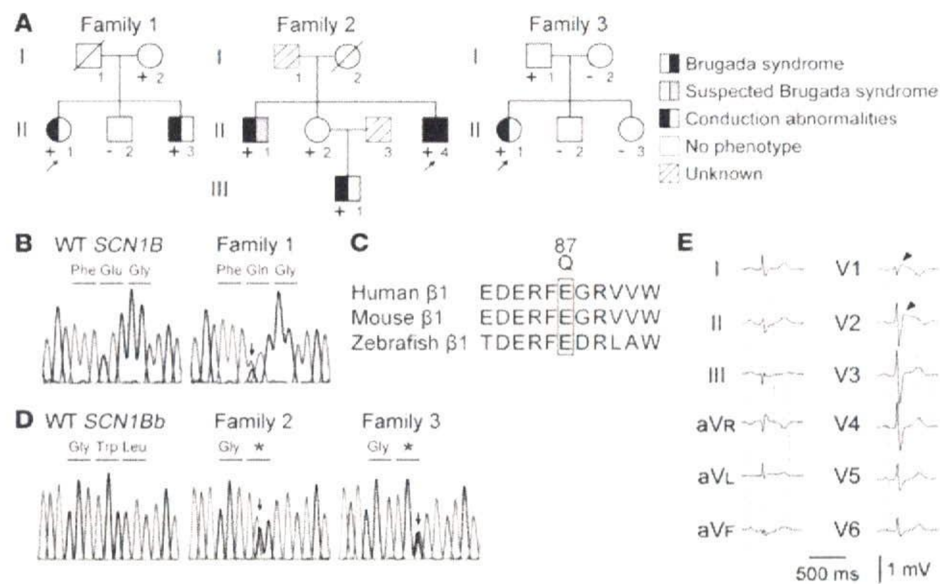

Figure 2

SCN1B mutations found in patients with Brugada syndrome and conduction disease. (**A**) Pedigrees and phenotypes of the families affected by Brugada syndrome and/or conduction disease. Individuals carrying the mutation are indicated (+). Individuals who tested negative for the mutation are indicated (–). Individuals I-1 from family 1; and I-1, I-2, and II-3 from family 2 did not undergo genetic testing. Arrows indicate probands. (**B**) The c.259G→C mutation in *SCN1B* resulting in p.Glu87Gln found in family 1. (**C**) Alignment of β1 across species showing the high conservation of Glu87. (**D**) The c.536G→A (middle) and c.537G→A (right) mutations in exon 3A of β1B, both resulting in p.Trp179X found in families 2 and 3, respectively. (**E**) Twelve-lead ECG from the proband of family 2 (II-4). The arrowheads indicate ST-segment elevation typical of Brugada syndrome.

human heart. Both β1 and β1B transcripts were detected in right and left ventricles and in Purkinje fibers (Figure 3). The β1 transcript level was higher in Purkinje fibers (which make up the conduction system in the ventricle) than left- (2.4-fold; $P < 0.05$) and right-ventricular (1.6-fold; P = NS) free walls. β1B transcript levels showed an even greater difference: Purkinje fibers versus left- (4.8-fold; $P < 0.001$) and right-ventricular (3.7-fold; $P < 0.001$) free walls. Levels of both transcripts were also slightly (but not statistically significantly) higher in right- versus left-ventricular free wall (1.5-fold and 1.3-fold for β1 and β1B transcripts, respectively).

Cellular electrophysiology. The effects of mutant and WT β1 and β1B variants on $Na_V1.5$ sodium current were assessed using the whole-cell patch-clamp technique in transfected CHO cells. As described in Methods, bicistronic expression vectors encoding a reporter (GFP or DsRed) with or without β subunits were cotransfected with expression vector encoding $Na_V1.5$. Currents were compared in cells transfected with *SCN5A* alone or *SCN5A* plus WT, mutant, or both β subunits.

Figure 4A shows representative current traces in cells expressing $Na_V1.5$ alone and $Na_V1.5$ plus WT or mutant β1B (p.Trp179X β1B) or their combination; current densities at –30 mV are summarized in Figure 4B. Coexpression of $Na_V1.5$ with WT β1B significantly increased sodium current density over $Na_V1.5$ alone, by 69%, while currents recorded with p.Trp179X β1B coexpression were no different from $Na_V1.5$ alone. Similarly, while coexpression of WT subunit with $Na_V1.5$ shifted the voltage dependence of both activation and inactivation to more negative potentials compared with those with $Na_V1.5$ alone, no such shift was observed with the mutant (Figure 4C and Table 1). This result indicates that while WT β1B modulates $Na_V1.5$ gating (in a fashion similar to WT β1; see below), the mutant exerts no such effect. Coexpression of WT or mutant β1B with $Na_V1.5$ did not alter recovery from inactivation (Figure 4D and Table 1).

To examine whether expression of the mutant influences the effect of WT β1B on $Na_V1.5$ current (e.g., to produce a dominant negative action), cells were transfected with $Na_V1.5$ and varying amounts of WT and p.Trp179X β1B. Figure 4B shows that the sodium current increase over $Na_V1.5$ alone recorded with transfection of 1 μg of both β1B subunit constructs was identical to the increase with that of 1 μg of WT β1B. In addition, the increase in sodium current recorded with transfection of 0.5 μg of both β1B subunit constructs was 51% of that with 1 μg of β1B alone. These data indicate that p.Trp179X β1B does not exert a dominant negative effect on WT β1B function and further support the finding that the mutant, unlike WT, does not affect sodium channel function.

Figure 5A shows representative current traces of $Na_V1.5$ and $Na_V1.5$ coexpressed with WT and/or mutant β1 (p.Glu87Gln β1); current densities are summarized in Figure 5B. Coexpression of $Na_V1.5$ with WT β1 significantly increased sodium current density at –30 mV, by 76%, while coexpression with mutant β1 (p.Glu87Gln β1) did not increase the sodium current. The increase in sodium current recorded with coexpression of $Na_V1.5$ and 1 μg of both WT and p.Glu87Gln β1 (+20%) was markedly smaller than the increase with coexpression of $Na_V1.5$ with 1 μg WT β1 alone (+76%), indicating that this mutant exerts a dominant negative effect on WT β1 function. Figure 5C shows that WT β1 produced negative shifts in the voltage dependence of $Na_V1.5$ activation and inactivation similar to those observed with WT β1B. p.Glu87Gln β1 shifted the voltage dependence of inactivation to negative potentials (similar to WT β1) but did not alter the voltage dependence of activation (Table 2). Coexpression of WT or mutant β1 with $Na_V1.5$ did not alter recovery from inactivation (Figure 5D and Table 2).

Since Glu87 is located in a region of the protein common to both β1 and β1B, we also studied the effects of p.Glu87Gln β1B on $Na_V1.5$ current properties (Supplemental Figure 1 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI33891DS1). While WT β1B increased $Na_V1.5$ current by 69% (Figure 4), p.Glu87Gln β1B did not increase the sodium current compared with $Na_V1.5$ alone. Similarly, WT β1B produced a negative shift in voltage dependence of both activation and inactivation (Table 1), while p.Glu87Gln β1B shifted only the voltage dependence of inactivation compared with $Na_V1.5$ alone. As with the other β subunit

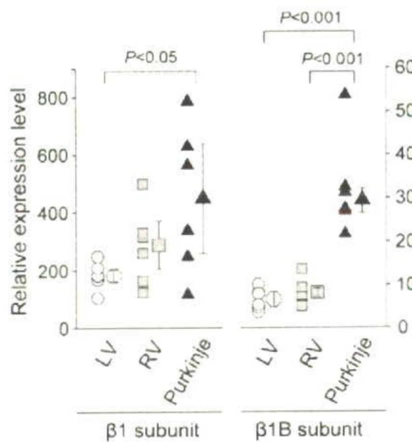

Figure 3
Expression profile of β1 and β1B transcripts in nondiseased human ventricular tissue as determined by quantitative real-time PCR. Relative expression levels of the β1 and β1B subunits are presented, normalized to those of *HPRT1* in LV (circles), RV (squares), and Purkinje fibers (triangles). Tissues for each group were collected from 6 human donors (nondiseased hearts, $n = 6$). Data points indicate the average of 2 measurements in each tissue sample. Larger symbols and error bars indicate median ± median absolute deviation for all samples.

constructs studied, there was no change in recovery from inactivation. Thus, the effects of p.Glu87Gln were comparable in the β1 and the β1B backbones.

Since Glu87 is located in a region of the protein common to both β1 and β1B, we also studied the effects of p.Glu87Gln β1B on $Na_V1.5$ current properties (Supplemental Figure 1 and Supplemental Table 1). While WT β1B increased $Na_V1.5$ current by 69% (Figure 4), p.Glu87Gln β1B did not increase the sodium current compared with $Na_V1.5$ alone. Similarly, WT β1B produced a negative shift in voltage dependence of both activation and inactivation (Table 1), while p.Glu87Gln β1B shifted only the voltage dependence of inactivation compared with $Na_V1.5$ alone. As with the other β subunit constructs studied, there was no change in recovery from inactivation. Thus, the effects of p.Glu87Gln were comparable in the β1 and the β1B backbones.

Discussion

In this study, we provide what we believe to be the first report of mutations in *SCN1B* sequences encoding the β1 and β1B transcript variants in patients with conduction disease and/or Brugada syndrome. Further, we provide new data indicating that β1 and β1B transcripts in the heart vary by region; greater expression in Purkinje fibers is consistent with the conduction system phenotype we describe in mutation carrier patients. Finally, we demonstrate that the β1 and β1B variants modulate function of the major cardiac sodium channel α subunit $Na_V1.5$ and that the identified *SCN1B* mutations blunt or inhibit this effect.

