Functionally-Validated *SCN5A* Variants Allow Interpretation of Pathogenicity and Prediction of Lethal Events in Brugada Syndrome

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

ABSTRACT

Aims

The prognostic value of genetic variants for predicting lethal arrhythmic events (LAEs) in Brugada syndrome (BrS) remains controversial. We investigated whether the functional curation of *SCN5A* variations improves the prognostic predictability.

Methods and results

Using a heterologous expression system and whole-cell patch clamp, we functionally characterised 22 variants of unknown significance (VUS) among 55 *SCN5A* mutations previously curated using *in silico* prediction algorithms in Japanese BrS registry (n=415). According to the loss-of-function (LOF) properties, *SCN5A* mutation carriers (n=60) were divided into two groups: LOF-*SCN5A* mutations and non-LOF *SCN5A* variations. Functionally proven LOF-*SCN5A* mutation carriers (n=45) showed significantly severer ECG conduction abnormalities and worse prognosis associated with earlier manifestations of LAEs (7.9%/year) than *in silico* algorithm-predicted *SCN5A* carriers (5.1%/year) or all BrS probands (2.5%/year). Notably, non-LOF *SCN5A* variation carriers (n=15) exhibited no LAEs during the follow-up period. Multivariate analysis demonstrated that only LOF-*SCN5A* mutations and a history of aborted cardiac arrest were significant predictors of LAE. Gene-based association studies using whole-exome sequencing data on another independent *SCN5A* mutation-negative BrS cohort (n=288) showed no significant

enrichment of rare variants in 16,985 genes including 22 non-*SCN5A* BrS-associated genes as compared with control (n=372). Furthermore, rare variations of non-*SCN5A* BrS-associated genes did not affect the LAE-free survival curves.

Conclusion

In vitro functional validation is key to classifying the pathogenicity of *SCN5A* VUSs and for risk stratification of genetic predictors of LAE. Functionally proven LOF-*SCN5A* mutations are genetic burdens of the sudden death in BrS, but the evidence for other BrS-associated genes is elusive.

Keywords: Brugada syndrome; *SCN5A* mutations; lethal arrhythmia; variants of unknown significance; whole-exome sequencing; patch-clamp

Translational Perspectives

SCN5A mutations are associated with the risk of lethal arrhythmia in Brugada syndrome (BrS), however, nearly 70% of BrS-associated *SCN5A* rare variations registered in ClinVar are classified as variants of unknown significance, requiring curation strategies to accurately differentiate pathogenic and benign variations to predict patient prognosis. As a monogenic trait, functionally validated loss-of-function *SCN5A* mutations, but not rare coding variants of other BrS-associated genes, are genetic risks of lethal arrhythmias in

BrS. Although the contribution of polygenetic factors in BrS warrants further investigations, these results may help to develop a new personalised risk stratification paradigm for sudden cardiac death.

INTRODUCTION

Brugada syndrome (BrS) is a rare heritable arrhythmia characterised by the coved-type ST segment elevation in the right precordial leads and an increased risk of sudden cardiac death due to lethal ventricular arrhythmia.[1] Mutations in *SCN5A*, encoding cardiac sodium channel (Nav1.5), are identified in approximately 20% of cases; however, the predictive value of *SCN5A* mutations for subsequent lethal arrhythmic events (LAEs) remains controversial. Specifically, *SCN5A* mutations were not associated with LAE in European BrS cohorts,[2, 3] whereas our Japanese multicentre BrS cohort study has previously demonstrated that 60 probands carrying 55 different *SCN5A* mutations exhibited their first LAE at younger ages (34 versus 42 years) than probands without *SCN5A* mutations (n=355).[4] Observations consistent with those of the latter study were reported in an Italian BrS cohort.[5] The selection bias of patients[4] or demonstrated transethnic differences in the phenotypic severity of BrS, and the frequency of *SCN5A* variations[6] may underlie the discrepancy of the aforementioned studies. Nevertheless, and more importantly, *SCN5A* mutations often exhibit incomplete penetrance in BrS, and this gene is associated with a relatively high background rate of rare missense variants in the general population (2%–5%). Moreover, efforts to enhance the classification of *SCN5A* missense variants using protein topology-driven estimated predictive assessments or *in silico* prediction algorithms are limited compared with those for other major cardiac channelopathy genes, such as

KCNQ1/KCNH2.[7] Therefore, risk assessment of *SCN5A* variants using phenotypic data of a single variant carrier remains challenging, and an inaccurate classification of rare variants might have obscured the prognostic value of *SCN5A*.

