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# SCN1B gene variants in Brugada Syndrome: a study of 145 SCN5A-negative patients

SUBJECT AREAS:  
ARRHYTHMIAS  
GENETICS RESEARCH

Received  
15 May 2014

Accepted  
14 August 2014

Published  
25 September 2014

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Brugada syndrome is characterised by a typical ECG with ST segment elevation in the right precordial leads. Individuals with this condition are susceptible to ventricular arrhythmias and sudden cardiac death. The principal gene responsible for this syndrome is *SCN5A*, which encodes the  $\alpha$ -subunit of the Nav1.5 voltage-gated sodium channel. Mutations involving other genes have been increasingly reported, but their contribution to Brugada syndrome has been poorly investigated. Here we focused on the *SCN1B* gene, which encodes the  $\beta$ 1-subunit of the voltage-gated sodium channel and its soluble  $\beta$ 1b isoform. *SCN1B* mutations have been associated with Brugada syndrome as well as with other cardiac arrhythmias and familial epilepsy. In this study, we have analysed *SCN1B* exons (including the alternatively-spliced exon 3A) and 3'UTR in 145 unrelated *SCN5A*-negative patients from a single centre. We took special care to report all identified variants (including polymorphisms), following the current nomenclature guidelines and considering both isoforms. We found two known and two novel (and likely deleterious) *SCN1B* variants. We also found two novel changes with low evidence of pathogenicity. Our findings contribute more evidence regarding the occurrence of *SCN1B* variants in Brugada syndrome, albeit with a low prevalence, which is in agreement with previous reports.

**B**rugada syndrome (BrS) is an arrhythmogenic cardiopathy characterised by a typical ECG pattern with ST segment elevation in the right precordial leads and a predisposition to malignant events in the absence of structural heart disease<sup>1</sup>. The genetic basis of this condition is heterogeneous, and inheritance autosomal dominant<sup>1,2</sup>. Due to incomplete penetrance and variable expressivity, however, familial associations often are not recognised. The first gene identified in association with BrS was *SCN5A*, which encodes for the  $\alpha$ -subunit of the cardiac voltage-gated sodium channel (Nav1.5)<sup>3</sup>. Loss-of-function mutations in *SCN5A* account for an estimated 15% to 30% of BrS cases<sup>2,4</sup>.

In a minority of patients, mutations have been found in genes encoding subunits of the cardiac calcium or potassium channels or other proteins involved in the regulation of cardiac sodium current or the maintenance of the resting membrane potential<sup>5</sup>.

Crotti and coworkers<sup>6</sup> recently reported on the contribution of 12 known BrS-susceptibility genes in one cohort of patients. *SCN5A* was confirmed as the major contributing gene the other 11 genes together accounted for less than 5% of the cases in the study and also displayed lower penetrance. Consequently, the authors suggest that routine genetic testing in the case of BrS should be limited to *SCN5A*, and should be extended to other genes only in the presence of peculiar features (e.g., to calcium channel subunits in patients presenting with short QT-associated BrS)<sup>6,7</sup>.

However, because studies on the minor BrS susceptibility genes are relatively few, it is unclear when testing is appropriate.

