Research paper

**GSTM3 variant is a novel genetic modifier in Brugada syndrome, a disease with risk of sudden cardiac death**

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**ABSTRACT**

**Background:** Brugada syndrome (BrS) is a rare inherited disease causing sudden cardiac death (SCD). Copy number variants (CNVs) can contribute to disease susceptibility, but their role in Brugada syndrome (BrS) is unknown. We aimed to identify a CNV associated with BrS and elucidate its clinical implications.

**Methods:** We enrolled 335 unrelated BrS patients from 2000 to 2018 in the Taiwanese population. Microarray and exome sequencing were used for discovery phase whereas Sanger sequencing was used for the validation phase. HEK cells and zebrafish were used to characterize the function of the CNV variant.

**Findings:** A copy number deletion of **GSTM3** (chr1:109737011-109737301, hg38) containing the eighth exon and the transcription stop codon was observed in 23.9% of BrS patients versus 0.8% of 15,829 controls in Taiwan Biobank (**P** < 0.001), and 0% in gnomAD. Co-segregation analysis showed that the co-segregation rate was 20%. Patch clamp experiments showed that in an oxidative stress environment, **GSTM3** down-regulation leads to a significant decrease of cardiac sodium channel current amplitude. Ventricular arrhythmia incidence was significantly greater in **gstm3** knockout zebrafish at baseline and after flecainide, but was reduced after quinidine, consistent with clinical observations. BrS patients carrying the **GSTM3** deletion had higher rates of sudden cardiac arrest and syncope compared to those without (OR: 3.18 (1.77/5.74), **P** < 0.001; OR: 1.76 (1.02/3.05), **P** = 0.04, respectively).

**Interpretation:** This **GSTM3** deletion is frequently observed in BrS patients and is associated with reduced INa, pointing to this as a novel potential genetic modifier/risk predictor for the development of the electrocardiographic and arrhythmic manifestations of BrS.

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1. Introduction

Brugada Syndrome (BrS) is a cardiac channelopathy associated with an increased risk of sudden death. The prevalence of BrS is estimated to be 1–5 per 10,000 people in Caucasians but is higher in...
Research in Context section

Evidence before this study

Unexpected sudden cardiac death in the young (SCDY) always make people, especially parents, grieve over their lost tremendously. Brugada Syndrome (BrS) is one of SCDY, an inheritable cardiac channelopathy associated with an increased risk of young sudden death or syncpe, and a distinct electrocardiogram (ECG) pattern consisting of a right bundle-branch block with ST segment elevation in V1, and V2 in the absence of any structural heart disease. The average age at diagnosis is 40 ± 22 years, and it is male-predominant. The prevalence of BrS is estimated to be 1–5 per 10,000 people in Caucasians but is higher in Southeast Asians (12 per 10,000) [1,2]. The average age at diagnosis is 22 years, and it is male-predominant [3].

BrS-causing gene responsible for approximately 20% of BrS cases in Caucasians and 7.5–8% of cases in Han Chinese. Although several susceptibility genes have been identified, a genetic cause remain unknown in approximately 80% of BrS patients. Copy number variants (CNVs) can contribute to disease susceptibility, but their role in Brugada syndrome (BrS) is unknown.

Added value of this study

In this study, we enrolled 335 unrelated BrS patients from 2000 to 2018 in the Taiwanese population using a 2-stage approach with extreme phenotype sampling strategy. We performed microarray and exome sequencing for discovery phase whereas Sanger sequencing were used for validation phase. We performed patch clamp study using HEK293 cells and gstm3 knockout zebrafish experiments to characterize the CNV function. We identified a diallelic deletion of GSTM3 contains the eighth exon and the transcription stop codon, and functional studies showed that this GSTM3 deletion is associated with reduced cardiac sodium channel current. In this Taiwanese BrS patient cohort, the frequency of a copy number deletion of GSTM3 was observed in 23.9% of 301 BrS patients without SCN5A mutations versus 0.8% of 15,829 ancestry-matched healthy controls in Taiwan. Intriguingly, the GSTM3 deletion is not reported in the large dataset based on whole-genome sequences (>10,000 individuals), suggesting that it is closely associated with BrS. We also found that BrS patients carrying the GSTM3 deletion had higher rates of sudden cardiac arrest and syncope compared to those without.