The American Collage of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG-AMP) guidelines provide approaches for a more appropriate classification of pathogenic variants, and "functional studies supporting a deleterious effect (PS3)" are assigned one of four criteria with "strong evidence" of pathogenicity.[8] Loss-of-function (LOF) of cardiac Na current (I_{Na}) due to *SCN5A* mutations is the primary pathophysiology underpinning BrS. A previous functional study has shown that patients carrying null *SCN5A* mutations are associated with more frequent episodes of syncope and more severe conduction abnormalities than those with other types of *SCN5A* mutations.[9] More recently, Glazer *et al*. have shown that patch clamp enables reclassification of variants of unknown significance (VUSs) of *SCN5A*.[10] These studies suggest that the *in vitro* functional re-evaluation of *SCN5A* variations may improve LAE predictability in BrS. In the present study, we have conducted a PubMed search and re-evaluated the function of 55 *SCN5A* rare variants previously curated via multiple *in silico* prediction algorithms in a Japanese BrS cohort[4] and determined whether the functionally proven *SCN5A* mutations may improve the predictive value for LAE in patients with BrS.

Increasing evidence suggests that BrS is unlikely to be a Mendelian monogenic disease but rather an oligogenic disorder involving multiple rare and nonrare variants, as well as structural abnormalities and inflammation, contributing to the underlying basis of disease.[11] A previous international genome-wide association study had identified three common independent susceptibility variants close to *SCN5A*, *SCN10A*, and *HEY2*,[12] and to date, more than 20 genes have been reported to be associated with BrS.[13] Although no significant enrichment of these rare coding variants, except for *SCN5A*, were observed in BrS cases[14] and the ClinGen consortium recently reported *SCN5A* as the only causative gene with definitive evidence for the diagnosis of BrS,[13] the predictive value of non-*SCN5A* coding variants for the long-term prognosis of BrS–i.e. LAE and sudden cardiac death–has never been evaluated. In this study, we performed whole-exome sequencing on a distinct *SCN5A*-negative BrS cohort to identify novel pathological rare variants and assessed if non-*SCN5A* rare coding variants contribute to the genetic burden of sudden death in BrS.

METHODS

Patients and study cohorts

The diagnosis of BrS was made according to the criteria of a consensus report,[15] and LAE was defined as sudden cardiac death, cardiac arrest, ventricular tachycardia (VT)/ventricular

fibrillation (VF), or appropriate discharge of implantable cardioverter defibrillator (ICD). Clinical characteristics including time to first LAE and electrocardiographic (ECG) parameters were obtained as previously described.[4] This study was approved by the institutional review board (National Cerebral and Cardiovascular Center, R19048) and local ethics committee of each institution. All participants in the cohorts provided written informed consent before clinical and genetic investigations in accordance with the Declaration of Helsinki.

This study consisted of two independent Japanese multicentre BrS cohorts, specifically BrS cohort-I (415 probands)[4] and BrS cohort-II (288 unrelated probands), and 372 ethnic-matched controls (Figure 1, Table 1). In BrS cohort-I, 415 BrS probands were assigned as *SCN5A*-mutation carriers (*SCN5A*(+); n=60) and *SCN5A*-mutation negative probands (*SCN5A*(-); n=355) based on Sanger sequencing as previously described.[4] We enrolled independent Japanese BrS probands (cohort-II), whose negative *SCN5A* genotype statuses were determined in advance by Sanger sequencing. BrS cohort-II and the control Japanese subjects were subjected to whole-exome sequencing and gene-wise association test. *In silico* prediction of *SCN5A* variants was performed using seven algorithms as previously described.[4,7] Further information is provided in the Supplemental Methods.