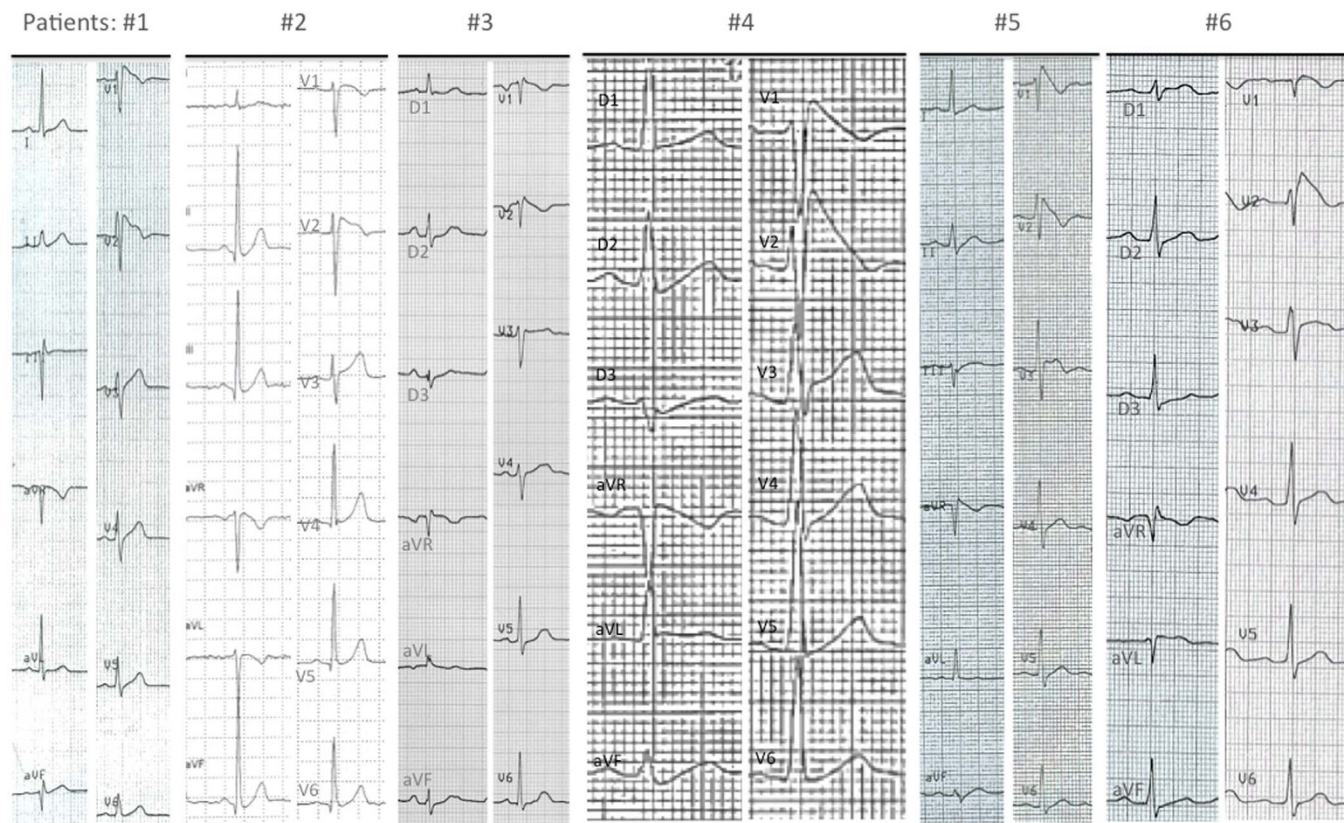
In this study, we focus on the minor BrS susceptibility gene *SCN1B* whose alternatively spliced mRNAs encode the  $\beta$ 1 subunit of the voltage-gated sodium channel (NM\_001037, NP\_001028, isoform A) and its soluble  $\beta$ 1b isoform (NM\_199037, NP\_950238 isoform B)<sup>8</sup>. Both isoforms are expressed in human heart and brain. The transmembrane  $\beta$ 1 subunit is known to modulate the sodium channel through non-covalent interaction with the