Implications of all the available evidence

We propose that our finding have both diagnostic and risk stratification clinical impacts for patients with BrS. Our study identified a deletion of GSTM3 in BrS patients, which is associated with reduced I Na, suggesting that the deletion could be a genetic modifier of the BrS phenotype. This study drives the understanding of this disease forward. This variant may be a novel genetic modifier/risk predictor for the development of the electrocardiographic and arrhythmic manifestations of BrS. It could be used as a risk predictor in patients with BrS for clinical practice. The gene could also be a potentially future therapeutic target and clinical genetic testing for patient care.

Southeast Asians (12 per 10,000) [1,2]. The average age at diagnosis is 40 ± 22 years, and it is male-predominant [3].
SCN5A, which encodes the cardiac sodium channel, is the major BrS-causing gene responsible for approximately 20% of BrS cases in Caucasians and 7.5–8% of cases in Han Chinese [4,5]. Although several susceptibility genes have been identified [1,6,7], a genetic cause remains unknown in approximately 80% of BrS patients. One primary reason for this lack of causal certainty is the fact that a large proportion of patients is likely to represent non-Mendelian cases with oligogenic inheritance [8]; phenotyping errors, inadequate sensitivity of screening methods, and mutations in non-coding regions or in unknown genes are additional sources of causal uncertainty. Another possibility may be the presence of copy number variations (CNVs) in genes affecting the onset of BrS, which are known to play a role in cardiovascular diseases [9,10]. However, there is limited evidence regarding whether CNVs are important in BrS.

Here, we conducted a genome-wide CNV study in BrS patients without SCN5A mutations using a multi-stage study design with extreme phenotype sampling strategy [11,12]. We initially used genome-wide microarray to screen CNV regions in a case-control design, then used whole exome sequencing (WES) to fine-tune the length of candidate CNV regions in BrS patients because the candidate CNV regions were too long for Sanger sequencing technology. Thereafter, we validated the candidate CNV region in an independent BrS patient cohort using Sanger sequencing. We compared the frequency of identified CNV regions in the healthy populations using in-house controls from Taiwanese, the Taiwan Biobank (TWB), and the genome aggregation database (gnomAD). Finally, we used cell and zebrafish models to investigate the functional role of the identified CNVs in BrS patients.

2. Materials and methods

2.1. Study subjects

We consecutively recruited 335 unrelated patients with BrS from 2000 to 2018 in the Taiwanese population in Taiwan; 76 were identified via symptoms of sudden cardiac arrest (SCA) or syncope early in the study period (2000–2010) and 259 more, both symptomatic and asymptomatic, were identified later (2011–2018), after the SADS-TW BrS registry increased awareness of BrS [13]. Aborigines were excluded from this study. BrS was diagnosed by 2 independent cardiologists using established criteria (Shanghai BrS Score ≥ 3.5) [14]. Since SCN5A is the major BrS-causal gene [4], we screened it first. Peripheral blood samples were collected from all participants. Mutations or SNPs in the SCN5A gene were screened using direct sequencing. We followed the primers and PCR conditions published by Wang et al. [15] to perform genotyping in all amino acid-coding exons and intron borders of SCN5A. Amplicons were purified by solid-phase extraction and were bidirectionally sequenced using a PE Biosystems Taq DyeDeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, CA, USA). Sequencing reactions were separated on a PE Biosystems 373A/3100 sequencer, and the results were compared with a reference sequence from GenBank and the TWB. The pathogenicity of a mutation was defined by American College of Medical Genetics and Genomics guidelines [16]. Exclusion of 34 BrS patients with SCN5A mutations left 301 unrelated BrS patients enrolled in this CNV study.

The prevalence of BrS is lower (0.05–0.1%) [1,17] than that of common diseases (e.g., hypertension (28–31%) [18]). To overcome the small case number, we used a 2-stage study design with extreme phenotype sampling strategy and 2 independent cohorts with increasing sample size which attempted to maximize power and efficiency [11,12] (Fig. 1). In stage I, we initially selected 66 BrS patients (cohort 1, discovery cohort) to discover candidate CNV regions by microarray and WES. In stage II, cohort 2 (replication cohort, 235 unrelated BrS) were used to confirm the significant CNVs using PCR-based genotyping assay and Sanger sequencing. This study was approved by the ethics committee of National Taiwan University Hospital. All participants gave informed consent before participating in the study.
2.2. Microarray experiments, exome sequencing and CNV analysis

Omni1-Quad BeadChip microarrays (Illumina, USA) were performed according to the manufacturer’s instructions. The data were submitted to the Gene Expression Omnibus (accession number GSE46348). To evaluate whole-genome CNV regions, raw intensity data from 1.14 million SNPs and CNV probes were imported into Partek Genomics Suite software (Partek Inc., USA) to perform CNV analysis. The criteria used for identifying CNV regions are shown in the Supplementary Note and the accuracy of the segmentation algorithm has been discussed in a previous study [19]. Lastly, Ingenuity Pathway Analysis (Ingenuity Systems, Inc., USA) was performed to characterize the biological functions of genes located in CNV regions. We used WES to fine-tune the length of candidate CNV regions. The detailed procedures of WES are described in the Supplementary Note. The CNVkit (v0.9.4) algorithm was used to obtain genome-wide CNV regions of each sample while the flat reference was set as the identical coverage in all samples [20]. A CNV region was defined as a deletion if its copy number was less than 1.2.