[Figure 1, Table 1]

Assignment and functional evaluation of 22 variants of unknown significance

We performed public database screening and PubMed literature search for 55 variations reported in BrS cohort-I and identified 22 VUSs. To functionally evaluate the Na channel properties of 22 VUSs, we constructed human *SCN5A* expression plasmids of VUSs using site-directed mutagenesis, and I_{Na} of HEK293T cells were recorded using the whole-cell patch-clamp technique using a heterologous expression system. After analysing the biophysical properties of 22 VUS channels, the 55 variants were categorised into two groups, LOF and non-LOF, according to the presence or absence of significantly reduced peak I_{Na} density than wild-type (WT) *SCN5A*, respectively. Detailed information is provided in the Supplemental Methods.

Statistical analyses

Quantitative variables are shown as the mean±standard deviation (SD) unless otherwise stated. Statistical significance was set at $P<0.05$. For the statistical analysis of continuous variables with a normal distribution, one-way analysis of variance followed by Bonferroni's post-hoc comparison tests were used. The cumulative probability of an index LAE over the course of patient follow-up or their entire lifetime was determined using Kaplan–Meier methods for each subgroup, and the difference in survival rates was analysed using a log-

rank test. Univariate analysis using a Cox proportional-hazards model was performed to determine variables that improve the prediction of LAE. Independent variables with $P<0.05$ in the univariate analyses were included in the multivariate analysis. Statistical analyses were performed using the R programme (ver 4.0.2) and SPSS statistical package (ver 26).

RESULTS

Functional classification of BrS-associated 55 *SCN5A* variations

A PubMed search had identified 21 publications that described the biophysical properties of 22 *SCN5A* variations (17 missense, one in-frame deletion, and four nonsense variations) (Figure 1, Supplemental Table S1). Moreover, among 55 variations, 11 were novel null variants (two nonsense, six frame-shift, three canonical splice site) classified as PVS1 (very strong evidence of pathogenicity) according to the ACMG-AMP guidelines.[8] Accordingly, the remaining 22 missense variations were assigned as VUS (Table 1). The functional properties of each VUS were analysed using whole-cell patch-clamp assays (Supplemental Figure S1) and categorised into two groups according to the degree of peak I_{Na} reduction: LOF (significantly reduced I_{Na} density compared with WT; n=13), and Non-LOF (no significant difference compared with WT; n=9), and the border zone of LOF and Non-LOF was 53.2%–65.6% (Supplemental Table S2). Since the experimental conditions of current study and previous patch-clamp studies were largely similar (Supplemental Table S3), both data

were combined with null PVS1 mutations (n=11) to classify a total of 55 variants according to their biophysical properties as follows: LOF (n=40) and Non-LOF (n=15) (Figure 2, Supplemental Table S4). The locations of the 55 variants, illustrated based on Nav1.5 protein topology, exhibited diffuse distribution within the entire protein.

[Figure 2]

Correlation between functional severity of *SCN5A* variations and ECG parameters

Among different ECG parameters, cardiac conduction properties (P, QRS, S durations and PQ interval) were significantly prolonged in the LOF compared to the Non-LOF or *SCN5A*(-) (Supplemental Figure S2). However, no significant differences were observed in these parameters between Non-LOF and *SCN5A*(-), suggesting that the conduction parameters reflect the severity of sodium channel dysfunction associated with *SCN5A* variations (Supplemental Table S5). Alternatively, other electrophysiological and clinical findings were largely comparable among the three groups (Supplemental Figure S3).

Lethal arrhythmic events associated with the severity of sodium channel dysfunction

A total of 62 probands (15%) developed LAEs during the mean follow-up period of 72 months. Notably, none of the Non-LOF probands developed LAEs during follow-up. (Figure

3) Furthermore, LOF exhibited significantly more frequent total lifetime events and ICD implantation than Non-LOF. Most LAEs (n=56, 90%) were terminated by appropriate ICD discharges, and LAE-free rates by Kaplan–Meier analysis was comparable regardless of the ICD discharge. These data suggest that an appropriate ICD discharge serves as a surrogate for sudden cardiac death in BrS (Supplemental Figure S4); therefore, it was hypothesized that the prognosis of BrS patients can be discriminated based on LOF properties of the *SCN5A* variants

[Figure 3]