**Table 1 | SCN1B variants identified in this study**

| Nomenclature            | Frequencies            |                    | Nucleotide conservation         |                                 |        |        | Prediction of pathogenicity of missense change |               |            |         | Predicted effect on splicing |                |                   |
|-------------------------|------------------------|--------------------|---------------------------------|---------------------------------|--------|--------|--|---------------|------------|---------|------------------------------|----------------|-------------------|
|                         | Variant <sup>1</sup>   | Isom? <sup>2</sup> | ESP (NCBI/GOV/mm/mM/M/WM) [ref] | MAF 1000 Genomes                | PhyloP | SIFT   | Polyphen2 (HumDiv/HumVar)                      | SNAP          | SNPs 3D    | Panther | MultiPred                    | NetGene        | SpliceView        |
| <b>g_3552179G&gt;T</b>  | c.40+15G>T             | A                  | rs72536351;G>T                  | G=232/T=58 G=0.8/<br>T=0.2      | 0.008  | 0.448  |  |               |            |         | Nsc                          | Nc             | Nc                |
| <b>g_3552345C&gt;A</b>  | c.44C>A (p.Ser151Y)    | B                  | #1                              | C=289/A=1 C=0.997/<br>A=0.003   | 0.992  | 2.142  | Dam/Ben<br>Dam/Dam                             | Neu<br>NotNeu | Ben<br>Ben | NotDel  | Nsc                          | Nc             | Nc                |
| <b>g_3552478G&gt;A</b>  | c.448+140G>A           | A                  | #2                              | G=289/A=1 G=0.997/<br>A=0.003   | 0      | -0.44  |  |               |            |         | Nsc                          | Nc             | Nc                |
| <b>g_35524824T&gt;C</b> | c.386G>A (p.Gly196Gly) | A                  | rs5742440;T>C                   | T=162/C=128 T=0.559/<br>C=0.441 | 0      | -0.602 | Ben/Ben  | NotNeu        |            | NotDel  | Nsc                          | Nc             | New acceptor site |
| <b>g_35524836G&gt;A</b> | c.629T>C (p.Leu210Pro) | A                  | #3                              | G=289/A=1 G=0.997/<br>A=0.003   | 0.001  | 0.205  | Ben/Ben  | NotNeu        |            | NotDel  | New acceptor site            | Nc             | New acceptor site |
| <b>g_35524939C&gt;A</b> | c.641G>A (p.Arg214Gln) | A                  | rs67701503;C>A                  | C=227/A=63 C=0.783/<br>A=0.217  | 0.133  | 1.174  | Ben/Ben  | NotNeu        |            | NotDel  | Nsc                          | Nc             | Nc                |
| <b>g_35524944G&gt;C</b> | c.448+130T>C           | A                  | rs67486287;G>C                  | G=228/C=62 G=0.786/<br>C=0.214  | 0.129  | 0.448  | Ben/Ben  | NotNeu        |            | NotDel  | Nsc                          | Nc             | Nc                |
| <b>g_35530073T&gt;C</b> | c.749G>C (p.Arg250Thr) | A                  | rs16969830;T>C                  | T=280/C=10 T=0.966/<br>C=0.034  | 0.014  | 0.205  | Ben/Ben  | NotNeu        |            | NotDel  | New acceptor site            | Nc             | New acceptor site |
| <b>g_35530162C&gt;A</b> | c.590C>A (p.Ala197Asp) | A                  | #4                              | C=289/A=1 C=0.997/<br>A=0.003   | 0      | 1.416  | Dam/Ben  | NotNeu        | Dam        | Del     | Nsc                          | Nc             | Nc                |
| <b>g_35530514T&gt;G</b> | c.59125T>G             | A                  | rs28365107;T>G                  | T=286/G=41 T=0.986/<br>G=0.014  | 0.006  | -0.198 |  |               |            |         | Nsc                          | New donor site | Nc                |
| <b>g_35530525C&gt;A</b> | c.59114C>A             | A                  | rs28365109;C>A                  | C=286/A=4 C=0.986/<br>A=0.014   | 0.006  | 0.044  |  |               |            |         | Nsc                          | Nsc            | Nc                |
| <b>g_35530580G&gt;A</b> | c.632G>A (p.Cys211Tyr) | A                  | #5                              | G=289/A=1 G=0.997/<br>A=0.003   | 0.001  | 0.992  | Dam/Dam  | NotNeu        | Dam        | Del     | New donor site               | nc             | Nc                |
| <b>g_35530641G&gt;A</b> | c.*5+31G>A             | A                  | rs28365108;G>A                  | G=288/A=2 G=0.993/<br>A=0.007   | 0.001  | -0.198 |  |               |            |         | Nsc                          | nc             | Nc                |
| <b>g_35530690C&gt;G</b> | c.*611C>G              | A                  | rs28365105;C>G                  | C=266/G=24 C=0.917/<br>G=0.083  | 0.037  | -0.037 |  |               |            |         | Nsc                          | Nsc            | Nc                |
| <b>g_35530737T&gt;C</b> | c.*42T>C               | A                  | rs2278995;T>C                   | T=226/C=64 T=0.779/<br>C=0.221  | 0.148  | -1.005 |  |               |            |         | Nsc                          | nc             | Nc                |
| <b>g_35530771G&gt;T</b> | c.*735T>C              | A                  | #6                              | G=289/T=1 G=0.997/<br>T=0.003   | n.r.   | 0.016  |  |               |            |         | Nsc                          | nc             | Nc                |
| <b>g_35530781A&gt;C</b> | c.*769G>T              | A                  | rs2278996;A>C                   | A=224/C=66 A=0.772/<br>C=0.228  | 0.146  | -0.198 |  |               |            |         | Nc                           | Nsc            | Nc                |
| <b>g_35530797A&gt;T</b> | c.*102A>T              | A                  | rs72550274;A>T                  | A=288/T=2 A=0.993/<br>T=0.007   | 0.004  | -1.57  |  |               |            |         | Nc                           | Nsc            | Nc                |
| <b>g_35531222T&gt;C</b> | c.*527T>C              | A                  | rs41275828;T>C                  | A=277/C=13 A=0.955/<br>C=0.045  | 0.021  | 0.772  |  |               |            |         | Nc                           | Nc             | Nc                |
| <b>g_35531229C&gt;T</b> | c.*534C>T              | A                  | rs72550266;C>T                  | C=289/T=1 A=0.997/<br>T=0.003   | n.r.   | -0.924 |  |               |            |         | Nc                           | Nc             | Nc                |