2.3. Validation of identified CNV regions

To further minimize the chance that false associations arose as a result of technical genotyping artefacts [21], different platforms using PCR-based genotyping and Sanger sequencing were used to validate the results of microarray and WES. To confirm the region containing the deletion of GSTM3 identified by genome-wide microarray and WES, we designed PCR primers for the region with the lowest copy number of GSTM3. Forward and reverse primers used to amplify target regions are listed in Table S1, and the detailed procedures are given in the Supplementary Note.

2.4. Investigation of the identified CNV region in local controls and a public control

To evaluate the CNV frequency of GSTM3 in relatively healthy populations, we used 2 local controls and 1 public control. For the first local controls, we first performed PCR and Sanger sequencing in 997 ancestral-matched in-house controls. In-house controls were ancestral-matched individuals with no arrhythmia-related symptoms, normal coronary arteries by angiography, normal 12-lead electrocardiogram (ECG), and no family history of sudden cardiac death (SCD), BrS, or heart failure. For the second local controls, we analysed genotyping data from the TWB (20,117 participants; https://taiwanview.twbiobank.org.tw/search, Supplementary Note). The design of the TWB array was a joint effort of the TWB, the National Center of Genome Medicine (NCGM; http://ncgm.sinica.edu.tw/ncgm_02/index.html), and Affymetrix, Inc. To compare with other ethnicities, we used the gnomAD structural variants (SVs) database to examine the GSTM3 deletion frequency in major worldwide populations.

2.5. Basal expression of GSTM3 gene in human adult right ventricle

BrS is believed to be a right heart disease [22]. Although GSTM3 is expressed in human heart muscle [23], whether GSTM3 is specifically expressed in right ventricular cells has never been investigated before. We used a cDNA library from a healthy human adult right ventricle (Invitrogen) and diluted it to a working concentration of 100 ng/μL. Then, using primers for human β-actin (ACTB gene) and GSTM3, we performed PCR and separated the products on a 2% agarose gel to check the amplified band.

2.6. Western blot of GSTM3 proteins extracted from HEK 293 cells and HL-1 cells

HEK293 cells (derived from human embryonic kidney) were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin at 37 °C, 5% CO2. HL-1 cardiac muscle cells, an immortalised mouse atrial cell line, were cultured on a plate coated with gelatin/fibronectin, maintained in Claycomb medium containing 10% FBS, penicillin/streptomycin, L-glutamine, and 0.1 mM norepinephrine. Protein lysates (50 μg) were extracted from HEK293 cells, HL-1 cells, and mouse testis tissue (use as positive control), then separated by
SDS-PAGE, blotted with the GSTM3 antibody (Cusabio Technology, LLC), and detected using an enhanced chemiluminescence western blotting system (Amersham Biosciences). GSTM3 protein is endogenously expressed in HEK293 cells but not in HL-1 cells (Fig. S1). Therefore, we used HEK293 cells stably expressing Nav1.5 channel in further studies.

2.7. HEK293 cell culture and transfection

HEK293 cells stably express SCN5A (encoding the Nav1.5 channel), hereafter referred to as HEK Nav1.5 cells. HEK Nav1.5 cells were cultured in a controlled environment (5% CO₂, 37 °C) and maintained in DMEM (Euroclone, Italy) supplemented with FBS (10%), L-glutamine (2 mM), penicillin/streptomycin (100 U/mL, 100 μg/mL), and zeocin (200 μg/mL). The transfection was carried out using jetPRIME reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions. Cells were transfected with GSTM3 Silencer Select Pre-designed siRNA (20 nM; Ambion, Thermo Fisher Scientific, Italy) or with Silencer Select Negative Control #1 Pre-designed siRNA, a non-targeting siRNA providing a negative control to compare siRNA-treated samples (20 nM; Ambion, Thermo Fisher Scientific, Italy). Twenty-four hours after transfection, cells were harvested either to analyze the knockdown of endogenous GSTM3 protein levels by western blotting or for cytotoxicity assays, or re-seeded for electrophysiological recording.