Based on this assumption, we calculated the cumulative rate of an index LAE during the mean follow-up period of 72 months (range, 1–249 months) using the Kaplan–Meier method for several subgroups with different statuses with respect to Na channel properties (Figure 4). Patients carrying *SCN5A* rare variants (*SCN5A*(+); n=60) had a significantly higher annual LAE rate than *SCN5A*(-) (n=355; 5.1%/year versus 2.2%/year; P=0.017, Figure 4A, Table 2), as previously reported.[4] The estimated mean LAE-free periods (mean±standard error (SE)) for patients of *SCN5A*(+) and *SCN5A*(-) were 136.6±12.9 months and 210.8±6.0 months, respectively. As shown in Figure 4B, none of Non-LOF subgroup developed LAEs during the follow-up period, whereas the LOF subgroup had a significantly higher LAE rate

(7.9%/year, P=0.019; Figure 4B, Table 2, Supplemental Table S6) and a shorter LAE-free period (94.5±10.7 months). By combining Non-LOF and *SCN5A*(-) results, we re-evaluated the survival curves of patients with or without LOF-*SCN5A* mutations (Figure 4C) and found that the LOF subgroup exhibited a significantly higher annual LAE rate, and shorter estimated mean LAE-free period than the Non-LOF plus *SCN5A*(-) (2.1%/year, 208.8±5.9 months, n=370; P=0.0001, Figure 4C, Table 2). Qualitatively similar results were obtained from Kaplan-Meier analysis with shorter follow-up period (<107 months) (Supplemental Figure S5)

[Figure 4; Table 2]

Reclassification of *SCN5A* variations and the predictability of LAEs

Univariate analysis using a Cox proportional hazard model showed that the positive status of both functionally-validated LOF-*SCN5A* mutations and *in silico* algorithm-predicted rare *SCN5A* variations are significant predictors of LAE, but the former exhibited a higher hazard ratio than the latter (Table 3, Supplemental Table S6).[4] Multivariate analyses were then performed using independent variables with P<0.05 in Table 3A (with two different *SCN5A* statuses) . A history of aborted cardiac arrest was the strongest predictor of LAE regardless of the *SCN5A* status. Moreover, *SCN5A* variant status was a significant predictor of LAE, and

the predictive values of functionally-validated LOF-*SCN5A* mutations was higher than that of *in silico*-predicted rare *SCN5A* variations demonstrated previously.[4] In contrast, prolonged QRS, or documented atrial fibrillation were not predictors of LAE in BrS.

[Table 3]

Genome-wide screening and risk stratification of BrS-associated genes other than *SCN5A*

To determine which genes besides *SCN5A* carry burden of rare genetic variations in BrS cases versus controls, we performed whole-exome sequencing on a distinct Japanese cohort of *SCN5A*(-) BrS (BrS cohort-II, n=288) and controls (n=372). Then we performed genome-wide gene-wise association tests using rare variations using two different cut-off values of minor-allele frequency (<1% and <0.3%), however, we failed to identify novel genes significantly enriched with rare coding variations among the entire set of genes in BrS cohort-II (Supplemental Figure S6) or previously recognised 22 non-*SCN5A* BrS-associated genes.[13] (Supplement Tables S7, S8) We assessed whether rare coding variants of 22 BrS-associated genes with limited evidence modify the prognosis of BrS; lifetime cumulative LAE-free rates were calculated by the Kaplan–Meier method. However, Log-rank tests showed that these rare variants did not affect the age of initial LAE in the BrS cohort-II (Figure 5). Even when

focusing on genes that are known to modulate cardiac Na channel function, rare variants of these genes were not enriched in cases nor affected the prognosis of BrS cohort-II (Supplemental Figure S7, Supplemental Table S8). Thus, we find no evidence supporting an association between the BrS-associated non-*SCN5A* genes and sudden arrhythmic death.

[Figure 5]

DISCUSSION

In this study, we aimed to dissect the genetic basis of BrS by conducting electrophysiological evaluations of *SCN5A* variations and have demonstrated that functionally-validated LOF-*SCN5A* mutations, not rare coding variations of other BrS-related genes, are associated with genetic risks of lethal arrhythmia in BrS. In addition to a history of aborted cardiac arrest being the strongest, and most well-established predictor of future LAEs in patients with BrS, we have demonstrated, to the best of our knowledge, for the first time that LOF-*SCN5A* mutations are an independent and significant predictor of sudden death.

