Common Sodium Channel Promoter Haplotype in Asian Subjects Underlies Variability in Cardiac Conduction

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Background—Reduced cardiac sodium current slows conduction and renders the heart susceptible to ventricular fibrillation. Loss of function mutations in SCN5A, encoding the cardiac sodium channel, are one cause of the Brugada syndrome, associated with slow conduction and a high incidence of ventricular fibrillation, especially in Asians. In this study, we tested the hypothesis that an SCN5A promoter polymorphism common in Asians modulates variability in cardiac conduction.

Methods and Results—Resequencing 2.8 kb of SCN5A promoter identified a haplotype variant consisting of 6 polymorphisms in near-complete linkage disequilibrium that occurred at an allele frequency of 22% in Asian subjects and was absent in whites and blacks. Reporter activity of this variant haplotype, designated HapB, in cardiomyocytes was reduced 62% compared with wild-type haplotype (P=0.006). The relationship between SCN5A promoter haplotype and PR and QRS durations, indexes of conduction velocity, was then analyzed in a cohort of 71 Japanese Brugada syndrome subjects without SCN5A mutations and in 102 Japanese control subjects. In both groups, PR and QRS durations were significantly longer in HapB individuals (P≤0.002) with a gene-dose effect. In addition, up to 28% and 48% of variability in PR and QRS durations, respectively, were attributable to this haplotype. The extent of QRS widening during challenge with sodium channel blockers, known to be arrhythmogenic in Brugada syndrome and other settings, was also genotype dependent (P=0.002).

Conclusions—These data demonstrate that genetically determined variable sodium channel transcription occurs in the human heart and is associated with variable conduction velocity, an important contributor to arrhythmia susceptibility. (Circulation. 2006;113:338-344.)

Key Words: arrhythmia • conduction • death, sudden • genetics • ion channels

Sudden cardiac death (SCD) accounts for 20% of all mortality in Western countries. One key determinant of normal excitation and conduction of the cardiac impulse is the cardiac sodium channel, responsible for rapid depolarization in most cardiomyocytes. Reduced sodium current predisposes to SCD. For example, although sodium channel blockers have been used for antiarrhythmic therapy, the Cardiac Arrhythmia Suppression Trial (CAST) showed that these agents increase the incidence of SCD. Loss of function mutations in SCN5A, the cardiac sodium channel gene, causes ~20% of cases of the Brugada syndrome, which is associated with a high risk of SCD. Furthermore, there is evidence that such sodium channel mutations also may lead to enhanced fibrosis in myocardial tissue.
SCN5A Promoter Haplotypes Affect Conduction

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Methods

Identification of Polymorphisms

Resequencing 2.8 kb of the SCN5A promoter region in a single individual of Asian origin identified him as a homozygote for 6 DNA polymorphisms in the region: T–1418C, T–1062C, T–847G, G–354C, and C287T (Figure 1). The resequenced region encompassed positions −2190 to 613, relative to the major transcription initiation site of the SCN5A promoter, including 2.2 kb upstream of exon 1, exon 1 (which is 173 bp and noncoding), and the proximal 439 bp of intron 1. The fragment was amplified by long and accurate polymerase chain reaction (PCR; TaKaRa kit) with primers F1 and R1 (Data Supplement Table I; see http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.580811/DC1). Further studies described below established that these polymorphisms were common and in near-total linkage disequilibrium, thereby identifying 2 common haplotype blocks, designated HapA and HapB. We also detected a third combination of polymorphisms, designated HapC, in <1% of subjects. In addition to the study populations, 150 white and 100 black individuals were tested for these haplotypes.

Functional Analysis

Generation of Constructs

The 2.8-kb fragment described above was amplified from genomic DNA of HapA- and HapB-homozygous individuals. These fragments were cloned into the pGEM-T Easy vector (Promega), and inserts were subsequently subcloned into the pGL3-basic vector (Promega), which contains the firefly luciferase coding sequence, to generate SCN5A promoter–luciferase fusion constructs for reporter assays. These constructs were designated pGL3-Hap A and pGL3-Hap B.