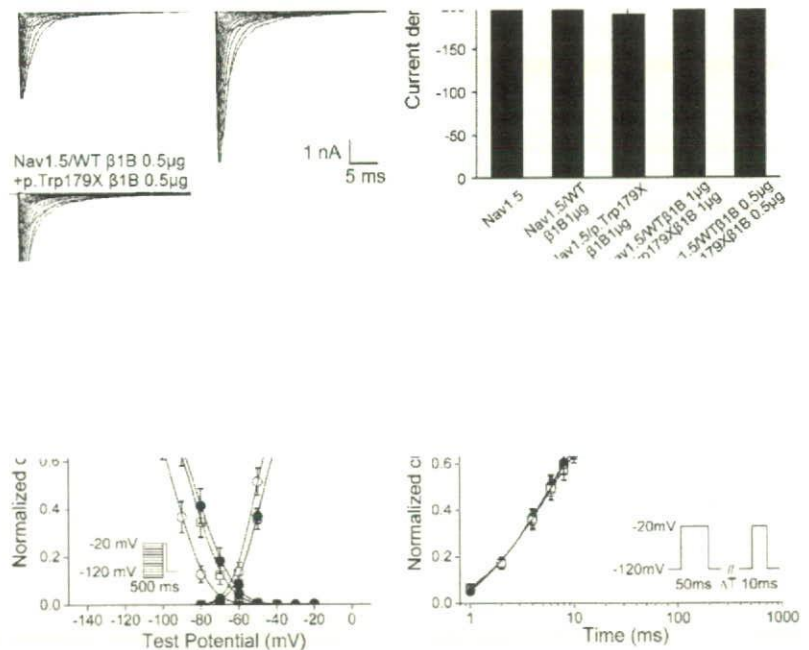

Figure 4
Electrophysiological characteristics of the p.Trp179X β1B mutant. (**A**) Representative traces of sodium current demonstrating an increase in sodium current with WT but not mutant subunit. (**B**) Sodium current density at –30 mV for $Na_V1.5$ alone ($n = 29$), $Na_V1.5$ coexpressed with WT β1B ($n = 28$), $Na_V1.5$ coexpressed with p.Trp179X β1B ($n = 18$), $Na_V1.5$ coexpressed with WT β1B plus p.Trp179X β1B (1 μg for each; $n = 14$), and $Na_V1.5$ coexpressed with WT β1B plus p.Trp179X β1B (0.5 μg for each; $n = 10$). (**C**) Voltage dependence of activation and inactivation. Filled circles, open circles, and squares indicate $Na_V1.5$ alone, $Na_V1.5$ coexpressed with WT β1B, and $Na_V1.5$ coexpressed with p.Trp179X β1B, respectively. The pulse protocol used to study the voltage dependence of inactivation is shown in the inset. (**D**) Recovery from inactivation. Biophysical properties are provided in Table 1.

Table 1
Biophysical parameters of WT and mutant β1B

	Voltage dependence of activation			Voltage dependence of inactivation			Recovery from inactivation		
	$V_{1/2}$, mV	*k*, mV	*n*	$V_{1/2}$, mV	*k*, mV	*n*	τ_f, ms (amplitude, %)[A]	τ_s, ms (amplitude, %)[A]	*n*
$Na_V1.5$	–46.2 ± 1.0	7.1 ± 0.4	29	–83.8 ± 1.8	7.6 ± 0.2	17	7.7 ± 1.1 (87.2 ± 1.1)	56.4 ± 9.8 (11.6 ± 1.0)	12
$Na_V1.5$/WT β1B	–50.6 ± 0.7[B]	6.3 ± 0.3	28	–94.2 ± 1.3[A]	7.6 ± 0.2	14	7.4 ± 1.0 (86.5 ± 1.2)	43.3 ± 8.6 (13.1 ± 1.1)	14
$Na_V1.5$/p.Trp179X β1B	–46.3 ± 1.3[C]	6.5 ± 0.4	18	–85.2 ± 2.0[B]	6.6 ± 0.3	15	8.2 ± 1.0 (91.8 ± 1.1)	58.0 ± 11.9 (7.8 ± 1.0)	12

Values are shown as mean ± SEM. [A]The percentages refer to the properties of the overall time constants contributed by the 2 components τ_f and τ_s. [B]$P < 0.01$ versus $Na_V1.5$ alone. [C]$P < 0.01$ versus $Na_V1.5$/WT β1B.

The 3 mutations were identified in 3 probands with conduction disease and/or Brugada syndrome as well as in other family members with or without these arrhythmia phenotypes. Formal linkage analysis was not possible because the families are too small and penetrance is incomplete. Thus, evidence in support of disease causality of these mutations (beyond their identification in subjects with clinical phenotypes) includes the findings that both β1 and β1B transcripts are expressed in heart and that the mutant subunits (p.Glu87Gln β1, p.Glu87Gln β1B, and p.Trp179X β1B) did not increase $Na_V1.5$ currents in heterologous expression experiments, while WT β1 and β1B did. Incomplete penetrance, a well-recognized feature of the monogenic arrhythmia syndromes (12, 23), was observed. For *SCN5A* mutations linked to Brugada syndrome, penetrance as low as 12.5% has been described (24). A role for sex, age, and genetic modifiers (e.g., common polymorphisms) is suspected (5, 25, 26), but the mechanisms for this common clinical finding remain poorly understood.

Two types of mutations were identified. The c.536G→A and c.537G→A mutations in exon 3A both result in a stop codon at position 179, predicted to generate a β1B protein lacking the transmembrane and cytoplasmic domains and thus unable to integrate into the sarcolemma and to associate with $Na_V1.5$. Thus, the a priori assumption is that a mutation such as this will cause disease by simple haploinsufficiency. The electrophysiologic data support this idea, since coexpression of p.Trp179X β1B failed to increase $Na_V1.5$ current and did not modulate the effect of the WT β1B protein. Furthermore, the voltage dependencies of activation and inactivation of $Na_V1.5$ coexpressed with p.Trp179X β1B were the same as those for $Na_V1.5$ alone, in contrast to the shifts observed with WT β1B. While *Scn1b*-knockout mice display clear ECG changes (27), studies with young (17- to 18-day-old) heterozygotes identified no difference from WT. Since age-related changes in conduction are a recognized feature of cardiac conduction disease and conduction delay is one of the proposed mechanisms of Brugada syndrome (2, 28), aging may be important for the β subunit–mediated phenotype.

On the other hand, the c.259G→C mutation leads to an amino acid substitution (p.Glu87Gln) within the extracellular domain of the

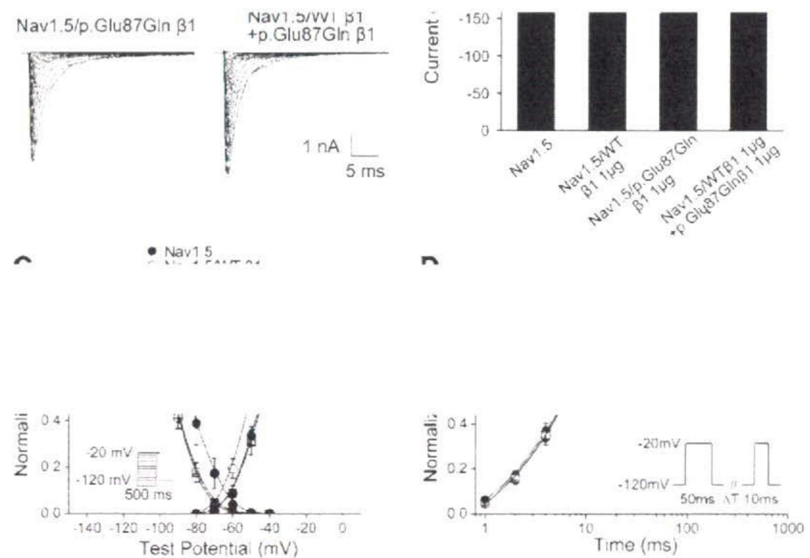

Figure 5
Electrophysiological characteristics of the p.Glu87Gln mutant. (**A**) Representative traces of sodium current. (**B**) Current density at –30 mV for $Na_V1.5$ alone (n = 13), $Na_V1.5$ coexpressed with WT β1 (n = 17), $Na_V1.5$ coexpressed with p.Glu87Gln β1 (n = 18), and $Na_V1.5$ coexpressed with WT β1 plus p.Glu87Gln β1 (n = 15). (**C**) Voltage dependence of activation and inactivation. Filled circles, open circles, and squares indicate $Na_V1.5$ alone, $Na_V1.5$ coexpressed with WT β1, and $Na_V1.5$ coexpressed with p.Glu87Gln β1, respectively. (**D**) Recovery from inactivation. Biophysical properties are provided in Table 2.

Table 2
Biophysical parameters of WT and mutant β1

	Voltage dependence of activation			Voltage dependence of inactivation			Recovery from inactivation		
	$V_{1/2}$, mV	k, mV	n	$V_{1/2}$, mV	k, mV	n	τ_f, ms (amplitude, %)[A]	τ_s, ms (amplitude, %)[A]	n
$Na_V1.5$	−46.1 ± 1.7	7.8 ± 0.4	13	−85.1 ± 3.2	7.3 ± 0.5	12	8.2 ± 1.2 (88.2 ± 1.3)	52.4 ± 7.9 (11.0 ± 1.3)	9
$Na_V1.5$/WT β1	−50.6 ± 1.4[B]	7.3 ± 0.4	17	−92.6 ± 1.4[A]	6.4 ± 0.2	12	7.5 ± 1.1 (84.2 ± 1.3)	43.1 ± 4.6 (14.1 ± 1.3)	13
$Na_V1.5$/p.Glu87Gln β1	−44.9 ± 1.4[C]	7.7 ± 0.4	18	−92.5 ± 1.7[A]	6.8 ± 0.2	12	7.7 ± 1.1 (89.1 ± 1.1)	52.0 ± 9.5 (10.4 ± 1.1)	10

Values are shown as mean ± SEM. [A]The percentages refer to the properties of the overall time constants contributed by the 2 components τ_f and τ_s. [B]$P < 0.05$ versus $Na_V1.5$ alone. [C]$P < 0.05$ versus $Na_V1.5$/WT β1.

protein. The electrophysiological data demonstrate that the mutant subunit did modulate $Na_V1.5$ gating (shift in the voltage dependence of inactivation, in either the β1 or β1B background), supporting the idea that it associates with $Na_V1.5$ at the cell surface. In addition, in contrast to the p.Trp179X β1B, p.Glu87Gln did exert a dominant negative effect on the WT subunit. Thus, the 3 mutations lead to a decrease in $Na_V1.5$ current through somewhat different mechanisms. This reduction of current is consistent with the conduction disease and Brugada syndrome phenotypes of the patients.

Normal impulse propagation in the atria, ventricles, and Purkinje network is critically dependent on normal sodium channel function. Dysfunction of the sodium channel leads to conduction delay, and loss-of-function mutations in *SCN5A* have been described in isolated conduction disease unassociated with structural heart disease (2, 3). Thus, our finding of *SCN1B* mutations associated with reduced sodium current in patients with conduction disease is consistent with previous studies of the mechanism of this disorder. The preferential expression of the β1 and β1B transcripts in human Purkinje fibers further supports the prominent conduction delay seen as part of the clinical phenotypes.

Loss-of-function mutations in *SCN5A* were the first reported cause of the Brugada syndrome (4). These mutations reduce sodium current by reducing $Na_V1.5$ cell surface expression and/or altering gating (4, 5, 29). A common view is that in epicardial cells, this reduction in sodium current produces marked action potential shortening, attributed to an "unopposed" early transient outward potassium current. By contrast, reduction of sodium current in endocardial cells is thought to produce only modest action potential shortening. The resultant increased heterogeneity of repolarization predisposes to rapid reentry, resulting in ventricular fibrillation (4, 30). A common feature in Brugada syndrome – consistent with reduced sodium current – is slowed conduction (28, 31). Indeed, an alternate proposed mechanism suggests that the characteristic right-precordial ST-segment elevation on the ECG and initiation of arrhythmias is attributable primarily to right-ventricular outflow tract conduction delay (28). The trend to higher expression levels of β1B in right ventricle may thus contribute to the Brugada syndrome phenotype.