Advances in genetic sequencing have increased the potential yield of genetic testing, while raising the clinical dilemma of the discovery of many VUSs compromising the accuracy of variant interpretation. The pathogenicity of *SCN5A* variants in BrS has often

been unknown or disputed; 67.5% of the total 1,140 BrS-associated *SCN5A* variations submitted to ClinVar are classified as either of uncertain significance, no assertion provided, or conflicting interpretations.[16] These VUSs are often specific to a particular family, and their penetrance and expressivity are highly variable in BrS,[1] hampering the annotation of their pathogenicity through segregation analysis. In the ACMG-AMP guidelines, the evidence level of pathogenicity for "absent in population databases" is assigned as moderate (PM2), while that of "*in silico* prediction algorithms" is assigned as supporting (PP3). Specificity of *in silico* algorithms to predict the pathogenicity of missense variants is generally low despite their high sensitivity,[8] resulting in the overprediction of missense variations as deleterious. Recent studies, using purely *in silico* analyses, including systematic evaluation using the ACMG-AMP guidelines, failed to predict the disease risk of *SCN5A* variants in BrS.[17, 18] These results support the observation of our study that 27% of the *SCN5A* missense VUSs (15/55) were overpredicted *in silico*, therefore implicating the need for additional reliable tools to improve the annotation of pathogenicity for large numbers of *SCN5A* VUSs. In this study, we propose that the functional evaluation of *SCN5A* VUSs using a patch-clamp study might be an efficient strategy to aid the differentiation of malignant variants associated with predisposition to sudden death from those that are innocuous (Graphic abstract).

Among the 55 functionally reclassified *SCN5A* variants, including 22 VUSs, most (40 variants, 73%, LOF) showed a significant reduction in the peak I_{Na} than WT-*SCN5A*, which was associated with more severe abnormalities in ECG conduction parameters (Supplemental Figure S2), and an earlier manifestation of LAEs (Figure 4). Note that our *in vitro* functional classification of *SCN5A* variants according to the significant I_{Na} density reduction (LOF vs Non-LOF) successfully dissected the cumulative risk of LAEs in the 60 carriers during the follow-up period (Figure 4B). The close relationship between the degree of *SCN5A* Na channel dysfunction and the phenotypic severity has been previously reported; *SCN5A* truncation mutation carriers were found to have more syncopal episodes and prolonged cardiac conduction abnormalities than missense mutation carriers.[9] Another Italian study of 92 BrS patients identified four *SCN5A* mutations (R104Q, L276Q, E1225K, and A1428S) in 12 patients with LAE during follow-up,[5] and our dataset included BrS probands carrying the identical LOF mutations (Supplemental Table S4). These observations further support the notion that LOF-*SCN5A* mutations are phenotypically malignant and associated with LAE, while the reduction in peak I_{Na} density serves as the principal predictor of BrS disease risk. Further functional evaluations and larger scale clinical studies involving more *SCN5A*-positive cases are warranted to prove this hypothesis.

Although more than 20 non-*SCN5A* associated genes have been recognised in BrS, precise interpretation of the pathogenicity of rare variations of these genes is often challenging. Using rare variant burden analysis of BrS-associated genes, Le Scouarnec *et al*. identified a significant enrichment of *SCN5A* coding variants only in BrS cases than controls, but not those of other BrS-associated genes.[14] Using an evidence-based ClinGen approach, Hosseini *et al*. concluded that *SCN5A* is the only gene classified with definitive evidence of disease causality in BrS.[13] Herein, we used whole-exome sequencing in a larger cohort of BrS patients lacking *SCN5A* mutations and demonstrated that the rare coding variations of non-*SCN5A* BrS-associated genes were neither enriched in BrS (Supplemental Figure S6), nor modified the long-term prognosis of BrS patients (Figure 5). Our data further support the notion that LOF-*SCN5A* mutations, but not rare coding variants of other BrS-susceptible genes, are the genetic burden of LAE in BrS.