 Reporter Activity

Reporter activity was assayed in neonatal mouse cardiomyocytes and in Chinese hamster ovary cells as described in detail previously. In brief, 1 µg pGL3-Hap A or pGL3-Hap B was transfected into neonatal mouse cardiomyocytes or Chinese hamster ovary cells. In each experiment, 0.05 µg pRL-TK plasmid (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 with the Dual-Luciferase Reporter Assay System (Promega). The pGL3-basic (promoterless) plasmid was tested in each experiment; its activity level served as the baseline.

Study Participants

Participants in the clinical study were ascertained at the National Cardiovascular Center (Osaka, Japan). All protocols (including molecular screening) were reviewed and approved by the Ethical Review Committee of the National Cardiovascular Center, and informed consent was obtained from all individuals.

The control population consisted of 102 subjects drawn from mutation-negative relatives in congenital long-QT syndrome families in which the causative mutation had been identified. Only 1 person was drawn from each family. There were 67 male and 35 female subjects ranging from 9 to 69 years of age; mean age was 40±14 years (mean±SD).

The Brugada syndrome population included 80 patients diagnosed with Brugada syndrome, defined as type 1 “coved” ST-segment elevation in V1 through V3 in 70 patients, induced by sodium channel blocker in 10 patients. In all patients, physical examination, chest roentgenogram, laboratory values, echocardiography with wall motion analysis, and Doppler screening excluded structural heart disease. Aborted cardiac arrest or ventricular fibrillation (VF) was documented in 30 patients, syncope was identified in 20, and 30 were asymptomatic. All patients had previously been screened for SCN5A coding region mutations, and a mutation had been identified in 9 patients. The patient group included 76 male and 4 female subjects ranging from 1 to 76 years of age (mean±SD, 47±16 years).

ECG Phenotypes

ECGs were assessed by an investigator (W.S.) who was blinded to age, gender, and genetic and clinical information. Phenotypes assessed included RR interval, PR interval measured in lead II (PRII), QRS interval measured in leads V1 (QRSv1) and V6 (QRSv6), ST amplitude at J point (STJ), and ST amplitude at 80 ms after the end of the QRS (ST80). The effects of intravenous administration of sodium channel blockers on these ECG parameters were examined in 49 of 80 Brugada syndrome patients. Pilsicainide (maximum 1 mg/kg at a rate of 0.1 mg · kg⁻¹ · min⁻¹) was used in 37 patients, flecainide (maximum 2 mg/kg at a rate of 0.2 mg · kg⁻¹ · min⁻¹) was used in 9 patients, and disopyramide (maximum 2 mg/kg at a rate of 0.2 mg · kg⁻¹ · min⁻¹) was used in 3 patients.

Genotyping

Genomic DNA was prepared from blood leukocytes. Genotyping for the T–1418C and T–1062C single nucleotide polymorphisms (SNPs) was performed by restriction fragment length polymorphism analysis after PCR amplification with Earl and HaeIII, respectively. PCR primers used to amplify the 161-bp fragment encompassing the T–1418C SNP were F2 and R2, and those used to amplify the 123-bp fragment encompassing the T–1062C SNP were F3 and R3 (Data Supplement Table II). Genotyping for the other 4 polymorphisms (T–847G, 835insGC, G–354C, and C287T) was done by DNA resequencing of both strands. PCR primers used to amplify the 638-bp fragment encompassing the T–847G, 835insGC, G–354C, and C287T polymorphisms were F4 and R4; those used to amplify the 599-bp fragment encompassing the C287T polymorphism were F5 and R5.

Statistical Analysis

Using the individual genotypes for the 6 polymorphisms, we estimated haplotype frequencies using an E-M algorithm. The haplotype frequencies were used to calculate the probabilities of the haplotype pairs compatible with the genotype combinations of the multiple heterozygous patients using Bayes' theorem. Observed haplotype pair frequencies were compared with those expected under Hardy-Weinberg equilibrium in the Brugada syndrome population and control population separately with a χ² test. To compare haplotype pair frequencies among Brugada syndrome patients and control subjects, Fisher’s exact test was used.