<sup>1</sup> Variants are listed according to their genomic position.  
<sup>2</sup> Each variant is described as the expected change on transcript A [NM\_001037, NP\_001028] (above the line) and transcript B [NM\_199037, NP\_950238] (under the line) and, when present, the corresponding isoforms.  
<sup>3</sup> Specific patients are referred to only for putatively pathogenic variants. As discussed in the text, pathogenicity predictions are indicated according to the output of each bioinformatic tool: Ben: Benign, Dam: Damaging, Del: deleterious, Nc: no changes, Neu: neutral, Not del: not deleterious, Not neu: Not neutral, Not tol: Not tolerated, n.r.: not reported, n.s.c.: no substantial changes, Tol: tolerated.  
<sup>4</sup> Data from the NHLBI GO Exome Sequencing Project (ESP) are reported as: observed genotypes i.e., homozygosity for minor (mm) or Major (MM) allele, and heterozygosity (mM). 'tot': number of samples sequenced for each position. 'avr. read depth': the average sample read depth.



**Figure 1** | The diagnostic ECG of the six patients who were carriers of an *SCN1B* variant. Patients #1, #2 and #3 had a spontaneous type 1 pattern, while patients #4, #5 and #6 had type 1 ECG induced during ajmaline test. Cardiac conduction properties in patients with *SCN1B* mutation were always in the normal range. HV intervals and PR and QRS intervals at ECG are reported here. Patients: HV interval (ms); PR (ms); QRS (ms). Patient #1: not available; 160; 80. Patient #2: 37; 160; 80. Patient #3: 43; 160; 80. Patient #4: 48; 160; 80. Patient #5: not available; 180; 80. Patient #6: 45; 200; 90. No patient had pauses observed at Holter monitoring.

$\alpha$  subunit. In addition to cardiac excitability, the  $\beta 1$  subunit has multiple roles involving neural excitability, cell-cell adhesion, cell migration and neurite outgrowth, and pathfinding<sup>9,10</sup>. Less is known about the soluble  $\beta 1b$  peptide encoded by the alternative splice variant retaining intron 3<sup>11</sup>.

