinc-finger transcription factor, Zinc family member 2)
c.-225-1744 G>C	0.89 ± 0.09	1.10 ± 0.12	—	IK1 (Ikaros zinc-finger family)
c.-225-1657 A>G	0.80 ± 0.11	0.71 ± 0.11	—	ATF (Activating transcription factor); PAX3 (Pax-3 paired domain protein)
c.-225-1315 G>T	0.92 ± 0.21	0.55 ± 0.07*	—	—
c.-225-1294 T>G	0.84 ± 0.08	0.9 ± 0.07	ILF2 (interleukin enhancer binding factor 2, a protein subunit of nuclear factor 90 complexes)	SRY (sex-determining region Y gene product); CABL (C-abl DNA-binding sites)
c.-225-988 G>T	0.91 ± 0.17	0.87 ± 0.23	—	—
c.-225-775 T>A	0.82 ± 0.04	0.76 ± 0.04*	—	NFY (nuclear factor Y)
<i>Exon 1</i>				
c.-206 G>A	0.78 ± 0.03*	0.84 ± 0.08	CDE (cell cycle-dependent element)	—
<i>Intron 1</i>				
c.-53+114 C>T	0.92 ± 0.16	0.79 ± 0.08	—	—
c.-53+167 G>T	0.58 ± 0.09*	0.47 ± 0.09*	—	—
c.-53+274 G>A	1.23 ± 0.13	0.98 ± 0.06	—	—
<i>Haplotype</i>				
2-change: c.[-225-1823 C>T;-225-834 T>C]	0.86 ± 0.07	0.84 ± 0.18	NRF1 (Nuclear respiratory factor 1); HELT (Hey-like bHLH-transcriptional repressor)	NGFIC (nerve growth factor induced protein C); WT1 (Wilms tumor suppressor)
6-change			ZNF76 (a human ortholog of signal transducer and activator of transcription of <i>Xenopus</i>); 3WT1 sites; 3 ZF5 (Zinc finger/POZ domain transcription factor) sites	E2F (E2F, involved in cell-cycle regulation)

Activity relative to wild type: see Methods.
**P* < 0.05.

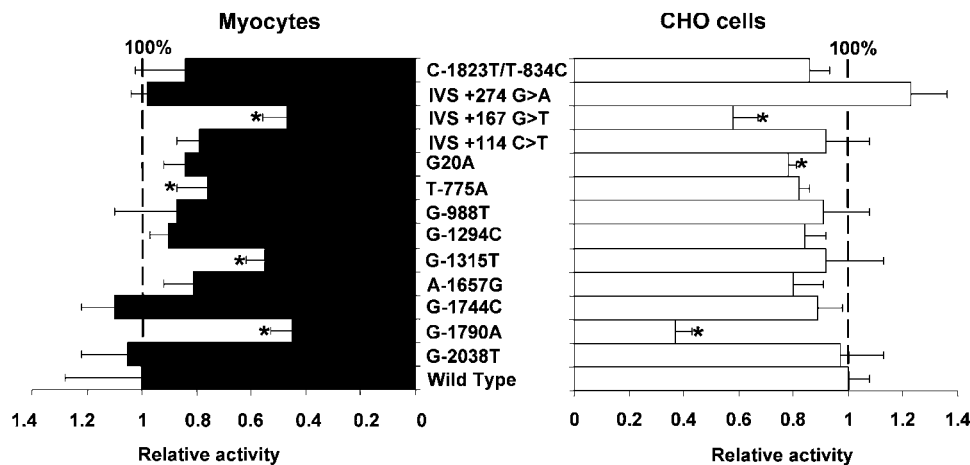


Figure 1 Functional analysis of DNA variants identified in the promoter and flanking regions in neonatal mouse cardiac myocytes and CHO cells. Transcriptional activities for mutant constructs are shown relative to wild type (see Methods). Firefly luciferase expression levels, which report the activities of the inserted *SCN5A* sequence, were normalized with the co-expressed Renilla luciferase activities to control for transfection efficiency. Data are presented as mean ± SEM and each data point was derived from at least six separate transfection experiments.