All quantitative phenotypes were normally distributed, and data are expressed as mean±SD. Continuous ECG phenotypes were compared between mutation–negative Brugada syndrome patients, SCN5A mutation–positive Brugada syndrome patients, and control subjects by ANOVA adjusted for age and gender, followed by a post hoc test for pairwise comparisons. Student t tests were used.

Figure 1. Haplotypes identified in the cardiac sodium channel gene (SCN5A) promoter. Nucleotide variations are indicated by their position relative to the major transcription initiation site (+1), with the most frequent nucleotide given below and the least frequent nucleotide given above the position. *Frequency in the Japanese (control) population.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Nucleotide Variation</th>
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<tbody>
<tr>
<td>A</td>
<td>T T T T G C</td>
</tr>
<tr>
<td>B</td>
<td>G C G G G T</td>
</tr>
<tr>
<td>C</td>
<td>C T T T C C</td>
</tr>
</tbody>
</table>

Frequency: Haplotype A 75.5%, Haplotype B 24%, Haplotype C 0.5%
to compare the after-drug-challenge continuous ECG phenotypes between SCN5A mutation–negative and –positive Brugada syndrome patients. Correlations between quantitative phenotypes before and after sodium channel blockade are expressed as Pearson correlation coefficients (r). For comparison of the proportion of male subjects, Fisher’s exact test was used.

The effect of haplotype pairs on the continuous ECG phenotypes was tested in the Brugada syndrome patients and control subjects separately by ANOVA with adjustment for age and gender. The 9 SCN5A mutation–positive Brugada syndrome patients were treated as a separate category (7 HapA/HapA homozygotes, 2 HapA/HapB heterozygotes, pooled). The 2 individuals with the rare HapC variant (1 patient from each group) were excluded from analyses. In all analyses, the proportion of variance attributable to the haplotype pair (R²) was calculated and corrected for the effects of age and gender.

Differences in reporter gene expression activity between HapA and HapB were examined for statistical significance with Student’s t test. Throughout, values of P<0.05 were interpreted as being significant. All statistical analyses were done with SAS software (version 9, SAS Institute).

Multiple Testing
When a Bonferroni correction for the 24 statistical models is used to compare the continuous ECG phenotypes, the significance level for the overall probability values is 0.002. Similarly, the Bonferroni-corrected significance levels for the pairwise comparisons between 3 and 4 groups is 0.017 and 0.008, respectively.

Results
Haplotypes
The 6 polymorphisms were in near-complete linkage disequilibrium, with only 2 (similar) discordant haplotypes (of 364; <1%), each occurring in 1 subject from each population. We designated HapA containing all common alleles and HapB containing all minor alleles (Figure 1). The discordant haplotype was designated HapC. The estimated frequencies of HapA, HapB, and HapC were 0.755, 0.240, and 0.005 in the control subjects and 0.782, 0.211, and 0.007 in the SCN5A mutation–negative Brugada syndrome patients, respectively.

Haplotype distributions were in Hardy-Weinberg equilibrium (P>0.05) in both populations. No significant difference in haplotype frequencies was observed between the Brugada syndrome group and the control subjects. The haplotypes were absent in white and black samples.

Functional Analysis
In cardiomyocytes, reporter activity of HapB was markedly reduced, by 62%, compared with HapA: 5.5±0.4 (mean±SE) versus 14.5±2.8 (normalized activity units; n=9 each; P=0.006; Figure 2). A similar trend was seen in the noncardiac cells: 2.7±0.3 versus 3.6±0.3 (n=13 each; P=0.04; Figure 2).

Phenotypic Characteristics of the Control and Brugada Syndrome Patient Populations
The decreased reporter activity for HapB suggested that individuals carrying this promoter haplotype would display ECG-detectable conduction slowing. Accordingly, the relationships between genotype and ECG intervals were evaluated in the control and Brugada syndrome populations.