*SCN1B* mutations were originally identified in patients with genetic epilepsy with febrile seizures plus spectrum disorders (GEFS+)<sup>9,12–14</sup>. Subsequently, mutations of *SCN1B* were found to be associated with various arrhythmic phenotypes, such as cardiac conduction disease<sup>15</sup>, sudden infant death syndrome (SIDS)<sup>16</sup>, atrial fibrillation<sup>17,18</sup> and long QT syndrome<sup>19</sup>, thus supporting the role of  $\beta 1$  and/or  $\beta 1b$  in normal cardiac electrical activity.

Screening for *SCN1B* mutations has been extended through a variety of studies involving patients from different populations<sup>6,15,16,18,20–22</sup>. The current study aims to determine the prevalence and spectrum of *SCN1B* genetic variants in 145 unrelated *SCN5A*-negative BrS patients recruited at a single centre.

## Results

Table 1 shows the twenty observed single nucleotide substitutions (listed in order of genomic position) and their predicted effects on either of the two transcripts - *SCN1B* and *SCN1Bb* - and the encoded  $\beta 1$  and/or  $\beta 1b$  subunits. Also listed are the incidence of each substitution among the 145 patients and their presence in online database and literature listings, their conservation among species, as well as *in silico* predictions of their pathogenicity. Sixteen of the substitutions have previously been reported as polymorphisms or as rare genetic variants.

Two of the previously undescribed changes and two of the known changes are predicted to affect only one of the polypeptide isoforms

(two the  $\beta 1$ , one the  $\beta 1b$ ) or both isoforms (one change). The other two unreported changes, one synonymous and one in the 3'UTR, show weaker hints of pathogenicity. The main clinical features of the carrier subjects and molecular details of the six changes are reported below and in Figure 1.

A previously unreported C>A transversion in exon 2, which we interpreted as likely to be pathogenic, resulting in the substitution of tyrosine for serine at position 15 of both *SCN1B* isoforms (p.Ser15Tyr-*SCN1B*; p.Ser15Tyr-*SCN1Bb*), was identified in patient 1. The patient is a 52-year-old man with a spontaneous BrS type 1 ECG pattern in the 2nd intercostal space who also had a syncopal episode at 35 years of age. He denied having palpitations, thoracic pain, or seizures. His paternal uncle died suddenly at age 40. Serine 15 is predicted by the SignalP 4.1 algorithm (<http://www.cbs.dtu.dk/services/SignalP/>) to belong to the signal peptide<sup>8,23</sup>. Five of the six bioinformatic tools used in this study predicted that the amino acid substitution would be benign. However, these methods may be of limited use for changes in signal peptides because of the low sequence conservation and lack of three-dimensional structure of these regions. In fact, when considering the role of signal peptides in protein biosynthesis, the substitution of a serine with a bulkier tyrosine is expected to affect the processing of the precursor polypeptide chains and to result in deficiency of both  $\beta 1$  isoforms<sup>23</sup>. Additionally, the absence of this variant in the Exome Sequencing Project (ESP)<sup>24</sup> population, and in 1000 Genomes<sup>25</sup>, supports its pathogenicity.

A synonymous G>A transition in exon 3A that we considered to be of unknown significance (p.Gly196Gly-*SCN1Bb*) was identified in patient 2. He is a 50-year-old man with BrS type 2 ECG at baseline. Spontaneous BrS type 1 ECG was documented via 12-lead 24-hour Holter monitoring. The death of his father at 32 years of age was



ascribed to valvular heart disease. In view of the mounting evidence of the contribution of synonymous variants to human disease<sup>26</sup>, we considered the possibility that the variant may affect splicing, mRNA stability or exerts a different pathogenic effect. These latter scenarios are still possible despite the fact that *in silico* programs did not predict any effect on the splicing function. Notably also this variant was absent in control populations.