The absence of LOF-*SCN5A* mutations in a given patient with BrS does not necessarily suggest a benign prognosis since the disease presentation is affected by several factors, including age, sex, common single nucleotide polymorphisms (SNPs) near *SCN5A/SCN10A/HEY2* genes,[12] and structural abnormalities including fibrosis and inflammation.[11] Considering that most (~80%) patients with BrS are mutation-negative, it is speculated that the genetic risk of sudden death is also determined by both monogenic factors (rare LOF-*SCN5A* mutations) and polygenic factors (unidentified common

variants) (Graphic abstract). Although SNPs associated with sudden death or lethal arrhythmia have not been elucidated in BrS, it is possible that the polygenetic contribution of BrS-associated common SNPs in *SCN5A*-negative BrS may be equivalent to or even greater than in *SCN5A*-positive BrS.[19]

Study limitations

Patients in this study were exclusively of Japanese descent, and limited in number; therefore, our study should be replicated using larger cohorts of different ethnicities. Electrophysiological properties of the variants were analysed based on heterologous expression; however, some *SCN5A* variants might exhibit different properties in HEK293T cells as compared with those in cardiomyocytes or *in vivo*.[20]

CONCLUSIONS

In vitro functional validation is a key method to classify the pathogenicity of *SCN5A* VUSs. Functionally-validated LOF-*SCN5A* mutations contribute to the genetic burden of sudden death in BrS. Integrating the genetic information of LOF-*SCN5A* mutations with other rare or polygenic common risk variations, which are currently unknown, may help to develop a new personalised risk stratification paradigm for the complex oligogenic disease, BrS.

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FIGURE LEGENDS

Figure 1. **Flowchart of this study**

Patients of BrS cohort-I were assigned to groups of loss-of-function (LOF) *SCN5A* mutation carriers (N=45), non-LOF *SCN5A* variation carriers (N=15), and *SCN5A*-mutation negative patients (*SCN5A*(-), N=355) by *in silico* curation, PubMed search and functional evaluation using patch clamp. Numbers of unique variations (n) and patients (Pt) are shown where a duplication was identified. BrS cohort-II consists of independent BrS probands carrying no *SCN5A* rare variations.

Figure 2. **Functional classification of 55 *SCN5A* rare variations of Japanese BrS cohort-I**

A. Whole-cell currents of rare *SCN5A* variants and wild-type (WT) Nav1.5 channel were recorded (inset and Supplemental figure S1) from HEK293T cells, and the percentage peak current densities versus WT were plotted. Variants were classified into loss-of-function (LOF; significantly reduced peak current density than WT; n=40) or Non-LOF (not significantly different from WT; n=15). Check marks indicate variants of unknown significance (VUSs) for which patch-clamp was performed in this study (n=22), and asterisks represent variations of previous literatures whose precise current density data are unavailable (n=10).

B. Location of 55 *SCN5A* variants of LOF and Non-LOF are shown with topological representation of Nav1.5.

Figure 3. **Association of clinical events in BrS patients with distinct Na channel function properties**

A. All events and **B**. lethal arrhythmic events (LAE) in lifetime; **C.** LAE and **D**. syncope during follow-up; **E**. ICD discharge and **F**. ICD implantation were compared among BrS patients with LOF (n=45), Non-LOF (n=15), and *SCN5A*(-) (n=355). Statistical analysis was performed using Fisher's exact test with Bonferroni adjustment.

Figure 4. **Kaplan–Meier analysis of LAE-free survival during follow-up in BrS cohort-I**

A. LAE-free survival during the follow-up period in BrS probands carrying *SCN5A* rare variations (All *SCN5A*; n=60) and *SCN5A*(-) (n=355). Confidence bands indicate 95% pointwise CI. **B.** Time course of BrS patients with LOF-*SCN5A* mutations (LOF, n=45), and Non-LOF (n=15). Non-LOF probands have no LAEs during the follow-up period. **C**. LAE-free survival of LOF (n=45) vs Non-LOF plus *SCN5A*(-) (n=370). The dissociation between two survival curves is more pronounced than that in panel A.

Figure 5. Kaplan–Meier analysis of lifetime LAE-free survival in BrS cohort-II with or without rare variants of BrS-associated genes

LAE-free survival of BrS cohort-II probands were comparable regardless of the presence of rare coding variants of 22 non-*SCN5A* BrS-associated genes with two different minor allele frequencies (MAF) (A:<1%, B:<0.3%).