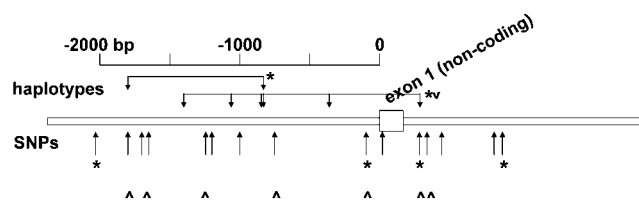


Figure 2 Summary of *SCN5A* promoter polymorphisms. The diagram indicates the genomic structure of the *SCN5A* promoter and flanking regions, and the positions of DNA variants identified in this study. * Indicates a minor allele frequency >4% in ≥1 screened population; ^ indicates variant reporter activity <80% or >120% of wild-type control in myocytes.

location, and chronology of gene expression. Functional polymorphisms in transcriptional regulatory regions may affect numerous processes in mRNA biogenesis and metabolism, including processing, trafficking, stability, and translational control. Sequence variants in regulatory regions are thus logical candidates for modulating human physiology, disease susceptibility, and response to environmental stressors. DNA polymorphisms in 5' regulatory regions have been linked to a variety of human disorders including lung cancer,³⁰ depression,^{31,32} and decreased activity of CYP3A4.³³ Previous studies from our laboratory and elsewhere³⁴ have characterized the modulating components of *SCN5A* core promoter in human and rat and have validated three conserved GC boxes that resemble the consensus Sp1 recognition site. Although Sp1-binding sites have been implicated as disease-associated 'hot spots',^{35–38} the DNA variant screening efforts in this study did not identify any functional polymorphisms occurring in these Sp1-binding sites and the core promoter region. The absence of loss-of-function DNA variants in the core promoter of *SCN5A* suggests that the functional components in *SCN5A* core promoter are highly selectively constrained.

Changes in *cis*-acting response elements that lead to differences in protein abundance are thought to act by altering the binding of *trans*-acting proteins (transcription factors). Identifying transcription factors that bind to specific DNA regions to generate transcriptional activity *in vivo* remains a major challenge in this area. One approach is to analyze potential binding sites using sequence-based programs. Accordingly, we screened the variant sites identified here in *SCN5A* by using MatInspector, a software tool that utilizes a library of transcription factor-binding sites to predict matches in specific DNA sequences.^{39,40} Interestingly, 2/15 and the two-change haplotype were predicted to alter transcription factor-binding sites (Table 3). Among them, we observed that the c.-225–1790 G>A variant, which significantly reduced expression activity, results in the generation of a GATA1-binding site and elimination of a putative ZIC2 site. ZIC2 belongs to the strong transcriptional activator

Cys₂–His₂ family of zinc-finger transcription factors.^{41,42} Elimination of this site could therefore hypothetically lead to loss of *SCN5A* expression. The previously reported six-change haplotype results in elimination of a predicted E2F-binding site and generation of three novel WT1 sites and two ZF5 sites, which is also consistent with the *in vitro* expression activity data and the loss-of-function phenotype observed in individuals carrying this haplotype allele (Table 3). Further investigation of these DNA–protein interactions predicted '*in silico*' will be needed to validate their biological significance.

The polymorphisms we identify here may be associated with variability in basal electrocardiographic indices such as QRS duration, a manifestation of ventricular conduction velocity; this was a major effect of the six-change haplotype variant we have recently reported.²⁴ On the other hand, such variants may be clinically silent in the basal state, but still modulate sodium-channel-related physiology in the face of environmental stressors such as transient myocardial ischemia or exposure to sodium channel-blocking drugs. Some of the variants identified here did not alter promoter activity *in vitro* and hence may simply be non-functional polymorphisms.

In summary, DNA polymorphisms displaying variant *in vitro* activity have been identified in the promoter region of the cardiac sodium channel gene: 2/88 (2.3%) BS patients and 11/1033 (1.1%) control individuals have alleles in this region of *SCN5A* that alter sodium channel function. The data further reinforce the concept that sodium channel function varies on a transcriptional level even among apparently normal subjects.

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