This idea is further supported by functional studies in a single large kindred in which a *GPD1L* mutation was linked to Brugada syndrome: coexpression of mutant *GPD1L* with $Na_V1.5$ was reported to decrease sodium current, consistent with the observation that loss-of-function mutations in *SCN5A* cause Brugada syndrome (12). In principle, reduction in L-type calcium current might also produce differential effects in epicardial and endocardial sites and thus cause Brugada syndrome; rare kindreds with this mechanism have now been described (14).

Conduction disease was observed in families 1 and 3, while in family 2, mutation carriers presented either solely with conduction disease or conduction disease in combination with ECGs typical of Brugada syndrome. This phenomenon of overlapping clinical phenotypes is common in individuals with *SCN5A* mutations leading to loss of sodium channel function (6, 7), and conversely, in vitro electrophysiologic analysis of *SCN5A* mutations linked to Brugada syndrome or isolated conduction disease consistently reveals loss of $Na_V1.5$ channel function (2, 4). Indeed, a single mutation segregating in a given family can lead to conduction disease in some family members and Brugada syndrome in others (6, 7). What determines the ultimate phenotype – Brugada syndrome versus isolated conduction disease – is unknown. Sex, age, and genetic modifiers (e.g., common polymorphisms) have been proposed as modulators of the clinical phenotypes (5, 25, 26).

The reported effects of β1 on $Na_V1.5$ channels are controversial (32). Some groups have reported that β1 increases $Na_V1.5$ currents with or without affecting voltage dependence or channel kinetics, while others have reported no effect of β1 on $Na_V1.5$ current (20, 33–37). The β1B variant has to date only been studied in coexpression studies with the neuronal sodium channel $Na_V1.2$ (encoded by *SCN2A*), where it was shown to increase sodium current and cause a small negative shift in voltage dependence of activation (19). In our experiments, WT β1 and β1B had similar effects on $Na_V1.5$ current: both increased sodium currents and led to hyperpolarizing (negative) shifts in voltage dependence of activation and inactivation.

Not only were the effects of the WT β subunits on $Na_V1.5$ current similar, but the effects of the p.Glu87Gln mutation in the β1 background (p.Glu87Gln β1) were also similar to those in the β1B background (p.Glu87Gln β1B). Although the β1 and β1B variants share the same topology (an N-terminal extracellular immunoglobulin domain, a transmembrane domain, and a C-terminal cytoplasmic domain), their sequence identity is limited to the extracellular immunoglobulin domain; the C-terminal half of β1B, residues 150–268, has only approximately 17% amino acid sequence identity with β1 (19). Taken together, the data suggest that the molecular determinants of β1 and β1B modulation of $Na_V1.5$ cell-surface expression and gating likely reside in the extracellular immunoglobulin domain. This is in line with previous studies of skeletal muscle ($Na_V1.4$ encoded by *SCN4A*) and neuronal ($Na_V1.2$) sodium channel α subunits that have shown that deletion of the intracellular domain of the β1 subunit has no effect on its modulation of α subunit function, whereas deletions within the extracellular domain block modulation (38–40). Alternatively, specific residues may not be as important as preservation of overall structural motifs, as suggested by the data of Zimmer

and Benndorf, who reported that the β1 subunit modulates $Na_V1.5$ via the membrane anchor plus additional intracellular or extracellular regions (41).

In addition to modulation of sodium channel α subunit expression and function, other roles have been suggested for β subunits: these include acting as adhesion molecules or as participants in signal transduction (16, 32). The different transmembrane and C-terminal domains of β1 and β1B might therefore lead to participation in different signaling pathways. For instance, phosphorylation of the tyrosine at position 181 of the β1 C terminus regulates its interaction with ankyrin-G (42), which is thought to be critical for ankyrin-G localization within cardiomyocytes (intercalated discs versus T tubules). β1B lacks this tyrosine in its C-terminal domain, so a role for β1B as a modulator of this function seems less likely.

Another mechanism regulating function of β subunits is the potential for cleavage by β-site amyloid precursor protein–cleaving enzyme (BACE1) and γ-secretase, resulting in the release of the N- and C-terminal fragments (43). The processed C-terminal fragment of β2 and β4 has been reported to be associated with cell adhesion, migration, and morphogenesis in neuronal cells as well as regulation of the expression level of the neuronal sodium channel $Na_V1.1$ (44–46). Thus, p.Trp179X β1B may result in absence of functions depending on the generation of a β subunit C-terminal fragment by BACE1. However, a role for BACE1 cleavage has not been studied in either human β1 subunit or cardiomyocytes, and the cleavage site located at the common juxtamembrane domain in β1 and β1B is not conserved between human and mice (19, 43).

Mutations in *SCN1B* have been previously reported in generalized epilepsy with febrile seizures plus (47), and β1-null mice exhibit a severe seizure disorder and die at approximately 3 weeks of age (48). In addition, these mice exhibit bradycardia and prolonged rate-corrected QT intervals (27). These changes suggest that β1 plays an important role in the murine heart, although it is possible that the changes are a consequence of the severe overall developmental phenotype in this model (48). To our knowledge, defects in cardiac function have not been investigated in *SCN1B* mutation carriers presenting with epilepsy (32, 49). Conversely, we have observed no neurological phenotype in our patients. Four *SCN1B* mutations have been linked to epilepsy to date (47, 49, 50), all of which localize to the extracellular immunoglobulin-like fold of the protein, as does the p.Glu87Gln mutation reported here. One additional possible link between the cardiac and neurological phenotypes associated with β1 mutations is the syndrome of sudden unexpected death in epilepsy (SUDEP) (51), where a role for cardiac bradyarrhythmias has been proposed (52).

To date, *SCN5A* mutations are the most common cause identified in cases of Brugada syndrome, and *SCN5A* is the only identified causative gene in conduction disease (2, 11). However, *SCN5A* mutations are not identified in the majority of patients, and it has been reported that the frequency of mutations in other implicated genes (*CACNA1C, CACNB2b, GPD1L*) is also low in Brugada syndrome (12–14). In this study, *SCN1B* mutations were identified in less than 1% of probands with Brugada syndrome and less than 5% of probands with conduction disease and thus account for a small subset of these inherited arrhythmic syndromes.

A conventional heterologous mammalian expression system was used for functional assessment of the mutations. The environment in this approach is different from that in native cardiomyocytes, and other proteins known to associate with the sodium channel complex, such as other β subunits, are generally absent. Despite these limitations, the in vitro characteristics of the mutations were concordant with the phenotype observed in the patients, which, in combination with the genetic data presented, supports the hypothesis of a causal relationship between the mutations and disease.

In summary, we have for the first time to our knowledge identified *SCN1B* mutations in families with conduction disease and Brugada syndrome. We have shown expression of the β1 subunit transcript and the alternate β1B subunit transcript variant in human heart and demonstrated reduced $Na_V1.5$ sodium current as a result of loss or altered β subunit modulation of Nav1.5 current. These findings implicate *SCN1B* as a disease gene for human arrhythmia susceptibility.

Methods

Study populations. The study populations consisted of: (a) unrelated Brugada syndrome probands identified and characterized at the Academic Medical Center, Amsterdam (n = 38), l'institut du thorax, Nantes, (n = 216), and the Niigata University Graduate School of Medical and Dental Sciences (n = 28); and (b) patients with cardiac conduction disease were identified and characterized at the Academic Medical Center (n = 2), l'institut du thorax (n = 39), and Ege University School of Medicine (n = 3). The study was performed according to a protocol approved by the Medical Ethical Committee, Academic Medical Center, Amsterdam; Comité de Protection des Personnes, Nantes; Ege University Research Ethics Committee; and Medical Research Ethics Committee, Niigata University Graduate School of Medical and Dental Sciences. Informed consent was obtained from all patients. Coding region and splice site mutations in *SCN5A* had been previously excluded in all probands by single-strand confirmation polymorphism analysis, denaturing HPLC sequencing, or direct sequencing using primers in flanking intronic sequences.

Mutation analysis. Probands with Brugada syndrome and cardiac conduction disease were screened for mutations in regions of the *SCN1B* gene encoding β1 and β1B, except for Japanese probands, who were screened only in the regions of *SCN1B* gene encoding β1B. Screening for mutations was performed by PCR amplification of coding regions and flanking intronic sequences, followed by direct sequencing of amplicons on an ABI PRISM 3730 DNA Sequence Detection System (Applied Biosystems). Primer sequences are listed in Supplemental Table 2.

Control populations. We screened randomly selected and unrelated white Dutch individuals (n = 176); white individuals (n = 702) selected from the KORA S4 survey, which included population-based southern German individuals (n = 4,261) surveyed between 1999 and 2001 (53); unrelated white Turkish individuals (n = 150); and 4 different ethnic groups (white, African American, Hispanic, Asian; n = 94 for each group) from the Coriell Cell Repositories. The Coriell samples were resequenced as described above by the J. Craig Venter Institute through the NHLBI DNA Resequencing and Genotyping Program. The other control samples were genotyped at the identified mutation sites.

Subunit mRNA abundances in human cardiac tissue. Real-time RT-PCR was used to quantify subunit abundances. Assays were conducted in nondiseased human hearts obtained from the University of Szeged, Szeged, Hungary, that were technically unusable for transplantation based on logistic considerations (54). Before cardiac explantation, organ donor patients did not receive medication except dobutamine, furosemide, and plasma expanders. The investigations conformed to the principles outlined in the Helsinki Declaration of the World Medical Association. All experimental protocols were approved by the Ethical Review Board of the Medical Center of the University of Szeged (no. 51-57/1997 OEJ). The left ventricles from 6 donors and the right ventricles from 6 donors were dissected and stored in cardioplegic solution at 4°C for approximately 4–8 hours before being

frozen in liquid nitrogen. Purkinje fiber mRNA was extracted from false tendons dissected from the ventricles of 6 donors. Further information on the donors is presented in Supplemental Table 3.

Total RNA from each cardiac sample was isolated and DNase treated with the RNeasy Fibrous Tissue Mini Kit (QIAGEN) following the manufacturer's instructions. The quality of total RNA was assessed with PAGE (2100 Bioanalyzer; Agilent Technologies). Absence of genomic DNA contamination was verified by PCR. First-strand cDNA was synthesized from 2 μg total RNA with High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed on a TaqMan system with predesigned 6-carboxyfluorescein–labeled (FAM-labeled) fluorogenic TaqMan probe and primers for β1, custom-designed TaqMan probe and primers for β1B (located in the retained segment of intron 3), and 1× TaqMan Universal PCR Master Mix (Applied Biosystems). PCR efficiency of the β1 and β1B fluorescent probes was estimated at approximately 98%. After 2 minutes at 50°C and 10 minutes at 95°C, 40 cycles of amplification were performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data were collected with instrument spectral compensation by Applied Biosystems SDS 2.1 software and analyzed with the Ct relative quantification method (55). Fluorescence signals were normalized to the housekeeping gene hypoxanthine phosphoribosyl transferase 1 (*HPRT1*). For each sample, β1 and β1B transcripts were quantified in duplicate. The values were averaged and then used for the $2^{-\Delta Ct}$ × 100 calculation, where $2^{-\Delta Ct}$ corresponds to expression relative to *HPRT*. Primer and probe sequences are listed in Supplemental Table 4.