2.8. Western blot of GSTM3 protein extracted from HEK NAV1.5 cells transfected with siRNA specific for GSTM3 or with a non-targeting negative control siRNA

Cells were lysed and cytoplasmic proteins were extracted using a NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, Italy). Protein concentrations were determined using Bradford assay reagent (Pierce Coomassie Plus Protein Assay; Fisher Scientific, Italy). Twenty-five μg of cytoplasmic protein were separated on homemade gels (12% acrylamide) in a standard running buffer and then transferred onto nitrocellulose membranes (GE Healthcare, Euroclone, Italy). Membranes were blocked with 5% non-fat dry milk in 1 × Tris-buffered saline containing 0.1% Tween 20 (TTBS 0.1%) for 90 min at room temperature, washed 3 times in TTBS 0.1%, and then incubated overnight at 4 °C with anti-α-tubulin (1:500 in TTBS 0.1% plus milk 5%, rabbit polyclonal; Cusabio Biotech Co, College Park, MD, USA) and anti-human glutathione S-transferase μ3 (1:1000 in TTBS 0.1% plus milk 5%, rabbit polyclonal; Cusabio Biotech Co, College Park, MD, USA). After 3 TTBS 0.1% washings, membranes were incubated for 90 min with an IgG HRP-conjugated antibody (donkey anti-rabbit, 1:5000 in TTBS 0.1% plus milk 1%; Santa Cruz Biotechnology) and then washed again 3 times in TTBS 0.1%

Proteins were detected using an enhanced chemiluminescence detection kit (Supersignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Italy) in an ImageQuant LAS 4000 instrument (GE Healthcare Life Sciences, Italy). Blots were analysed and quantified with ImageJ software.

2.9. Cytotoxicity assays

To measure the mitochondrial activity and membrane damage in HEK293 cells, 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) (MTT Formazan powder; Sigma-Aldrich, Italy) and lactate dehydrogenase (LDH) (LDH Cytotoxicity Detection KitPLUS; Sigma-Aldrich, Italy) assays were performed. To create conditions of oxidative stress, cells were incubated for 30 or 60 min either with medium or with medium plus 15 mM tert-butyl hydroperoxide (tBHP; Sigma-Aldrich, Italy). Absorbance and emission were measured with a multi-label spectrophotometer (VICTOR3, Perkin Elmer, USA) at 570 nm and 490 nm, respectively.

2.10. Electrophysiological experiments in HEK NAV1.5 cells

Patch clamp experiments were performed at room temperature in HEK Nav1.5 cells in whole cell configuration. The experimental details are described in the Supplementary Note. To induce conditions of oxidative stress, cells were incubated for 30 min at room temperature with an extracellular solution containing 95 mM N-methyl-D-glucamine, 20 mM NaCl, 5 mM CsCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, 5 mM glucose, and 15 mM tBHP, and recordings were obtained within 60 min from the beginning of treatment. The standard voltage clamp protocols have been previously described [25,26]. Membrane voltages were not corrected for liquid junction potential. To measure the kinetics of the onset of inactivation, the declining phase of I₅ Na traces recorded between –30 mV and 20 mV were fitted with a bi-exponential function. The number of cells is indicated in Table 3, and the "current density" column is greater than the "activation" column because not all cells could complete the protocol for the study of the activation properties. We did not consider applying TTX in order to define I₅ Na, as HEK Nav1.5 cells stably express Nav1.5 channel, and the comparison with the un-transfected HEK293 cells showed the absence of inward currents (Fig. S2).

2.11. Generation and characterization of CRISPR/Cas9-mediated GSTM3 knockout male adult zebrafish

All experimental procedures on zebrafish were approved by the committee for the use of laboratory animals at National Taiwan University, Taipei, Taiwan (IACUC Approval ID: 103; Animal Use document no. 102) and carried out in accordance with the guidelines of the Animal Welfare Act. The details of generation and characterization of GSTM3 knockout adult male zebrafish using CRISPR/Cas9 are described in the Supplementary Note. Briefly, among the 3 guide RNAs (gRNAs) tested, only the exon 5-targeted gRNA effectively induced distinct changes in melting curves compared to the untreated groups (Fig. S3A). We thus raised only the exon 5 gRNA-treated F0 embryos to adulthood and then crossed them with WT fish to obtain F1 embryos. The F1 embryos were raised to 2 months old to collect genomic DNA from their tail fins. We screened for the GSTM3 mutation carriers by high resolution melting analysis, raised them to adulthood, and cross-bred them to obtain the F2 generation. The zygosity of the F2 fish was determined by capillary electrophoresis. We performed PCR using F2 fish tail fin genomic DNA to obtain amplicons from 24 fish. As shown in Fig. S3B, the WT fish showed only one band of 250 base pairs. In contrast, the homozygous mutant fish also had one band with a reduced size, which indicated a potential deletion in the GSTM3 gene. The heterozygous fish contained both bands with reduced intensity. To confirm the deletion in GSTM3, we sequenced those amplicons and found that all GSTM3+/- fish had a 7 bp deletion (---TCCGAA---) at the gRNA binding site compared to the sequence of WT fish (Fig. S3C).