ECG data are shown in Table 1. As expected, Brugada syndrome patients had significantly longer conduction intervals (PR₉, QRSᵥ₁, QRSᵥ₆) and greater ST-segment elevation (ST₉, STᵢ₀) compared with control subjects. Heart rate was not significantly different between the 2 populations. In addition, we found differences between SCN5A mutation–positive and SCN5A mutation–negative Brugada syndrome patients similar to those previously reported: Mutation-positive subjects had significantly longer baseline PR and QRS intervals and longer RR intervals. Data on the subset of Brugada syndrome patients who underwent drug challenge are presented in Table 2. For all ECG parameters investigated, highly significant (P<0.0001) correlations were present between measures before and after drug challenge (Table 2). As previously reported, SCN5A mutation–positive patients displayed longer PR and QRS intervals after challenge with sodium channel blockers compared with SCN5A mutation–negative patients.

Haplotype Pair Effects
PR and QRS durations were significantly longer in HapB individuals in both study populations (Brugada syndrome and control subjects: P=0.002 for PR₉; P<0.0001 for QRSᵥ₁, and QRSᵥ₆; Figure 3). In the control population, PR₉, QRSᵥ₁, and QRSᵥ₆ intervals showed a gene-dose effect, being longest in HapB homozygotes, intermediate in HapA/HapB heterozygotes, and shortest in HapA homozygotes. A similar pattern was observed in the SCN5A mutation–negative Brugada syndrome patient group. As discussed earlier, these analyses excluded data in the 2 individuals with HapC. PRᵢ₀, QRSᵥ₁, and QRSᵥ₆ means (±SD) per haplotype group for the 2 populations are listed in the Data Supplement Table II. Both the overall and pairwise probability values were highly statistically significant even after correction for multiple testing.

The amount of variance (R²) in PR and QRS intervals explained by the haplotype pair after correction for age and gender is shown in Table 3. As can be seen, a significant proportion of variance in PR and QRS intervals, both at baseline (both groups) and after drug challenge (Brugada syndrome group), was attributable to the haplotype. No significant association was found between haplotype and RR, STᵢ, and STᵢ₀ in either population (data not shown).

Drug Challenge and Haplotype
The haplotype pairs were also highly associated with conduction intervals (PRᵢ₀, QRSᵥ₁, QRSᵥ₆) after sodium channel
blockade in 44 SCN5A mutation–negative Brugada syndrome patients who underwent drug challenge (for PR, QRSv1, QRSv6, P<0.0001; Figure 3). PR, QRSv1, and QRSv6 means (±SD) per haplotype group are listed in the Data Supplement Table II. Here also, overall and pairwise probability values were highly statistically significant even after correction for multiple testing.

In addition, the extent of QRS widening (ΔQRS) after drug challenge was genotype dependent, and a gene-dose effect was also observed (ΔQRSv6; HapB/HapB = 30 ms [mean±SD]; HapA/HapB = 24.2±7.9; HapA/HapA = 17.8±7.2; P=0.002; Figure 4). A similar trend was seen for extent of PR widening (ΔPR) after drug challenge (ΔPRv1; HapB/HapB = 40 ms; HapA/HapB = 33.8±13.2; HapA/HapA = 28.6±8.3; P=0.05).

Discussion
We demonstrate that a set of 6 SCN5A promoter polymorphisms found in Asian subjects are in near-complete linkage disequilibrium, have a significant impact on sodium channel expression in vitro, account for a large proportion of variance in ECG conduction parameters in 2 independent Japanese populations, and represent pharmacogenetic markers predicting variable drug response.

Twin studies have identified strong genetic effects for ECG parameters, including PR and QRS durations. Indeed, associations have been reported between ECG parameters and single coding region nonsynonymous (amino acid–changing) SNPs in ion channel genes. However, common functional variants in regulatory regions that strongly modulate basal ECG intervals have not previously been identified. A preliminary report has suggested an association between a potassium channel promoter polymorphism and QRS axis in women only. Only recently has the concept of tightly linked polymorphisms (constituting a haplotype block) been applied to understanding variability in cardiac electrophysiology. In 1 study, a small degree of variance (<1%) in QT interval in a central European population could be attributed to single SNPs and haplotype blocks in 4 potassium channel genes.

<table>
<thead>
<tr>
<th>TABLE 1. Baseline ECG Characteristics of the Control and Brugada Syndrome Patient Populations</th>
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<tr>
<td>Control Subjects</td>
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<td>n</td>
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<td>Male, n (%)</td>
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<tr>
<td>ST&lt;sub&gt;is&lt;/sub&gt;, mV</td>
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Values are given as mean±SD. *Below the Bonferroni-corrected overall or pairwise significance levels (see Multiple Testing).