Generation of expression vectors. Full-length human β1 cDNA (GenBank accession number NM_001037) subcloned into a bicistronic vector also carrying the cDNA for enhanced eGFP (pEGFP-IRES; BD Biosciences — Clontech) was supplied by Alfred George Jr. (Vanderbilt University, Nashville, Tennessee, USA). Full-length human β1B cDNA (GenBank accession number NM_199037) was cloned from human ventricular mRNA, supplied by Katherine Murray (Vanderbilt University). The β1B cDNA was subcloned into a pEGFP-IRES vector (BD Biosciences — Clontech). Mutant constructs were prepared using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The inserts were subsequently sequenced to ensure that there was no other mutation besides the intended one.

Transient transfection in CHO cells. For functional analysis, cultured CHO cells were transiently transfected with the constructs described above using FuGENE 6 (Roche Applied Science). Constructs encoding β1 or β1B subunits (1 μg, unless otherwise specified) were cotransfected with the pBK-CMV vector (1 μg; Stratagene) encoding *SCN5A* (GenBank accession number NM_000335), supplied by Alfred George Jr. To study dominant negative effects, mutant β1 or β1B construct (0.5 μg or 1 μg) was cotransfected with the same amount of construct for WT β1 or β1B subunit that had been subcloned into a bicistronic vector also carrying cDNA for red fluorescent protein from *Discosoma* version T3 (pDsRed-IRES; supplied by Alfred George Jr.) along with *SCN5A* (1 μg). When *SCN5A* was transfected without β subunits, the plasmid encoding the eGFP (pEGFP-IRES; BD Biosciences — Clontech) with no β subunit insert was cotransfected. Cells were grown for 48 hours after transfection before study.

Electrophysiology. Cells displaying green fluorescence were chosen for study; in experiments with transfection of both WT and mutant β subunits, cells displaying both green and red fluorescence were chosen. Sodium currents were measured at room temperature using the whole-cell configuration of the patch-clamp technique with an Axopatch 200B amplifier (Molecular Devices). The extracellular bath solution contained (in mmol/l): 145 NaCl, 4.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4 (NaOH). Patch pipettes (~1.5 MΩ) contained (in mmol/l): 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.4 (CsOH). Currents were filtered at 5 kHz and digitized at 50 kHz. Cell capacitance and series resistance were compensated for by at least 80%. Voltage control, data acquisition, and analysis were accomplished using pCLAMP 9.2 and Clampfit 9.2 software (Molecular Devices).

Sodium current properties were determined by voltage clamp protocols as shown in the relevant figures. Cells were held at -120 mV, and currents were elicited with 50-ms depolarizing pulses from -80 to 60 mV in 10-mV increments. Voltage dependence of inactivation was studied using 500-ms prepulses from -120 to -20 mV in 10-mV increments, followed by a test pulse to -20 mV. The rate of recovery from inactivation was examined by 50-ms conditioning pulse to -20 mV from a holding potential of -120 mV, followed by a varying recovery duration and a 10-ms test pulse to -20 mV. All currents were normalized to cell capacitance. The voltage dependence of sodium current was determined by fitting a Boltzmann function ($y = [1 + \exp\{(V - V_{1/2}) / k\}]^{-1}$), yielding the voltage required to achieve half-maximal conductance or channel availability ($V_{1/2}$) and slope factor (k). The time constants of recovery from inactivation were determined using a double-exponential function ($y = A_f\{1 - \exp(-t / \tau_f)\} + A_s\{1 - \exp(-t / \tau_s)\}$), where τ_f and τ_s are the time constants of fast and slow components, and A_f and A_s are the fractions of the fast and slow components.

Statistics. Electrophysiological data are expressed as mean ± SEM. Gene expression data are expressed as median ± median absolute deviation. All statistical analyses were conducted with SPSS version 12.0. To test for significant differences among groups, an unpaired 2-tailed t test or ANOVA was used. The level of statistical significance was $P < 0.05$.

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Atrial Fibrillation in Patients With Brugada Syndrome

Relationships of Gene Mutation, Electrophysiology, and Clinical Backgrounds

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Objectives The goal of our work was to examine the relationships of atrial fibrillation (AF) with genetic, clinical, and electrophysiological backgrounds in Brugada syndrome (BrS).

Background Atrial fibrillation is often observed in patients with BrS and indicates that electrical abnormality might exist in the atrium as well as in the ventricle. *SCN5A*, a gene encoding the cardiac sodium channel, has been reported to be causally related to BrS. However, little is known about the relationships of atrial arrhythmias with genetic, clinical, and electrophysiological backgrounds of BrS.

Methods Seventy-three BrS patients (49 ± 12 years of age, men/women = 72/1) were studied. The existence of *SCN5A* mutation and clinical variables (syncopal episode, documented ventricular fibrillation [VF], and family history of sudden death) were compared with spontaneous AF episodes. Genetic and clinical variables were also compared with electrophysiologic (EP) parameters: atrial refractory period, interatrial conduction time (CT), repetitive atrial firing, and AF induction by atrial extra-stimulus testing.

Results Spontaneous AF occurred in 10 (13.7%) of the BrS patients and *SCN5A* mutation was detected in 15 patients. Spontaneous AF was associated with higher incidence of syncopal episodes (60.0% vs. 22.2%, $p < 0.03$) and documented VF (40.0% vs. 14.3%, $p < 0.05$). *SCN5A* mutation was associated with prolonged CT ($p < 0.03$) and AF induction ($p < 0.05$) in EP study, but not related to the spontaneous AF episode and other clinical variables. In patients with documented VF, higher incidence of spontaneous AF (30.8% vs. 10.0%, $p < 0.05$), AF induction (53.8% vs. 20.0%, $p < 0.03$), and prolonged CT was observed.

Conclusions Spontaneous AF and VF are closely linked clinically and electrophysiologically in BrS patients. Patients with spontaneous AF have more severe clinical backgrounds in BrS. *SCN5A* mutation is associated with electrical abnormality but not disease severity. (J Am Coll Cardiol 2008;51:1169–75) © 2008 by the American College of Cardiology Foundation

Brugada syndrome (BrS) is a distinct form of idiopathic ventricular fibrillation (VF) characterized by a unique electrographic (ECG) pattern consisting of a right bundle branch block-like morphology and ST-segment elevation in precordial leads (1–3). In addition to the ventricular arrhythmias, atrial arrhythmias are also often observed in this syndrome (4–6), indicating that electrical abnormality might exist in the atrium as well as in the ventricle. We, therefore, speculated that patients with BrS and spontaneous atrial fibrillation (AF) have more advanced disease process.

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The human cardiac sodium channel (*SCN5A*) is responsible for the fast depolarization upstroke for the cardiac action potential (7). Mutations in *SCN5A* have been previ-

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Abbreviations and Acronyms

AF = atrial fibrillation

BrS = Brugada syndrome

CS = coronary sinus

CT = conduction time

EP = electrophysiology/electrophysiological

ERP = effective refractory period

FH = family history of sudden death

ICD = implantable cardioverter-defibrillator

PCR = polymerase chain reaction

RAA = right atrial appendage

RAF = repetitive atrial firing

SCN5A = pore-forming region of the human cardiac sodium channel

VF = ventricular fibrillation

ously discovered in a wide spectrum of cardiac rhythm disorders: the long QT syndrome (8), BrS (7), sick sinus syndrome (9,10), cardiac conduction defect (11), and AF (12). In patients with BrS, *SCN5A* mutations have been reported to be causally linked to familial BrS (7,13). However, little is known about the relationships of atrial arrhythmias with genetic, clinical, and electrophysiological (EP) backgrounds. We, therefore, examined the relationships between genetic, EP, and clinical variables to AF in BrS patients.

Methods

Patient population and clinical data collection. Patients diagnosed with BrS in our hospital between 1997 to 2006 were studied. All of the tests that were performed were approved by the medical ethical review committees of our hospital. Informed consent was obtained from all patients. Clinical data, including data on age at diagnosis, gender, family history, documented VF, syncopal episodes, and implantable cardioverter-defibrillator (ICD) implantation, were obtained from patient records. Family history of sudden death (FH) was defined as unknown sudden death at less than the age of 50 years. All patients showed a typical ECG "Brugada pattern," which was defined previously (1). If the standard ECG pattern showed a type 2 or 3 Brugada pattern, 1 mg/kg of pilsicainide (a pure sodium channel blocker) was intravenously administered for 10 min with continuous monitoring in the intensive care unit and it was confirmed that the Brugada pattern had changed to a type 1 pattern.

Evaluation of incidence of AF. The occurrence of spontaneous AF was evaluated by clinical follow-up (every month), in which the patient's symptoms were observed and 24-h Holter recordings without any drugs were performed. Continuous ECG monitoring was also performed for 2 to 3 weeks during admission.

Analysis of *SCN5A* mutation. This study was performed in compliance with guidelines for human genome studies of the Ethics Committee of Okayama University. Informed consent was obtained from all patients. All exons of SCN5A were amplified by polymerase chain reaction (PCR) from deoxyribonucleic acid (DNA) isolated from peripheral leukocytes of the patients. Genomic DNA was extracted from peripheral blood leucocytes using a DNA extraction kit (Gentra, Minneapolis, Minnesota) and was stored at −30°C until use.

Twenty-seven exons of the *SCN5A* gene were amplified with previously reported intronic primers (14). *SCN5A* gene exon 1 is a noncoding region, and this region was not analyzed in this study. Exons 6, 17-1 Sense, 21, and 25 were not able to be amplified sufficiently by the primers, and we designed new intronic primers. The following primers were used in this study: 5′-GTT ATC CCA GGT AAG ATG CCC-3′ (sense) and 5′-TGG TGA CAG GCA CAT TCG AAG-3′ (antisense) for exon 6, 5′-AAG CCT CGG AGC TGT TTG TCA CA-3′ (sense) for exon 17-1, 5′-TGC CTG GTG CAG GGT GGA AT-3′ (sense) and 5′-ACT CAG ACT TAC GTC CTC CTT C-3′ (antisense) for exon 21, and 5′-TCT TTC CCA CAG AAT GGA CAC C-3′ (sense) and 5′-AAG GTG AGA TGG GAC CTG GAG-3′ (antisense) for exon 25. Polymerase chain reaction was performed in 25-μl reaction volumes containing 50 ng of genomic DNA, 20 pmol of each primer, 0.8 mM dNTPs, 1 X reaction buffer, 1.5 mM $MgCl_2$, and 0.7 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California) or TAKARA Taq (Takara Bio Inc., Otsu, Shiga, Japan). All PCR products were purified with a PCR products pre-sequencing kit (Amersham Life Science, Buckinghamshire, United Kingdom), reacted with a Big Dye Terminator FS ready-reaction kit (Applied Biosystems), and analyzed on an ABI PRISM3130xl sequencer (Applied Biosystems). Mutations were analyzed at least 3 times by independent PCR amplification and sequencing. Polymerase chain reaction products were subjected to single-strand conformation polymorphism analysis followed by direct sequence analysis.