A likely pathogenic G>A transition in exon 3A (p.Arg214Gln-*SCN1Bb*) was identified in patient 3, a 58-year-old woman with BrS type 1 ECG at baseline, who experienced a syncopal episode during emesis. This variant has previously been described in patients affected by BrS<sup>6,16,18,22</sup> as well as in cases of SIDS<sup>16</sup>, lone atrial fibrillation<sup>18</sup>, and various neurological disorders<sup>11</sup>. It also has been reported in the dbSNP, ESP (23 out of 6956 alleles) and 1000 Genomes (MAF=0.1%) databases. Further evidence that this variant may indeed be pathogenic, even if observed in the general population, comes from functional studies. Co-expression of mutant p.Arg214Gln-*SCN1Bb* with *SCN5A*/WT or *KCND3*/WT was shown to result in a decrease in the peak of sodium current density and an increase in the transient outward potassium current (I<sub>to</sub>), respectively, compared with the wild-type<sup>16</sup>.

A new rare variant that we considered likely to be pathogenic was found at the exon/intron 4 junction, a C>A transversion resulting in the substitution of aspartate for alanine at position 197 of the *SCN1B* transcript. It was identified in a 35-year-old man who experienced two episodes of neuromediated syncope after having a cough (patient 4). His baseline ECG showed a 2 mm J point elevation in V2 and BrS type 1 pattern was observed during ajmaline challenge. Head-up tilt test showed cardio-inhibitory syncope. Programmed electrical stimulation did not induce ventricular arrhythmias. A loop recorder was implanted, which documented sporadic sinus pauses (maximum duration 3.5 s) without symptoms. The family history was negative for sudden cardiac death and epilepsy. The predicted effect at the protein level involves the transmembrane β1 isoform only (p.Ala197Asp-*SCN1B*). As cytosine 590 is the last nucleotide of exon 4, it cannot be excluded that substitution interferes with the splicing process and results in a disruptive effect on both isoforms.

A likely pathogenic G>A transition in exon 5 (p.Cys211Tyr-*SCN1B*) was identified in a 40-year-old man presenting with a BrS type 3 ECG at baseline (patient 5). His medical history was unremarkable. The patient denied having syncope, palpitations or seizures. A BrS type 1 ECG pattern was induced by ajmaline testing. There was no family history of sudden cardiac death or syncope. This variant involves a highly conserved amino acid and was predicted to be deleterious. It had not only been previously reported in a patient with partial epileptic seizures<sup>27</sup> but also in control individuals from the same population and is present in the dbSNP, ESP (4 out of 13006 alleles) and 1000 Genomes databases (MAF=0.1%). Considering the reduced penetrance in BrS, a possible pathogenetic or modifying role of this rare variant cannot be excluded.

A thymine for guanine substitution of unknown pathogenic significance in the 3' UTR of *SCN1B* isoform A (c.\*76G>T-*SCN1B*) was identified in a 48-year-old Colombian woman (patient 6). The patient complained of arrhythmic palpitations occurring over the last year that persisted for less than one hour. Her baseline ECG showed a BrS type 3 pattern and type 1 ECG was induced in leads V1 and V2 by ajmaline administration. During follow-up, the patient exhibited a spontaneous BrS type 1 ECG pattern during 12-lead 24-hour ECG recording. Ventricular arrhythmias were not induced during the electrophysiological study. Because 3' UTR is a known target for gene regulation by microRNAs, the variant isoform A could in some way alter protein synthesis. It should be noted that the c.\*76G>T variant is not reported in the 1000 Genomes, while the ESP database is not informative in this case because it does not consider the 3' UTR region.

## Discussion

In the last few years, knowledge of causative factors and mechanisms underlying BrS has mainly been focused on the *SCN5A* gene. However, much less is known about the other BrS susceptibility genes and several unsolved questions remain.

In this study we report our findings on the mutation screening of the *SCN1B* gene, encoding an essential subunit of the sodium channel in 145 unrelated *SCN5A*-negative BrS patients from a single centre registry. We found four likely pathogenic *SCN1B* variants, two of which were previously undescribed, including p.Ser15Tyr, which is to our knowledge the first reported signal peptide mutation of *SCN1B*. In addition, two changes with weaker evidence of pathogenicity were considered to be of unknown significance (Table 1).