<table>
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<tr>
<th>TABLE 2. Clinical Characteristics of the Brugada Syndrome Patients After Sodium Channel Blocker Challenge</th>
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<tr>
<td>Before and After Sodium Channel Blockade</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td>n</td>
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<tr>
<td>------------------</td>
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<tr>
<td>Male, n (%)</td>
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<tr>
<td>Age, y</td>
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<td>aQRSv1, ms</td>
</tr>
<tr>
<td>aQRSv6, ms</td>
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<tr>
<td>aST&lt;sub&gt;r&lt;/sub&gt;, mV</td>
</tr>
<tr>
<td>aST&lt;sub&gt;is&lt;/sub&gt;, mV</td>
</tr>
</tbody>
</table>

Values are given as mean±SD. Pearson correlation coefficients (r) observed between measures before and after sodium channel blocker challenge (P<0.0001). Mean baseline ECG parameters for the 44 SCN5A<sup>−/−</sup> and 5 SCN5A<sup>+/−</sup> patients (not shown) were very similar to those for the total patient group given in Table 1.

*Below the Bonferroni-corrected overall significance levels (see Multiple Testing).
In contrast, the SCN5A promoter haplotype we report here explained a remarkable proportion of variance in conduction parameters in the Japanese subjects studied (Table 3). Such associations could arise because the haplotypes studied are, in turn, in linkage disequilibrium with other functionally important variants in regulatory or other regions of the gene. However, in this case, the in vitro functional studies indicate that the effect is attributable to a variant within the haplotype block; at this point, the specific variant mediating this effect has not been identified.

A principal determinant of cardiac conduction in atrial and ventricular muscle is the sodium current; sodium channel blockers prolong PR and QRS durations, an effect also seen with loss of function mutations in SCN5A. Critical degrees of conduction slowing represent a final common pathway to VF, so dissection of the genetic determinants of cardiac conduction in the general population is a key step to understanding variable susceptibility to common arrhythmias resulting from conduction slowing, as in myocardial ischemia or heart failure. Thus, the data we present here implicate the SCN5A promoter variant HapB, which slowed conduction in normal subjects and exacerbated conduction slowing in those with Brugada syndrome, as a candidate modulator of variability in risk of SCD. Importantly, imposition of further depression of sodium channel function by administration of sodium channel blocking drugs further exacerbated conduction slowing in a gene-dose-dependent fashion. Studies in large numbers of subjects at risk for SCD are required to further establish the role of this and other regulatory region polymorphisms in modulating that risk.

Differences in disease penetrance and expression have been widely reported in the cardiac sodium and other channelopathies. Relatives carrying an SCN5A mutation identical to that of the proband may be clinically unaffected, and family members may display different phenotypes, eg, Brugada syndrome or conduction disease. Genetic variants like the one presented here are obvious candidate modulators of this variability in phenotypic expression. Interindividual variability also has been noted in response to pharmacological challenge with sodium channel blockers in Brugada syndrome patients. In some patients, even some carrying an SCN5A mutation, drug challenge fails to unmask a Brugada syndrome ECG. The significantly greater increases in PR and QRS durations with sodium channel blockade in HapB carriers thus identify variability in expression of the drug target, the sodium channel, as a key mediator of this variable drug effect. It is thus possible that other sodium channel blocker response phenotypes such as the increased mortality with sodium channel blockers in the CAST was determined by variable sodium channel expression. DNA samples from that important clinical trial were not archived, so this question will remain unanswered. More generally, the data

### Table 3. Variance Explained by the Haplotype Pair

<table>
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<tr>
<th></th>
<th>Control Subjects</th>
<th>Brugada Syndrome Baseline</th>
<th>Brugada Syndrome Drug Challenge</th>
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<tr>
<td>$R^2$, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR</td>
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<tr>
<td>QRS&lt;sub&gt;6&lt;/sub&gt;</td>
<td>48.5</td>
<td>24.9</td>
<td>36.2</td>
</tr>
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</table>