EP study. After obtaining written informed consent from patients, an EP study was performed as described previously (6,15,16) in all patients. In brief, after right femoral and right jugular venous access had been obtained, 3 quadripolar electrode catheters (6-F) with an interelectrode distance of 5 mm (EP Technologies, Boston Scientific, Inc., Sunnyvale, California) were positioned in the right atrial appendage (RAA), His bundle region, and right ventricle, and an octopolar catheter (6-F) with an interelectrode distance of 2.5 mm (EP Technologies, Boston Scientific, Inc.) was positioned in the coronary sinus (CS). To reduce the differences among patients, the proximal electrode of CS catheter was positioned at the CS ostium and the distal electrode was located at the lateral wall of the left atrium in all patients. An extra-stimulus (S2) was delivered after 8 beats of drive pacing (S1) at a basic cycle length of 600 ms. The S1-S2 interval was decreased in 10-ms steps until the effective refractory period (ERP) of the RAA was reached. Sinus node recovery time was also measured during the EP study.

The parameters during EP study were as follows: 1) ERP of the RAA by atrial extra-stimulus testing; 2) interatrial conduction time (CT) measured by CT from the stimulus at the right atrium to atrial deflection at the distal portion of the CS; 3) the duration of local atrial electrogram (A) recorded at atrial pacing site; 4) repetitive atrial firing (RAF)

defined as occurrence of 2 or more premature atrial complexes after atrial stimulation; and 5) induced AF defined as AF that was induced by extrastimulus and persisted for >30 s (6,17–19). If RAF or AF was induced during the paired pacing, S2 was no longer decreased and ERP was defined as the minimum S2 interval that induced RAF or AF.

Programmed electrical stimulation was also performed at the ventricle to induce VF. As described previously (15), programmed electrical stimulation was performed at an intensity twice threshold and 2-ms in duration through the distal electrodes in the right ventricular apex, free-wall region, septal region of the right ventricular outflow tract, and posterolateral wall of the left ventricle using pulse generator as described before. The protocol of ventricular stimuli included up to 3 extrastimuli at the basic cycle length of 600 ms and 400 ms and the minimum coupled extrastimuli of 180 ms.

Statistical analysis. Data are expressed as mean values ± standard deviation. Student *t* test was performed to test for statistical differences between 2 unpaired mean values, and categorical data and percentage frequencies were analyzed by the chi-square test (SPSS II for Windows, SPSS Inc., Chicago, Illinois). A value of $p < 0.05$ was considered to be statistically significant.

Results

Patients' characteristics. The population consisted of a total of 73 probands. None of the patients in this study were members of the same family. Patients' characteristics are summarized in Table 1. Spontaneous AF was documented in 10 (13.7%) of the patients and VF was documented in 13 (17.8%) of the patients. Nineteen (26.0%) of the patients had an FH, and syncopal episodes occurred in 20 (27.4%) of the patients. Gene analysis revealed that *SCN5A* mutation was present in 15 (20.5%) of the patients. Spontaneous type 1 ECG was observed in 23 (31.5%) of the patients. In EP study, VF was induced in 34 (47%) of the patients and 33 (45.2%) of the patients had received ICD implantation.

Table 1 Patients' Characteristics (n = 73)

Men/women	72/1
Age (yrs)	49.5 ± 12.0
Syncopal episode (%)	20 (27.4%)
Documented VF (%)	13 (17.8%)
Spontaneous AF (%)	10 (13.7%)
Family history of sudden death (%)	19 (26.0%)
SCN5A mutation (%)	15 (20.5%)
Spontaneous type 1 ECG	23 (31.5%)
VF induction during EP study (%)	34 (46.6%)
ICD implantation (%)	33 (45.2%)

Values are mean ± SD or number of patients.
AF = spontaneous documented atrial fibrillation; ECG = electrocardiogram; EP = electrophysiological; ICD = implantable cardioverter defibrillator; SCN5A = pore-forming region of the human cardiac sodium channel; VF = ventricular fibrillation.

Circadian variation of spontaneous AF and VF. Spontaneous AF episodes were detected at night (12:00 AM to 6:00 AM) in 7 (70%) of the 10 patients with documented AF and 3 of 10 patients in the daytime (6:00 AM to 6:00 PM). Documented VF episodes were observed in 13 patients (46 episodes). Among them, 7 patients (55%) (22 episodes [48%]) were detected at night (12:00 AM to 6:00 AM), and 2 patients (15%) (7 episodes [15%]) in the daytime (6:00 AM to 6:00 PM).

Clinical and genetic differences in BrS patients with AF. Clinical and genetic parameters were compared in BrS patients with spontaneous AF and those without spontaneous AF (Table 2). None of the patients in this study showed chronic AF. Age was not different between the groups. In the clinical parameters, syncopal episode, documented VF, and spontaneous type 1 ECG were observed in larger percentage of patients with spontaneous AF (syncope: 60.0% vs. 22.2%, $p < 0.03$; documented VF: 40.0% vs. 14.3%, $p < 0.05$; and spontaneous type 1 ECG: 60.0% vs. 27.0%, $p < 0.04$). However, FH, *SCN5A* mutation, and VF induction during EP study were not related to spontaneous AF episodes (Table 2).

EP parameters in BrS patients with AF. In EP study, there was no significant difference between the ERP of the RAA in the AF (+) group (254.3 ± 44.7 ms) and that in the AF (−) group (243.9 ± 25.5 ms). However, CT was more prolonged in the AF group at S1 (CT at S1: 138.4 ± 23.8 ms vs.122.3 ± 20.1 ms, $p < 0.03$) and at S2 (172.4 ± 33.3 ms vs. 154.2 ± 18.0 ms, $p < 0.03$). Sinus node recovery time was significantly prolonged in the AF (+) group (1,971 ± 1,007 ms vs. 1,288 ± 488 ms, $p < 0.01$). Other parameters, including RAF, induction of AF, and local atrial electrograms (A1: A at S1 and A2: A at S2) were not different between the groups (Table 2).

Clinical and EP parameters in BrS patients with *SCN5A* mutation. Next we examined the relationships of genetic mutation with clinical and EP parameters in patients with BrS. None of the clinical parameters (age, syncopal episode, documented VF, spontaneous AF, FH, spontaneous type 1 ECG, and ICD implantation) were different in patients with *SCN5A* mutation and patients without *SCN5A* mutation. However, AF induction (in 46.7% of the patients with *SCN5A* mutation and in 20.7% of the patients without *SCN5A* mutation, $p < 0.05$), CT at S1 (138.1 ± 18.1 ms with *SCN5A* mutation and 121.5 ± 20.9 ms without *SCN5A* mutation, $p < 0.03$), CT at S2 (167.9 ± 14.2 ms with *SCN5A* mutation and 153.4 ± 21.3 ms without *SCN5A* mutation, $p < 0.03$), local A2 (103.9 ± 17.4 ms with *SCN5A* mutation and 89.8 ± 18.7 ms without *SCN5A* mutation, $p < 0.03$), and sinus node recovery time (1,682 ± 1,036 ms with *SCN5A* mutation and 1,300 ± 433 ms without *SCN5A* mutation, $p < 0.04$) during EP study were significantly different between the groups (Table 3).

Table 2 **Characteristics of Patients With and Without AF**

	Without AF	With AF	p Value
Clinical/genetic parameters			
Number of patients (men/women)	63 (62/1)	10 (10/0)	
Age (yrs)	48.4 ± 11.5	53.7 ± 14.2	NS
Syncopal episode (%)	14 (22.2%)	6 (60.0%)	<0.03
Documented VF (%)	9 (14.3%)	4 (40.0%)	<0.05
Family history of sudden death (%)	17 (27.0%)	2 (20.0%)	NS
SCN5A mutation (%)	13 (20.6%)	2 (20.0%)	NS
Spontaneous type 1 ECG (%)	17 (27.0%)	6 (60.0%)	<0.04
VF induction during EP study (%)	29 (46.0%)	5 (50.0%)	NS
ICD implantation (%)	27 (42.9%)	6 (60.0%)	NS
EP parameters of the atrium			
RAF	31 (49.2%)	6 (60.0%)	NS
AF induction	14 (22.2%)	5 (50.0%)	NS
ERP (ms)	243.9 ± 25.5	254.3 ± 44.7	NS
CT at S1 (ms)	122.3 ± 20.1	138.4 ± 23.8	<0.03
CT at S2 (ms)	154.2 ± 18.0	172.4 ± 33.3	<0.03
A1 (ms)	65.7 ± 12.9	72.5 ± 20.4	NS
A2 (ms)	92.4 ± 18.9	99.2 ± 21.8	NS
A2/A1	1.42 ± 0.25	1.39 ± 0.24	NS
Sinus node recovery time (ms)	1,288 ± 488	1,971 ± 1,007	<0.01

Values are mean ± SD or number of patients.
A1 = local atrial potential at S1; A2 = local atrial potential at S2; CT = interatrial conduction time; ERP = effective refractory period; RAF = repetitive atrial firing; other abbreviations as in Table 1.

Clinical, genetic, and EP parameters in BrS patients with spontaneous type 1 ECG. Next we examined the relationship of the basal ECG pattern to the clinical, genetic, and EP parameters in patients with BrS. Spontaneous type 1 ECG was observed in 23 of the patients (31.5%) and drug (pilsicainide)-induced type 1 ECG (type 2 or 3 ECG before the drug administration) in the remaining 50 patients (68.5%) in this study. Spontaneous AF was significantly more observed in patients with spontaneous type 1 ECG (26.1% vs. 8.0%, $p < 0.04$). Documented VF tended to be more observed but not statistically significant (30.4% vs. 12.0%, $p = 0.06$). Other parameters including age, syncopal episodes, FH, frequency of *SCN5A* mutation, VF induction, ICD implantation, and

Table 3 **Clinical and EP Parameters in Patients With and Without *SCN5A* Mutation**

	SCN5A Mutaion (−)	*SCN5A* Mutaion (+)	p Value
Clinical parameters			
Number of patients (men/women)	58 (57/1)	15 (15/0)	
Age (yrs)	49.6 ± 11.3	47.5 ± 14.5	NS
Syncopal episode (%)	15 (25.9%)	5 (33.3%)	NS
Documented VF (%)	9 (15.5%)	4 (26.7%)	NS
Spontaneous AF (%)	8 (13.8%)	2 (13.3%)	NS
Family history of sudden death (%)	13 (22.9%)	6 (40.0%)	NS
Spontaneous type 1 ECG (%)	16 (27.6%)	7 (46.7%)	NS
VF induction during EP study (%)	30 (51.7%)	4 (26.7%)	NS
ICD implantation (%)	26 (44.8%)	7 (46.7%)	NS
EP parameters of the atrium			
RAF	29 (50.0%)	8 (53.3%)	NS
AF induction	12 (20.7%)	7 (46.7%)	<0.05
ERP (ms)	240.2 ± 24.2	264.5 ± 35.6	NS
CT at S1 (ms)	121.5 ± 20.9	138.1 ± 18.1	<0.03
CT at S2 (ms)	153.4 ± 21.3	167.9 ± 14.2	<0.03
A1 (ms)	64.5 ± 13.2	73.0 ± 11.4	NS
A2 (ms)	89.8 ± 18.7	103.9 ± 17.4	<0.03
A2/A1	1.41 ± 0.26	1.45 ± 0.20	NS
Sinus node recovery time	1,300 ± 433	1,682 ± 1,036	<0.04

Values are mean ± SD or number of patients.
Abbreviations as in Tables 1 and 2.