The WT GSTM3 protein contains 219 amino acids. The deletion results in a premature stop codon in the middle. The mutant GSTM3 has the native sequence of 106 amino acids at its N-terminus, followed by 25 mutated amino acids due to the frameshift. The amino acid sequence alignment of WT and mutant GSTM3 is presented in Fig. S4. The identified GSTM3+/- and GSTM3---/- fish were separated and used for further experiments.

2.12. Expression of GSTM3 in adult male zebrafish heart by real-time quantitative PCR

To examine expression of GSTM3, we isolated 3 hearts of adult male zebrafish. The hearts were homogenised to extract total RNA.
using TRIzol reagent (Invitrogen) for cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. The cDNA was then subjected to real-time quantitative PCR analysis (Applied Biosystems). Taqman reactions were performed using one-step RT-PCR Master Mix Reagents with the ABI PRISM 7700 Sequence Detection System and analysed using the Sequence Detection System software. Gene expression was normalised to a commonly used reference gene (EF1A, elongation factor 1α). Primers and probes were based on GenBank sequences (GSTM3, NM_001162851.1; and EF1A, NM131263.1).

The data were analysed using the relative gene expression (i.e., ΔΔCT) method, as described in Applied Biosystems User Bulletin No. 2 and explained further by Livak and Schmittgen [27]. Briefly, the data were presented as the fold change in gene expression normalised to EF1A and relative to a calibrator. The results are presented in Fig. S5.

3. Results

3.1. Patient characteristics

The demographic characteristics of the study cohorts are shown in Table 1. The gender of all cohorts was male-predominant, and 63.1% of BrS patients overall were symptomatic. As expected, the percentage of the symptomatic BrS patients was higher in cohort 1 than in cohort 2 (95.5% vs. 54%), whereas the percentage of asymptomatic BrS patients was higher in cohort 2 than in cohort 1 (4.5% vs. 46%) because of our study design. There were no differences in gender, family history of SCD, and spontaneous type 1 Brugada ECG among the 2 cohorts.

3.2. Discovery of the CNV regions

The genomic landscape of CNV regions in BrS patients is illustrated in Fig. S7. A total of 502 aberrant regions were observed, including 447 deletions and 55 amplifications. Importantly, no CNVs were detected in the SCNSA gene. The Refseq database revealed 91 deleted and 11 amplified genes in total (Table S2). Among these 102 CNV genes, Ingenuity Pathway Analysis revealed 5 significantly enriched canonical pathways (P<10⁻⁵, Table S3, Hypergeometric test). Notably, although the 5 pathways had different cellular functions, they were primarily identified on the basis of the same genes, particularly the GST mu family. Unexpectedly, all 5 genes in the GST mu family, including GSTM1-5, showed high frequencies of deletion, suggesting they make up a gene cluster deleted in BrS. Among the 5 genes, GSTM3 is the gene nearest to the breakpoint of the CNV region spanning across the GST mu family. Therefore, GSTM3 was selected for further investigations. Because the candidate CNV region of GSTM3 (chr1:109,733,932-109,739,407) was too long (>5 K bp) to use traditional Sanger sequencing to identify the breakpoints of this CNV region, we used WES technology to fine-tune this candidate CNV region in the unrelated 66 BrS patients instead. Fifteen of the 66 BrS patients (23.4%) had this GSTM3 deletion, and WES showed the length of the GSTM3 deletion (chr1:109,737,076-109,737,247, hg38) (Fig. 2A). Except for common variants (e.g., SNPs), no radical or missense mutations were identified in the GSTM3 gene.