Variants likely to be causative of BrS were observed with low prevalence in *SCN1B* (2.75%), which is in agreement with previous reports<sup>6,15,16,18,20–22</sup>.

The obviously low detection rate of putative *SCN1B* mutation is not surprising, if one considers the small size of the *SCN1B* gene. However, this may not be the only explanation because the proportion of *SCN1B*/*SCN1Bb* mutations found among BrS patients in the analysed DNA sequence of the gene (4 mutations/2866 bp) is half of that found in the *SCN5A* sequence that we analysed in an equal number of consecutive BrS patients (26/9576 bp - our unpublished data).

At the time of clinical diagnosis, no other case of Brugada ECG pattern was observed among the first-degree relatives of these six families. This restrained us from referring them to genetic counselling and testing. Therefore, it was impossible to perform co-segregation studies to support or weaken the pathogenicity of the identified changes. However, the absence of clinical signs of BrS indicates that *SCN1B* variants, if pathogenic at all, have a low penetrance, in accordance with previous studies<sup>6,15,16,18,22</sup>.

Within the limitations of a single gene analysis in a relatively small number of patients, the spectrum of *SCN1B* variants identified in this and previous studies can be interpreted by assuming that *SCN1B* mutations may have a causative or a modifier role in the pathogenesis of BrS. This and all previous studies have encompassed various missense changes as well as a null mutation and changes in noncoding regions. Pathogenesis may involve a loss of function mechanism, as established in the case of *SCN5A*<sup>28</sup>. This view may prompt functional studies to verify whether both *SCN5A* and *SCN1B* mutations act through a similar pathogenic derangement of the sodium channel, resulting in an increased risk of arrhythmia.

In keeping with its tissue expression, several studies have associated *SCN1B* mutations not only with cardiac arrhythmias<sup>6,15,16,18,20–22</sup> but also with various neurologic phenotypes including epilepsy<sup>9,12–14</sup>. The mechanism underlying the development of either brain and/or heart phenotypes is not known. A variety of genetic and non-genetic factors<sup>29</sup> such as temperature<sup>30</sup>, age, gender, and epigenetics, are mentioned frequently as possible modulators of the clinical phenotypes. To better investigate the variety of affected tissues and manifestations -probably deriving from distinct molecular pathogenic mechanisms- the bioinformatic approach should be extended to consider also the structural role of the affected residues and protein-protein interactions. Furthermore, more advanced functional studies based on cellular and animal models are needed; these should employ the same approaches used to investigate the neurologic *SCN1B* phenotypes<sup>9</sup>. The involvement of *SCN1B* in BrS underlines the relevance of a careful analysis of ECG patterns and cardiological symptoms in patients referred to neurology units. Conversely, neurological symptoms should be considered carefully in patients referred to cardiology units.

From a practical point of view, when a mixed cardiac and neurologic phenotype is found, the patients should undergo *SCN1B* genetic testing. However, no other special clinical signature shows up in our study.



In conclusion, our findings provide further evidence on the prevalence and type of the *SCN1B* variants. We also highlight the need to describe these genetic variants in an unambiguous format, according to the current nomenclature guidelines, as such clarity is necessary at both the clinical and laboratory level, mainly in view of the introduction of Next Generation Sequencing approaches in the molecular diagnosis of BrS.

## Methods

**Study Subjects.** The present study involves a cohort of 145 patients [115 males, mean age 44 years (range 6.1–68.4), 30 females, mean age 47.8 years (range 9.4–75.7)] referred to our Laboratory of Medical Genetics for genetic analysis. Most of the patients belong to the Piedmont Brugada Registry<sup>31</sup> established in 2001.