Table 4 Clinical, Genetic, and EP Parameters in Patients With and Without Spontaneous Type 1 ECG

	Type 2 or 3 ECG	Type 1 ECG	p Value
Clinical/genetic parameters			
Number of patients (men/women)	50 (49/1)	23 (23/0)	
Age (yrs)	49.7 ± 12.0	47.8 ± 12.0	NS
Syncopal episode (%)	12 (24.0%)	8 (34.8%)	NS
Documented VF (%)	6 (12.0%)	7 (30.4%)	NS (p = 0.06)
Spontaneous AF (%)	4 (8.0%)	6 (26.1%)	<0.04
Family history of sudden death (%)	13 (28.0%)	6 (26.1%)	NS
SCN5A mutation (%)	8 (16.0%)	7 (30.4%)	NS
VF induction during EP study (%)	20 (40.0%)	14 (60.9%)	NS
ICD implantation (%)	19 (38.0%)	14 (60.9%)	NS
EP parameters of the atrium			
RAF	26 (52.0%)	11 (47.8%)	NS
AF induction	11 (22.0%)	8 (34.8%)	NS
ERP (ms)	246.2 ± 27.4	242.9 ± 32.0	NS
CT at S1 (ms)	122.9 ± 22.8	128.6 ± 17.4	NS
CT at S2 (ms)	155.8 ± 22.3	157.6 ± 16.9	NS
A1 (ms)	65.3 ± 12.1	69.9 ± 15.8	NS
A2 (ms)	91.1 ± 18.4	99.2 ± 20.8	NS
A2/A1	1.4 ± 0.3	1.4 ± 0.2	NS
Sinus node recovery time (ms)	1,310 ± 460	1,523 ± 855	NS

Values are mean ± SD or number of patients.
Abbreviations as in Tables 1 and 2.

all EP parameters were not different between the groups (Table 4).

Clinical, genetic, and EP parameters in BrS patients with and without VF episodes. Finally, we examined the relationships of disease severity (documented VF) with other clinical, genetic, and EP parameters in BrS patients. Spontaneous AF was observed in a large percentage of patients with VF episodes (30.8%) in comparison with that seen in patients without VF episodes (10.0%) ($p < 0.05$), but the frequency of *SCN5A* mutation was not different between the groups (Table 5). Spontaneous type 1 ECG tended to be more observed in patients with VF episodes but not statistically significant ($p = 0.06$). As for the EP parameters, ERP at RAA was not different, but the rate of AF induction

Table 5 Clinical, Genetic, and EP Parameters in Patients With and Without Documented VF Episode

	Documented VF (−)	Documented VF (+)	p Value
Clinical/genetic parameters			
Number of patients (men/women)	60 (59/1)	13 (13/0)	
Age (yrs)	48.3 ± 12.0	52.8 ± 11.1	NS
Spontaneous AF (%)	6 (10.0%)	4 (30.8%)	<0.05
Family history of sudden death (%)	17 (28.3%)	2 (15.4%)	NS
SCN5A mutation (%)	11 (18.3%)	4 (30.8%)	NS
Spontaneous type 1 ECG (%)	16 (26.7%)	7 (53.8%)	NS (p = 0.06)
VF induction during EP study (%)	28 (46.7%)	6 (46.2%)	NS
ICD implantation (%)	20 (33.3%)	13 (100%)	<0.01
EP parameters of the atrium			
RAF	29 (48.3%)	8 (61.5%)	NS
AF induction	12 (20.0%)	7 (53.8%)	<0.03
ERP (ms)	242.0 ± 26.2	261.1 ± 34.8	NS
CT at S1 (ms)	121.9 ± 19.6	137.6 ± 24.6	<0.02
CT at S2 (ms)	153.7 ± 16.8	171.3 ± 33.9	<0.02
A1 (ms)	66.1 ± 14.1	68.6 ± 8.5	NS
A2 (ms)	91.6 ± 19.8	100.4 ± 15.0	NS
A2/A1	1.4 ± 0.3	1.5 ± 0.2	NS
Sinus node recovery time	1,313 ± 505	1,658 ± 937	NS

Values are mean ± SD or number of patients.
Abbreviations as in Tables 1 and 2.

was significantly higher (53.8% vs. 20.0%, $p < 0.03$) and CT was prolonged in patients with VF episodes (CT at S1: 137.6 ± 24.6 ms vs. 121.9 ± 19.6 ms, $p < 0.02$; CT at S2: 171.3 ± 33.9 ms vs. 153.7 ± 16.8 ms, $p < 0.02$) (Table 5). Sinus node recovery time was not different between the groups ($p = 0.07$).

Discussion

The present study demonstrated that BrS patients with spontaneous AF have more severe clinical and EP backgrounds but not associated with family history or mutations of the gene encoding the cardiac sodium channel, *SCN5A*. Electrical vulnerability across the heart may be closely associated with spontaneous AF and VF occurrence in BrS patients.

AF in BrS. It has been reported that spontaneous AF is often observed in patients with BrS. The incidence of AF in this syndrome has been reported to be 10% to 53% (1,4,6). In this study, the incidence of spontaneous AF was 13.7% and most cases (70%) were documented at night. Matsuo et al. (20) reported that VF in patients with BrS was most frequently detected in the midnight to early morning period during sleep. Our finding of a circadian pattern in spontaneous AF and VF episodes is in agreement with their findings, and these findings suggested that nocturnal vagal activity and withdrawal of sympathetic activity may play an important role in arrhythmogenesis in both AF and VF occurrence in this syndrome.

The treatment for AF in BrS is an important issue. It has been reported that quinidine sulfate, isoproterenol, cilostazole (1), and bepridil chloride (21,22) are recommended in Brugada patients with repeated VF by a mechanism of augmenting the calcium current or reducing the I_{to} current. In this study, none of the patients received antiarrhythmic drugs for AF because their episodes were paroxysmal with few symptoms. However, 2 AF patients that experienced recurrent VF episodes had received antiarrhythmic drugs to prevent recurrent VF (1 patient received quinidine sulfate 0.3 g and the other received bepridil hydrochloride 100 mg). While these patients never experienced AF episodes with taking these drugs, indicating antiarrhythmic drugs that were effective to prevent VF might be also effective in AF.

EP parameters in patients with BrS. It has also been reported that atrial vulnerability was increased in patients with BrS, compared with that in a normal control group (6). Among the various indexes of EP parameters, we found the interatrial conduction delay (CT) was significantly increased in BrS patients with AF, indicating that global conduction of the atrial myocardium was impaired. Interestingly, atrial vulnerability (induced AF) was more impaired in BrS patients with VF episodes, indicating that electrical vulnerability may be across the whole heart including the atrium and ventricle. The fact that patients with AF have more episodes of VF or syncopal episodes supports this possibility.

There was no difference in VF inducibility between the patients with and without documented VF. In this study, all patients who had documented VF experienced at least 1 VF episode before ICD implantation; therefore, asymptomatic patients never experienced VF attacks during the follow-up period after ICD implantation. These results indicate that VF inducibility during EP study has a low specificity to identify high-risk BrS patients as reported before (23).

***SCN5A* mutation is not associated with AF in BrS.** The gene encoding the cardiac sodium channel, *SCN5A*, has been reported to be linked causally to BrS. We speculated AF is more common in patients with *SCN5A* mutation, but we found no difference between patients with *SCN5A* mutation and those without *SCN5A* mutation in spontaneous AF episodes or in other clinical parameters (spontaneous VF, syncopal episode, FH, and spontaneous type 1 ECG). The reason is still unclear, but this finding is perhaps of most interest. These results indicate that a defect in the *SCN5A* gene is not associated with AF events or with VF events as was previously reported (1), suggesting that genetic analysis is not useful for risk stratification.

Clinical implications. This study showed that spontaneous AF and atrial vulnerability are important predictors of VF events that cause sudden cardiac death. The fifth-generation ICD is preferable for patients with BrS, even for BrS patients who have never experienced an attack of AF, because atrial vulnerability is common and AF could occur during the follow-up period.

Study limitations. The number of patients in this study was small, and further study is needed to reach definitive conclusion regarding the impact of AF episodes for BrS. Moreover, we analyzed only the coding regions of *SCN5A* for mutations in this study, and the possibility of mutations occurring in regions of the gene other than coding regions cannot be excluded. The functional impact has not been studied for all identified *SCN5A* mutations; therefore, a causal relationship in individual patients has not been proved yet.

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SCN5A variants in Japanese patients with left ventricular noncompaction and arrhythmia

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Abstract

Left ventricular noncompaction (LVNC) is a genetically heterogenous disorder. Mutations in the human cardiac sodium channel alpha-subunit gene (*SCN5A*) are involved in the pathophysiology of cardiac arrhythmias and cardiomyopathies. This study was performed to compare the frequency of *SCN5A* variants in LVNC patients with or without arrhythmias, and to investigate the relationship between variants and disease severity. DNA was isolated from the peripheral blood of 62 Japanese probands with LVNC, comprising 17 familial cases and 45 sporadic cases. Blood samples were screened for variants in *SCN5A* using single-strand conformational polymorphism analysis (SSCP) and DNA sequencing. Seven variants, rs6599230:G > A, c.453C > T, c.1141-3C > A, rs1805124:A > G (p.H558R), rs1805125:C > T (p.P1090L), c.3996C > T, and rs1805126:T > C were identified in 7 familial and 12 sporadic cases. The frequency of *SCN5A* variants was significantly higher in the patients with arrhythmias than those without (50% vs 7%; $P = 0.0003$), suggesting these variants represent a risk factor for arrhythmia and supporting the hypothesis that genes encoding ion channels are involved in LVNC pathophysiology. The LVNC patients with heart failure also had high occurence of *SCN5A* variants, suggesting the presence of *SCN5A* variants and/or arrhythmias increase the severity of LVNC.
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Keywords: Noncompaction; *SCN5A* variants; Arrhythmia; Heart failure

Introduction

Left ventricular noncompaction (LVNC) is characterized by persistence of multiple prominent ventricular trabeculations and deep intertrabecular recesses in the left ventricle and is defined as an unclassified cardiomyopathy [1]. To date, this disorder is considered an arrest in the morphogenetic process of myocardial compaction [2]. The clinical manifestations are not specific for this form of cardiomyopathy, with clinical overlap with dilated and hypertrophic cardiomyopathies, and are highly variable, ranging from asymptomatic to severe cardiac dysfunction leading to heart transplantation or death. Most LVNC patients also present with some form of arrhythmia [2].