Figure 3. SCN5A promoter haplotype effects on durations of QRS<sub>6</sub> and PRI<sub>1</sub> in Brugada syndrome patients at baseline and after challenge with sodium channel blocking agents and in non-Brugada syndrome control subjects. Patient numbers are indicated in parentheses. Genotype effects on QRS<sub>6</sub> were similar to those on QRS<sub>6</sub> because of a high correlation between these 2 parameters (Pearson's coefficient, $r=0.96$). Data are presented as mean±SD. For Bonferroni-corrected significance levels for pairwise comparisons, refer to the Multiple Testing section in Patients and Methods.

Figure 4. SCN5A promoter haplotype effects on extent of QRS (ΔQRS<sub>6</sub> and ΔQRS<sub>6</sub>) and PR (ΔPR<sub>1</sub>) widening after sodium channel blockade. AA, n=29; AB, n=13; BB, n=2. Data are presented as mean±SD. The Bonferroni-corrected significance level is 0.002.
indicate that sodium channel function is additively suppressed by drug challenge. Brugada syndrome mutations, and the HapB regulatory variant. Although a strong reduction in reporter gene activity was observed for HapB compared with HapA in vitro, the extent to which this reduction translates proportionately into reduced sodium channel density in vivo is unknown.

Brugada syndrome is endemic in Asia, where the disorder is also known as sudden unexplained nocturnal death syndrome; in fact, the incidence is higher in Asia than in the United States and Europe. Because HapB is common in Asians and absent in whites and has a large negative impact on cardiac conduction, a long-recognized feature of Brugada syndrome, it may logically contribute to differences in Brugada syndrome incidence as a function of ethnicity. In this study, PR and QRS durations in individuals matched for haplotype were consistently longer in the Brugada syndrome group compared with control subjects; thus, the greatest conduction slowing was in those subjects with Brugada syndrome and the HapB/HapB genotype. Indeed, control HapB/HapB subjects had longer QRS durations than did those with manifest Brugada syndrome and the common HapA/HapA genotype. Thus, although the minor allele is quite common, it alone may give rise to one part of the spectrum of loss of sodium channel function that constitutes the Brugada syndrome. However, data at this stage do not indicate that HapB per se leads to Brugada syndrome.

More generally, the data fit nicely the concept that individuals vary in their ability to maintain sodium channel function to protect against the arrhythmia-prone substrate and identify HapB as a variant that contributes to such variable “antifibrillatory reserve.”

Acknowledgments

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Disclosures

Drs Shimizu and Miyamoto are applying for a Japanese domestic patent based on this work. The other authors report no conflicts.

References


CLINICAL PERSPECTIVE

The sodium current determines conduction velocity in the heart, and reducing sodium current predisposes to VF. Sodium channel blockers increased sudden death after MI in CAST, and at least some cases of the Brugada syndrome, in which structurally normal hearts are prone to VF, are due to loss of function mutations in the cardiac sodium channel gene SCN5A. Thus, variability in the synthesis of sodium channels could contribute to variable conduction velocity in heart and to VF susceptibility. This study represents an important first step to testing that hypothesis. A set of 6 DNA variants were identified in the SCN5A promoter, the region of the gene directing transcriptional activity. The variants are common but only in Asian subjects and are in tight linkage disequilibrium; ie, subjects have either wild-type sequences or all 6 variants, defining a haplotype block called HapB here. HapB sequences not only reduced transcriptional activity in vitro but also predicted slower conduction velocity, assessed by PR and QRS durations, in both Japanese control and Brugada syndrome subjects. The longest QRS durations were in Brugada syndrome patients homozygous for HapB (7%) challenged with sodium channel blockers. Indeed, normal subjects homozygous for HapB had longer QRS durations than Brugada syndrome patients homozygous for wild-type sequences. These data support the idea that common SCN5A promoter variants modulate conduction velocity and thus susceptibility to VF in response to challenges such as other arrhythmogenic mutations, sodium channel blocking drugs, or acute ischemia. In addition, HapB may contribute to the higher prevalence of Brugada syndrome in Asians.
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