Every patient exhibited type 1 Brugada ECG pattern<sup>32</sup> either spontaneous (43%) or induced by sodium channel blockers (2/3 ajmaline, the remaining flecainide). Routine examinations, including echocardiography, ruled out any underlying structural heart disease. *SCN5A* testing by direct sequencing and MLPA (Multiplex Ligation-dependent Probe Amplification) resulted negative in all participants.

Clinical information and blood samples were collected for diagnostic testing in patients with spontaneous or drug-induced type 1 Brugada ECG. Part of the remaining DNA sample extracted from blood was conserved for second level analysis (i.e., screening of other candidate genes), depending on the specific written consent obtained in occasion of the pre-test genetic counselling session.

**Ethics Statement.** Written informed consent was obtained from each participant. Approval was obtained from the institutional review boards at the University of Torino Medical School. The study was carried out in compliance with the Helsinki Declaration. The experimental protocol was carried out in accordance with the approved guidelines.

***SCN1B* mutation screening.** The entire *SCN1B* coding sequence, including the alternatively spliced exon 3A and the 3'UTR, was amplified using primers designed on the flanking intronic or exonic sequences. Primers and PCR conditions are available on request. Sanger sequencing was performed by an external commercial service using standard protocols. Putative pathogenic variants were validated via bidirectional re-sequencing of an independent PCR amplification.

As approval of the *SCN1B* sequence in the Locus Reference Genomic<sup>33</sup> website is still pending, variants were numbered according to the NM\_0011037, NP\_001028 (*SCN1B* isoform A) and NM\_199037, NP\_950238 (*SCN1B* isoform B) RefSeqs from the Genome Reference Consortium Human Build 37.3 (NC\_000019.9) and described according to the Human Genome Variation Society guidelines<sup>34</sup>.

**Variants characterization and bioinformatics.** Each variant was classified as neutral polymorphism, putatively pathogenic, or of unknown pathogenicity. This classification was achieved by considering the following: (1) the reported frequency in the following databases: dbSNP (build 131; <http://www.ncbi.nlm.nih.gov/projects/SNP/>), HGMD® (<http://www.biobase-international.com/product/hgmd/>), 1000 Genomes (<http://www.1000genomes.org/>), NHLBI GO ESP (<http://evs.gs.washington.edu/EVS/>) [date (06, 2014) accessed]; (2) literature reports of presence in patients or healthy subjects; (3) evolutionary conservation of the involved residue at both nucleotide and amino acid level, calculated by multiple alignments of 46 vertebrate species and (4) *in silico* prediction of functional effect by SIFT (<http://sift.jcvi.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SNAP (<http://roslab.org/services/snap/>), SNPs3D (<http://www.snps3d.org/>), Panther (<http://www.pantherdb.org/>), MutPred (<http://mutpred.mutdb.org/>), NetGene (<http://www.cbs.dtu.dk/services/NetGene2/>), Splice View ([http://zeus2.itb.cnr.it/~webgene/wwwspliceview\\_ex.html](http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html)) and NNSplice ([http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)).

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## Acknowledgments

This work was subsidised in part by the CR Asti Foundation, PRIN Grant (2010BWy8E9\_006) of the Italian MIUR, Ricerca Finalizzata Regione Piemonte and “Piccoli Cuori Joy” Foundation.

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Study design: M.T.R., D.G., M.D.M., C.G. and F.G. Samples collection: G.M., M.T.R., N.C., P.C. and C.G. Gene analysis: M.T.R., S.M. and S.V. Data interpretation and analysis: M.T.R.,



D.G., M.D.M., S.M. and S.V. Manuscript preparation: M.T.R., D.G., M.D.M., C.G. All authors have read and approved the final manuscript.

### Additional information

Written informed consent was obtained from each participant. Approval was obtained from the institutional review boards at the University of Torino Medical School. The study was carried out in compliance with the Helsinki Declaration. The experimental protocol was carried out in accordance with the approved guidelines.

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Ricci, M.T. *et al.* *SCN1B* gene variants in Brugada Syndrome: a study of 145 *SCN5A*-negative patients. *Sci. Rep.* 4, 6470; DOI:10.1038/srep06470 (2014).



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