LVNC, without other morphologic cardiac abnormalities, was first described echocardiographically in 1984 [3].

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In recent years, defects in several genes have been associated with LVNC. Recently, we identified novel sequence variants in *DTNA*, *TAZ*, and *LDB3* in patients with LVNC [4–6]. However, like other forms of inherited cardiomyopathy, LVNC is a genetically heterogeneous disease and can be inherited as an autosomal dominant or X-linked recessive disorder [4–6].

Mutations in the human cardiac sodium channel alpha-subunit gene (*SCN5A*) have been identified in patients with a range of arrhythmias including the long QT syndrome (LQTS) [7], Brugada syndrome [8], sudden unexplained nocturnal death syndrome [9], idiopathic ventricular fibrillation [10], congenital sick sinus syndrome [11], and cardiac conduction defects (CCD) [12], as well as sudden infant death syndrome [13]. Recently, variants in *SCN5A* [14,15] and a cardiac K_{ATP} channel gene (*ABCC9*)[16] have been reported in patients with dilated cardiomyopathy (DCM). The sodium channel plays a central role in the excitability of myocardial cells, by establishing a subtle equilibrium between depolarizing and repolarizing currents determining the action potential (AP) duration. Thus, variations in *SCN5A* may influence this equilibrium, even by weak effects on activity and/or the expression level of channels subunits. Single nucleotide polymorphisms (SNPs) in *SCN5A* have been implicated not only as the causes of inherited arrhythmic syndromes, but also as genetic risk factors for some acquired arrhythmias [17–20].

Therefore, we hypothesized that variations in genes encoding ion channels are implicated in the pathophysiology of LVNC in relation to the development of arrhythmias and the severity of disease. Here we report the analysis of the *SCN5A* gene in a large cohort of Japanese patients with LVNC, and present data supporting this hypothesis.

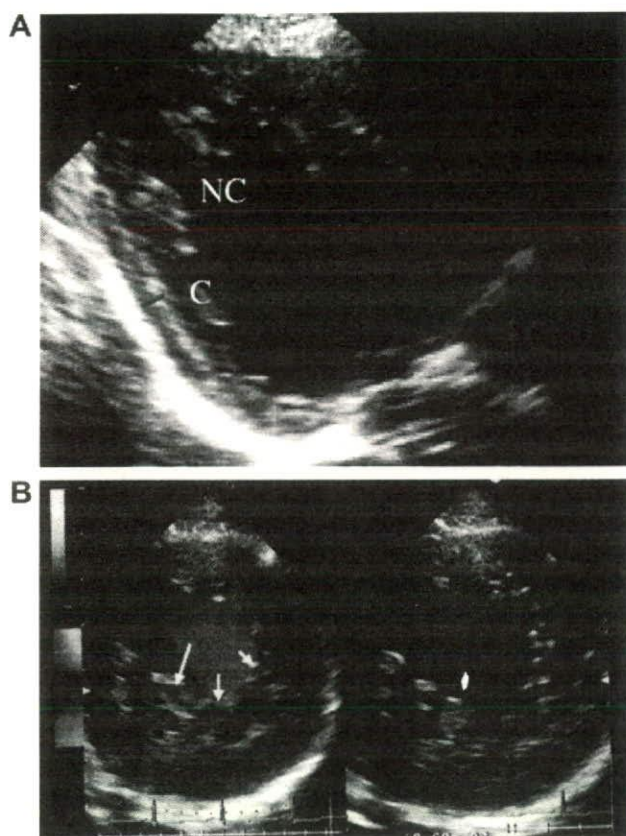

Fig. 1. (A) Two-dimensional echocardiogram of a patient with LVNC revealing noncompacted layer with intertrabecular recesses (NC) and outside the compacted layer (C), with an NC:C ratio >2.0. (B) Color Doppler echocardiogram of a LVNC patient demonstrating flow within deep intertrabecular recesses (arrow) in continuity with the left ventricular cavity. (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.)

Methods

Subjects and clinical diagnostic criteria

LVNC was diagnosed by echocardiographic criteria, including: (1) LV hypertrophy with deep endomyocardial trabeculations in ⩾1 ventricular wall segments, (2) reduced LV systolic function, (3) a two-layered endocardium with a noncompacted to compacted ratio of >2.0, and (4) deep recesses filled with blood from the ventricular cavity visualized on color Doppler imaging (Fig. 1) [4].

Initial clinical evaluations were performed without knowledge of genotype status. Once a proband was identified, a family history was obtained, and all potentially informative family members underwent clinical evaluation, including physical examination, chest radiograph, electrocardiogram (ECG), echocardiogram (2 dimensional and color Doppler) was used to evaluate the cardiac structure, LV size and function (shortening fraction and ejection fraction), and valve regurgitation (Fig. 1).

Peripheral blood samples were collected after written informed consent. Lymphoblastoid cell lines were established from the peripheral blood samples and then genomic DNA was isolated using QIAamp DNA extraction kits (Qiagen: Valencia, CA). The study was approved by the Research Ethics Committee of Toyama University Hospital.

Molecular genetic studies

The *SCN5A* gene was amplified by PCR from the genomic DNA of each of the probands. The PCR primers were designed to amplify each of the coding exons, as well as flanking intronic sequences, using the online utility Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and sequences from NCBI and Celera databases: the NCBI genomic DNA sequence file NT_022517.17 served as the primary source. PCR reactions were performed as previously described [6]: primer sequences and PCR conditions are available upon request. After PCR amplification, the samples were denatured by heating in a denaturing buffer and analyzed by SSCP [5].

Both normal and aberrant SSCP bands were cut directly from dried gels, purified, and sequenced according to the Applied Biosystems (ABI) Big Dye Terminator Cycle Sequencing protocol and analyzed using an ABI 310 Automated Sequencer. Sequencing results were compared with wild-type sequence published in NCBI by the BLAST comparative search algorithms (www.ncbi.nlm.nih.gov/BLAST/). Variations were confirmed by repeating the PCR from the genomic DNA template and sequencing the PCR products.

Statistical analysis

SNP frequencies in both of the groups were compared using the Chi square test. Relations between variants and arrhythmias were calculated by logistic regression. The differences were considered to be significant when $P < 0.05$.

Results

Genetic studies

The study cohort comprised 62 unrelated Japanese LVNC probands, which included 45 sporadic cases (S) and 17 familial cases (F): a further 8 affected family members were also recruited for genotype-phenotype correlations. Table 1 summarizes the probands sex, age at diagnosis, and clinical phenotype, and the *SCN5A* variants identified.

Six heterozygous and one homozygous (rs6599230:G > A) variants were identified in 7 familial and 12 sporadic cases (Tables 1 and 2). Of these, 4 are reported in the NCBI SNP database for *SCN5A* (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?geneId=6331&ctg=NT_022517.17&mrna=NM_198056.1&prot=NP_932173.1&orien=reverse), rs6599230:G > A, rs1805124:A > G (p.H558R), rs1805125:C > T (p.P1090L), and rs1805126:T > C. Two have been reported as SNPs (c.453C > T [21], c.1141-3C > A [22]). The last variant, c.3996C > T, was detected in a sporadic case of isolated LVNC with complete atrioventricular block (AVB): this variant has not been reported previously.

Association of SCN5A variants with arrhythmias

SCN5A variants were detected in 17 (50%) of the 34 probands with arrhythmias (7 familial cases and 10 sporadic cases) compared with 2 (7%) of the 28 probands without arrhythmias (Table 3): this difference was significant by Chi square analysis ($P = 0.0003$). In these 17 cases, a wide variety of arrhythmias were noted, including AVB ($n = 8$), atrial fibrillation (AF) ($n = 2$), LQTS ($n = 2$), Wolff-Parkinson-White syndrome (WPW) ($n = 5$), SSS ($n = 2$), ventricular tachycardia (VT) ($n = 7$), premature ventricular contraction (PVC) ($n = 10$), paroxysmal supraventricular tachycardia (PSVT) ($n = 3$).

While most of the variants were associated with multiple types of arrhythmia (Table 2), in the analysis of extended families multiple arrhythmia phenotypes were detected in the same family. For example for proband F6 (Fig. 2), who presented with LVNC with premature ventricular contraction (PVC), two variants were identified, rs1805126:T > C and p.P1090L. Both variants were inherited from his affected mother, who presented with LVNC with sick sinus syndrome (SSS). Using logistic regression analysis, patients who have the rs1805126:T > C variant have significantly high risk of arrhythmia (OR = 5.69 (1.05-30.84), $P = 0.044$).

We also investigated the correlation between the *SCN5A* variants and the severity of LVNC. Similar to the findings with arrhythmias, *SCN5A* variants were detected in 18 (53%) of the 34 patients who had developed heart failure, compared with just 1 (4%) of the 28 who had not. This was also a significant difference by Chi square analysis ($P = 0.0002$) (Table 4).

Discussion

During the more than ten years since mutations in the gene encoding the cardiac sodium channel, *SCN5A*, were first reported in 1995 [7], genetic alterations in *SCN5A* have been shown to influence the pathophysiology of cardiac arrhythmias, pharmacological sensitivities to antiarrhythmic drugs and recently cardiomyopathy [14]. Some common exonic SNPs were previously described as being able to produce subtle functional changes in channels physiology.

To date variations in three genes have been identified in patients with LVNC, *DTNA*, *TAZ* and *LDB3*.[4–6] Arrhythmias occur in more than 50% of patients with LVNC [23]. Since this phenotype does not correlate with the identification of gene variations, we hypothesized that variations in ion channel genes may contribute to the development of arrhythmias in LVNC patients regardless of the underlying cause of the disease. Therefore, we screened patients with LVNC for genetic variants in *SCN5A*. A significant number of genetic variations (both synonymous and non-synonymous nucleotide substitutions) were identified in the patient cohort. However, variations were detected significantly more frequently in patients who presented with arrhythmias than those who did not: 50% vs 7% ($P = 0.0003$).

Since LVNC patients with arrhythmias tend to have worse outcomes than patients without arrhythmias [23], we anticipated identifying a similar correlation between genetic variants and heart failure. Indeed *SCN5A* variants were detected in significantly more of the patients with heart failure, than this without: 53% vs 4% ($P = 0.0002$). Therefore, these data support the hypothesis that variations in ion channel encoding genes contribute to the underlying pathophysiology of LVNC and determining outcome.