3.3. Validation and replication

In cohort 2, the GSTM3 deletion was present in 23.4% of the 235 BrS patients using a PCR-based genotyping assay and direct sequencing (Fig. 2B and 2C). The length of the GSTM3 deletion is 291 basepairs (chr1:109,737,011-109,737,301, hg38). Importantly, this deletion region in GSTM3 contains exon 8 and the transcription stop codon (CCDS 812.1, hg38). In total, 23.9% of the BrS patients carried the GSTM3 deletion and 94.4% of the BrS patients with the GSTM3 deletion (68/72) were heterozygous carriers. Interestingly, when we screened this CNV in the 34 BrS patients excluded for having SCNSA

Table 1

Clinical characteristics of BrS patients in 2 independent cohorts.

|                  | Cohort 1 (N = 66) | Cohort 2 (N = 235) | All (N = 301) |
|------------------|-------------------|-------------------|--------------|
| **Age at diagnosis** (years) | 42.0 ± 12.9 | 45.0 ± 15.8 | 44.3 ± 15.2 |
| Gender (male) | 63 (95.4%) | 209 (89.9%) | 272 (90.4%) |
| **Presentations** | | | |
| SCA | 36 (54.5%) | 58 (24.7%) | 94 (31.2%) |
| Syncope | 27 (40.9%) | 69 (29.4%) | 96 (31.9%) |
| No symptoms | 3 (4.5%) | 108 (46.0%) | 111 (36.9%) |
| **Family history of SCD** | 13 (19.7%) | 49 (20.9%) | 62 (20.6%) |
| **Spontaneous type 1 Brugada ECG** | 59 (89.4%) | 176 (74.9%) | 235 (78.13%) |

BrS, Brugada syndrome; ECG: electrocardiogram; SCD: sudden cardiac arrest.
3.4. Evaluation of the GSTM3 deletion in the healthy controls

We evaluated the frequency of the GSTM deletion in 3 healthy controls (2 ancestral-matched local controls and 1 public CNV database). In the 2 local controls, the deletion was in 0.1% of 997 in-house unrelated controls whereas CNV analysis showed that 0.8% of the 15,829 ancestral-matched unrelated samples which passed the quality assessment have the GSTM3 deletion in the TWB, which were significantly lower than that in BrS patients (both \( P = 0.001 \), Proportional test). In the public CNV database, the WGS data from the gnomAD SVs database, which is 9% East Asian, showed that no such deletion in GSTM3 was reported in any samples out of 10,738 unrelated individuals, (https://gnomad.broadinstitute.org/region/1-110279562-110279935?dataset=gnomad_sv_r2).

3.5. Comparison of clinical demographics and severity of clinical presentation of the BrS patients with and without the deletion of GSTM3

The comparison of clinical characteristics between BrS patients with or without this deletion is shown in Table 2. In cohort 1 and cohort 2, there were no significant differences in age at diagnosis, gender, family history of SCD, or spontaneous type 1 Brugada ECG between the two cohorts. In cohort 1 and cohort 2, a higher percentage of BrS patients with deletion of GSTM3 experienced SCA compared to those without \( (P = 0.003, 0.007, \text{respectively, Fisher's exact test}) \). In total cohort, more BrS patients with deletion of GSTM3 experienced SCA and syncope than those without \( (P = 0.002, 0.04, \text{respectively, Fisher's exact test}) \).
3.7. Effect of the loss of GSTM3 on the cardiac sodium current under conditions of oxidative stress

Whole-cell patch-clamp data showed that Nav1.5 current density measured at −10 mV was not affected by the siRNA transfections (Fig. 4A-B; Table 3). As expected from the literature [32,33], a condition of oxidative stress, created by the application of 15 mM of tBHP, triggered a decrease in INa of about 60% both in control cells and in cells transfected with the siRNA negative control. However, when GSTM3 protein expression was silenced, tBHP reduced the INa density by approximately 75% (Fig. 4A-B; Table 3) and the onset of fast inactivation was accelerated (Fig. 3C).

Consistent with previous studies [34,35], no significant differences were found in activation after tBHP application (Fig. 5; Table 3), but tBHP exposure induced a significant negative shift in the availability of sodium channels (Fig. 5). When HEK Nav1.5 cells were treated with tBHP, V1/2 significantly shifted approximately 8 mV in the negative direction, and a similar shift was observed in cells transfected with the non-targeting siRNA (Table 3). This hyperpolarizing shift was even more dramatic (approx. −18 mV) when GSTM3 expression was reduced, after induction of oxidative stress (Fig. 5; Table 3). These data support the hypothesis that reduced expression of GSTM3 amplifies the response of Nav1.5 channels to oxidative stress, causing a more dramatic reduction in INa.