The H558R substitution is located in the Na^+ channel I, II interdomain cytoplasmic linker and previous functional studies have shown that the R558-encoding minor allele can dramatically alter the phenotype of true disease-causing *SCN5A* mutations [24]. It has been suggested that it modulates Na^+ channel functional changes caused by other variations, and plays a role in intragenic complementation [25,26]. H558R expressed in the different variant backgrounds has profoundly different effectd on function, even "loss of function."[27] In our two cases with the H558R variant the QT interval was normal, but they both had severe arrhythmias, one with PVC and the other WPW. The P1090L substitution was identified in a sporadic case with AF and a familial case with PVC (proband) and SSS (the proband's mother). P1090 is localized in the II, III interdomain cytoplasmic linker. As a relatively Asian-specific common polymorphism, P1090L has been shown to result in a significant negative shift of activation midpoint in the Q1077del background [27].

With the exception of the c.3996C > T variant, all of the other variants are commonly detected in the normal popu-

Table 1
Clinical and molecular data for probands with LVNC

ID	Sex	Age	Arrhythmia	Heart failure	SCN5A variant
F1	M	0y	PVC	+	No
F2	F	69y	—	+	No
F3	M	5y	AVB	+	No
F4*	F	1w	PVC	+	c.453C > T
F5	F	60y	AVB, VT	+	RS1805126:T > C
F6	M	4y	PVC, SSS	+	RS1805126:T > C, p.P1090L
F7	F	1y	—	—	No
F8	F	5y	AF	+	RS1805126:T > C
F9	M	3m	—	+	No
F10	M	13y	LQT	+	RS6599230:G > A, RS1805126:T > C
F11	F	29y	—	+	No
F12	F	1y	—	—	No
F13	F	3y	—	—	No
F14	M	4y	—	—	No
F15	F	N/A	PVC	+	RS1805126:T > C, RS6599230:G > A
F16	F	1m	—	—	No
F17*	M	13	WPW	+	RS1805124:A > G, c.1141-3C > A
S18	F	0.2y	PSVT, WPW	+	No
S19	M	12y	PVC	+	RS1805124:A > G, c.1141-3C > A, RS1805126:T > C
S20	F	12y	AVB	+	No
S21	M	7y	AVB	+	No
S22	F	1w	WPW	+	RS1805126:T > C
S23	N/A	N/A	WPW	—	No
S24	F	21y	VT	—	No
S25	M	3y	VT	+	No
S26	M	10d	PSVT, VT	+	No
S27	M	25y	AVB	—	No
S28	M	1y	AVB	+	No
S29	F	3m	PSVT	+	RS1805126:T > C
S30	M	10y	PVC	—	No
S31	F	12y	AF	+	p.P1090L, RS1805126:T > C
S32	F	1d	LQT	—	No
S33	F	14y	VT	—	No
S34	F	1y	PVC, VT	+	No
S35	N/A	N/A	—	—	No
S36	N/A	N/A	—	+	RS1805126:T > C
S37	N/A	N/A	—	—	No
S38	N/A	N/A	—	—	No
S39	N/A	N/A	—	+	RS1805126:T > C
S40	N/A	N/A	—	—	No
S41	M	1m	—	—	No
S42	M	2m	—	+	No
S43	M	0y	AVB	+	P1332P
S44	M	9m	PVC	+	c.1141-3C > A
S45	M	3y	—	+	No
S46	M	36y	VT	+	RS1805126:T > C
S47	M	N/A	SSS	+	RS1805126:T > C
S48	F	N/A	AVB	—	No
S49	F	1y	—	—	No
S50	M	8m	WPW	—	No
S51	M	0y	—	—	No
S52	M	8m	—	—	No
S53	F	22y	—	—	No
S54	F	4m	—	+	No
S55	M	15y	—	—	No
S56	M	5m	PVC	+	RS1805126:T > C
S57	M	17y	—	—	No
S58	M	1y	—	+	No
S59	F	1y	—	—	No
S60	F	0y	—	—	No
S61	M	43y	PVC	—	RS6599230:G > A
S62	F	16y	—	—	No

F, familial case; S, sporadic case; ID indicates identification; Age, proband's age at diagnosis; N/A, information unavailable; blank, no abnormality; +, the proband had heart failure; no, no variant was found. AVB AV block; AF, atrial fibrillation; LQT, long QT syndrome; WPW, Wolff-Parkinson-White syndrome; SSS, sick sinus syndrome; VT, ventricular tachycardia; PVC, premature ventricular contraction; PSVT, paroxysmal supraventricular tachycardia.

* Compound with *LDB3* variation [6].

Table 2
Summary of *SCN5A* variants detected

DNA variant (dbSNP ID)	Protein change	Exon	Family/case	Types of arrhythmia
87G > A (rs6599230)		Exon 2	F10,F15,S61	PVC, LQT
453C > T		Exon 4	F4	PVC
1141-3C > A		Intron 9	F17, S19, S44	PVC, WPW
1673A > G (rs1805124)	H558R	Exon 12	F17, S19	PVC, WPW
3269C > T (rs1805125)	P1090L	Exon 18	F6, S31	AF, SSS, PVC
3996G > A		Exon23	S43	AVB
5457T > C (rs1805126)		Exon28	F5, F6, F8, F10, F15, S19, S22, S29, S31, S36,S39, S46, S47, S56	PSVT, VT, PVC, AVB, LQT, SSS, AF, WPW

Abbreviations are defined in Table 1.

Table 3
Incidence of *SCN5A* variants in the cases with arrhythmia and in those without arrhythmia

	SCN5A Variant			(%)	No *SCN5A* variant			(%)
	Familial cases	Sporadic cases	Total		Familial cases	Sporadic cases	Total	
Arrhythmia	7	10	17	(50)	2	15	17	(50)
No arrhythmia	0	2	2	(7)	8	18	26	(93)
Total	7	12	19	(31)	10	33	43	(69)

($P = 0.0003$).

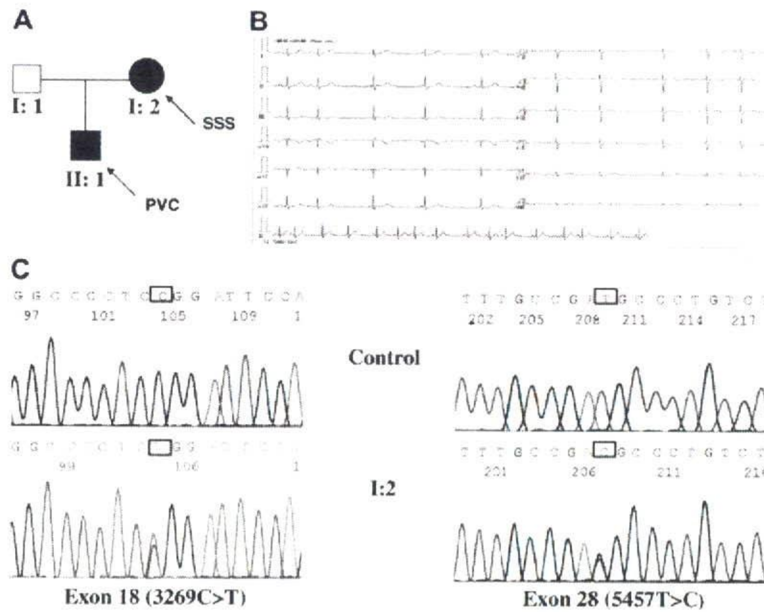

Fig. 2. (A) Pedigree of familial case 6. (B) ECG of the proband's mother who presented with SSS. (C) DNA sequence analysis of exon18 (left) and exon 28 (right) of *SCN5A* in a normal control (top) and in the proband's mother (I:2, bottom).

Table 4
Incidence of *SCN5A* variants in the cases with heart failure and in those without heart failure

	SCN5A Variant			(%)	No *SCN5A* variant			(%)
	Familial cases	Sporadic cases	Total		Familial cases	Sporadic cases	Total	
Heart failure	7	11	18	(53)	5	11	16	(47)
No heart failure	0	1	1	(4)	5	22	27	(96)
Total	7	12	19	(31)	10	33	43	(69)

($P = 0.0002$).

lation. Therefore, the question remains, how do these variants, most of which are synonymous substitutions, lead to abnormal arrhythmogenesis in the population? Regardless of their effect on the amino acid sequence, a significant number of single nucleotide substitutions alter mRNA splicing efficiency or accuracy [5]. Exonic splicing enhancer (ESE) and silencer (ESS) elements are present in most exons. Since ESEs are often distant from the canonical splice junctions, point mutations that inactivate an ESE can result in partial or complete exon skipping, affecting the structure or amounts of the expressed protein product. These splicing alterations may be incomplete, resulting in subtle changes in sodium channel expression that do not manifest changes in function in the normal heart, but in a pathological environment result in changes in cardiac repolarization, which consequently could modify the clinical expression of a latent LVNC pathogenic mutation. Rs1805126:T > C has been reported to be associated with short QTc intervals [18]. In our study, it related with the significantly high risk of the arrhythmia in LVNC patients. However, analysis using splice site prediction software (http://www.fruitfly.org/seq_tools/splice.html) or exonic splice enhancer prediction (http://genes.mit.edu/burgelab/rescue-ese/) does not predict any differences in mRNA splicing due to this SNP (data not shown).

Further, subtle changes in ion channel expression could be exacerbated by the presence of variants in proteins that interact with the ion channel. In most of the patients studied here, disease causing mutations have not been identified. Interestingly, multiple proteins containing PDZ domains have been associated with ion channel localization and function [28]. One such protein, LIM Domain Binding Protein 3 (LDB3), has been implicated in the pathogenesis of LVNC [6]. The p.D626N LDB3 variant was identified in two of the probands studied here (F4 and F17). In the first family, both the proband (F4) and her 1week old maternal twin sister carried the *LDB3* variant and the c.453C > T *SCN5A* variation. Shortly after birth, both were diagnosed with LVNC by echocardiography and have severe PVC. In the second family, the proband (F17) was initially diagnosed with isolated LVNC and WPW syndrome in a routine physical examination when he was 13 years old. He had the same variant in *LDB3*, as well as the p.H558R and c.1141-3 C > A variants in *SCN5A*. Thus it is possible to speculate that the presence of the D626N LDB3 variant, irrespective of whether it is disease-causing per se, could alter sodium channel expression, localization and/or function.

Study limitations

Due to the sensitivity of the PCR-SSCP analysis it is possible that the frequencies of SNPs reported here are lower than actually occur. However, as both patient groups were screened simultaneously without knowledge of phenotype, there would be no bias between groups for the variants identified. However, other variants may not have been detected for technical reasons and, it is possible, that they could have occurred more frequently in the groups of patients without arrhythmias. The mechanistic basis of the effect of these variants is largely unknown. Therefore, additional studies in which events such as intracellular acidosis or Ca overload can be induced may result in novel findings.

Conclusion

The prevalence of *SCN5A* variants is significantly higher in LVNC patients with arrhythmias than those without, supporting that the hypothesis that *SCN5A* variants increase arrhythmia susceptibility in LVNC. Further, the increased susceptibility to arrhythmias in turns leads to a greater risk of heart failure. We speculate that drugs targeted to the restoration of sodium channel function may offer a novel therapeutic option for patients with LVNC.

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