3.8. Comparisons of baseline ECG parameters and ventricular arrhythmic events before and after flecainide administration

Table S4 shows the ECG parameters and the number of WT, GSTM3+/− and GSTM3−/− zebra fish with ventricular arrhythmia (VA) before and after flecainide administration. The baseline PR interval, QRS duration, RR interval, and QTc interval were not significantly different among the three groups. After flecainide treatment (1 μM and 10 μM flecainide), there was a significant increase of PR interval and QRS duration in GSTM3+/− and GSTM3−/− zebrafish compared with WT. At baseline, the number of WT, GSTM3+/−, and GSTM3−/− zebrafish with VA was not different but the number of GSTM3+/− and GSTM3−/− zebrafish with VA significantly increased after flecainide administration (Fig. 6A-C). Interestingly, the number of GSTM3+/− and GSTM3−/− zebrafish with VA decreased after quinidine infusion (Fig. 6D-F; Table S5). The pharmacologic responses observed in GSTM3+/− and GSTM3−/− zebrafish are congruent with those encountered in clinical cases of BrS.

3.9. Comparison of arrhythmogenicity after programmed extra-systolic stimulation

Table S5 shows the number of WT, GSTM3+/− and GSTM3−/− zebrafish with inducible ventricular tachycardia (VT) or ventricular fibrillation (VF) after PES. After PES in the absence of drug treatment, the number of GSTM3+/− and GSTM3−/− zebrafish with inducible VT or VF was significantly higher than WT fish. With flecainide administration, the number of GSTM3+/− and GSTM3−/− zebrafish with inducible VT or VF increased significantly. In contrast, the number of zebrafish with inducible VT or VF among the 3 groups was not different after quinidine infusion except for 0.1 μM quinidine.

4. Discussion

Previous CNV studies in BrS patients focused on the SCN5A gene alone [36-40]. This is the first whole-genome CNV study to investigate the role of genomic CNVs in influencing susceptibility to BrS and to examine the role of CNV in risk stratification of BrS patients. We identified a diallelic CNV deletion of GSTM3 in 23.9% of Taiwanese BrS patients without SCN5A mutations. In contrast, the deletion of GSTM3 was rarely observed in 0.1% of in-house controls (N = 997) and 0.8% in
TWB samples (N = 15,829). Intriguingly, the GSTM3 deletion was not reported in the large dataset based on whole-genome sequences (>10,000 individuals), suggesting that it is closely associated with BrS. The value of genetic variants as a tool to evaluate recurrent arrhythmic risk in BrS is still undetermined [1]. In this study, although not all BrS families showed complete co-segregation of the GSTM3 deletion with BrS, the frequency of SCA in BrS patients with deletion of GSTM3 was significantly higher than in BrS patients without the deletion, suggesting that the clinical presentation of BrS patients with the deletion of GSTM3 may be more severe. We also observed that the frequency of VA was significantly higher in GSTM3 knockout zebrafish than in WT zebrafish. In other words, our findings suggest that deletion of GSTM3 may exert modulatory effect on arrhythmia risk in Taiwanese BrS patients, and provide a reference for risk stratification of BrS patients.

GSTM3 encodes a glutathione S-transferase involved in anti-oxidant defense, protecting the cells from oxidative stress [41]. The deleted region of GSTM3 contains the 8th exon and the transcription stop site. Thus, this deletion may result in failed transcription termination, leading to nonsense-mediated degradation of GSTM3 mRNA [42]. Cardiac oxidative stress caused by reactive oxygen species (ROS) has been demonstrated to play an important role in the mechanism of cardiac arrhythmia and SCD [43,44]. In excitable cardiac cells, ROS regulate both cellular metabolism and ion homeostasis. Increasing evidence suggests that elevated cellular ROS can cause alterations of membrane current in isolated cardiac myocytes [45] and abnormal Ca^{2+} handling, leading to arrhythmogenesis [43]. In particular, ROS induce a reduction in the total cardiac sodium current and a leftward shift in the availability curve [34,45]. The pathogenic mechanism underlying BrS may involve an outward shift in the balance of current in the early phases of the action potential in the epicardium of the right ventricular outflow tract, secondary to a decrease in inward current (e.g., INa) or an increase in outward current (e.g., IK-ATP). Oxidative stress, among other effects, produces both a decrease in INa and an increase in IK-ATP. Our data from HEK293 Nav1.5 cells showed that, upon treatment with the direct-acting oxidative agent tBHP, the reduction in INa is amplified when GSTM3 is reduced. A loss of INa has been shown to leave the transient outward current (Ito) less opposed, thus accentuating the epicardial action potential notch in the outflow tract of the right ventricle, leading to loss of the action potential dome and the development of phase 2 reentrant extra-systoles capable of precipitating VT/VF [46]. Reduced Ito has also been proposed to contribute to the manifestation of BrS by slowing impulse conduction into the right ventricular outflow tract (RVOT) [47]. Our findings suggest that a decrease in GSTM3 copy number can amplify the effect of oxidative stress on cardiac sodium current, leading to increased oxidative stress and arrhythmia risk in BrS patients.
ROS to reduce INa, thus predisposing patients to the development of BrS (Fig. 7). In the other hand, we found that Tpeak-Tend interval, representing the dispersion of repolarization,

increased in the untransfected cells compared to cells transfected with non-targeting siRNA (NEG CTRL) or siRNA specific for GSTM3 (siRNA GSTM3) in the absence (empty symbols) or in presence (filled symbols) of tBHP. After performed multiple testing correction, *P<0.005 vs the respective untreated condition; #P<0.005 vs CTRL + tBHP. In parentheses is the number of cells. (B) Current densities evoked by a voltage step at −10 mV (holding potential −100 mV) normalised by cell capacitance, in the absence (upper traces) or presence (lower traces) of tBHP. (C) Kinetics of the onset of fast inactivation. *P<0.005 after performed multiple testing correction. (ANOVA, followed by a modified t-test with Fisher LSD correction).

In Panel A and C, data are presented as mean values ± S.E.

Fig. 4. Effect of tBHP on the Nav1.5 peak current density and kinetics of inactivation. (A) Current-voltage relationship of peak inward currents in HEK Nav1.5 cells (CTRL), in HEK Nav1.5 cells transfected with a non-targeting siRNA (NEG CTRL) or siRNA specific for GSTM3 (siRNA GSTM3) in the absence (empty symbols) or in presence (filled symbols) of tBHP. After performed multiple testing correction, *P<0.005 vs the respective untreated condition; #P<0.005 vs CTRL + tBHP. Values are means ± S.E. *n=20, n=23 for GSTM3 (siRNA GSTM3) in the absence (empty symbols) or in presence (filled symbols) of tBHP.

Table 3

Properties of INa, current in control and GSTM3-silenced cells in the presence or absence of 15 mM tBHP.

|                        | Current density (pA/pF) | Activation Curve | Availability Curve |
|------------------------|------------------------|------------------|--------------------|
|                        | V1/2 (mV) | k           | V1/2 (%C0) (mV) | k           |
| CTRL                   | 75.1.8 ± 34.3 (n = 13) | 7.5 ± 8.0 | 54.0 ± 2.2 (n = 28) | 8.4 ± 0.4 |
| CTRL + tBHP            | 55.3 ± 16.1 (n = 13) | 9.1 ± 1.5 | 72.3 ± 0.9 (n = 6) | 8.2 ± 0.7 |
| NEG CTRL               | 146.9 ± 21.9 (n = 23) | 6.8 ± 1.7 | 67.0 ± 1.8 (n = 10) | 7.8 ± 0.3 |
| NEG CTRL + tBHP        | 24.1 ± 2.8 (n = 6) | 7.5 ± 2.8 (n = 15) | 7.4 ± 0.6 |
| GSTM3 siRNA            | 21.4 ± 32.0 (n = 20) | 8.06 ± 1.2 | 64.4 ± 1.6 (n = 19) | 8.2 ± 0.4 |
| GSTM3 siRNA + tBHP     | 22.8 ± 5.3 (n = 12) | 8.07 ± 1.02 | 82.8 ± 2.6 (n = 7) | 7.0 ± 0.2 |

V1/2, voltage of half-maximal activation; V1/2 (%) prepulse voltage where half-maximal inactivation occurred; k, slope factor. CTRL, untransfected cells; NEG CTRL, cells transfected with non-targeting siRNA. Values are means ± S.E. n is the number of cells patched from at least three different experiments. *P<0.005 vs the respective tBHP untreated condition; #P<0.005 vs CTRL + tBHP; §P<0.005 vs NEG CTRL + tBHP. (ANOVA, followed by a modified t-test with Fisher LSD correction).
In terms of clinical implications, our study identified a deletion of GSTM3 in BrS patients, which is associated with reduced INa, suggesting that the deletion could be a genetic modifier of the BrS phenotype. In addition, it could be used as a risk predictor in patients with BrS.

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**Declarations of Competing Interest**

The authors declared that they have no conflict of interests.

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**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ebiom.2020.102843](https://doi.org/10.1016/j.ebiom.2020.102843).

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