Compound heterozygous SCN5A gene mutations in asymptomatic Brugada syndrome child

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Abstract

Loss-of-function mutations in the SCN5A gene, encoding the cardiac Nav1.5 sodium channel, have been previously associated with Brugada syndrome (BrS). Despite the low prevalence of the disease, we identified a patient carrying two SCN5A mutations. We aimed at establishing a correlation between genotype, clinical phenotype and in vitro sodium current. A 3-year-old boy presented with right bundle branch block and ST-segment elevation. Genetic analysis and electrophysiology studies in transfected HEK293 cells were performed to identify possibly disease-causing variants and assess their effect on sodium channel function. Two SCN5A variants were identified: a new frameshift deletion causing premature truncation of the putative protein (c.3258_3261del4) and a missense substitution (p.F1293S). In vitro studies revealed that the truncated mutant did not produce functional channels and decreased total sodium current when co-expressed with p.F1293S channels compared to p.F1293S alone. In addition, p.F1293S channels presented with a steep slope of steady-state activation voltage-dependency, which was shifted towards more positive potentials by the co-expression with the truncated channel. p.F1293S channels also showed shift towards more positive potentials of the steady-state inactivation both alone and co-expressed with the deletion mutant.

Our data identified a severe reduction of sodium channel current associated with two distinct SCN5A changes. However, all mutation carriers were asymptomatic and BrS electrocardiogram was observed only transiently in the compound heterozygous subject. These observations underline the difficulty of genotype/phenotype correlations in BrS patients and support the idea of a polygenic disorder, where different mutations and variants can contribute to the clinical phenotype.

Introduction

Brugada syndrome (BrS) is an autosomal dominant arrhythmogenic syndrome that can be associated with ventricular fibrillation and sudden cardiac death in patients with structurally normal heart. BrS was first described in 1992 as a distinct clinical and electrocardiographic entity, characterized by ST-segment elevation in the right precordial leads (V1-V3) and right bundle branch block at surface electrocardiogram (ECG).1 However, the BrS ECG pattern is often concealed and can be unmasked using sodium channel blockers. To date, the implantable cardioverter defibrillator (ICD) represents the only viable option for symptomatic patients to prevent the occurrence of sudden death from malignant arrhythmias.2 However, new criteria for prognostic risk stratification of these patients are needed, especially for asymptomatic subjects.3 Despite the extraordinary progresses in characterizing BrS, still little is known about its natural history in the pediatric population. It has been however reported that BrS children display diagnostic and prognostic features similar to adult patients and may be as well subjected to sudden death.4

In 1998 Chen associated BrS with mutations in the SCN5A gene,5 encoding the α-subunit of the voltage-gated cardiac sodium channel Nav 1.5.6 However, only 20-25% of clinically diagnosed BrS patients carry a SCN5A mutation.7,8 At present, over three hundred SCN5A mutations have been associated with BrS (http://www.fsm.it/cardio/), which reduce the magnitude of the cardiac sodium current in functional studies by a variety of mechanisms.9-12 However, the emerging concept of arrhythmia genomics supports the idea of a complex disorder, where the co-segregation of different mutations and common genetic variants can contribute to the clinical phenotype.13 Here we report a three-years-old child diagnosed with BrS and carrying two different SCN5A mutations inherited from his asymptomatic parents. In addition, we describe the in vitro electrophysiological characterization of the two variants. The difficulty in correlating genotype with phenotype underlines the importance of widening the genetic screening of BrS patients to identify modifier variants.

Materials and Methods

Clinical phenotype

A 3-year-old boy of Caucasian ethnical origin was referred to our center for routine control related to occasionally detected congenital heart murmur. Echocardiography examination

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revealed oval foramen perviety without hemodynamic impairment, but was otherwise negative, with a conserved ejection fraction (75%). Chest X-ray was negative. ECG at admission showed sinus rhythm with focal right bundle branch block (Figure 1A). However, a second ECG showed a coved ECG ST elevation pattern (>2 mV) in V1 and V2 leads with T wave inversion in leads V1, V2 and V3, unmasking a typical type I BrS ECG pattern according to criteria indicated in the consensus report14 (Figure 1B). The duration of PR interval was 180-200 ms, with QRS=100 ms in V4. During 24 h Holter-ECG recording (mean frequency 90 bpm, minimum 82 bpm, max 139 bpm) a self-terminating asymptomatic episode of polymorphic ventricular tachycardia (130 bpm) was recorded. Also in consideration of the very young age, the patient was not subjected to ICD implantation and a close follow-up was proposed with serial ECG and 24 h Holter monitoring and prompt antipyretic treatment during fever and before immunizations. Proband parents were completely asymptomatic with normal echocardiography. However, both ECGs showed right ventricular conduction delay (Figure 1C and D). Unfortunately, they refused to undergo further clinical examination including flecainide testing. ECG parameters of all three family members are showed in Table 1.

### Genetic analysis

Written informed consent was obtained before genetic analysis. The investigation conformed to the principles outlined in the Declaration of Helsinki. Genomic DNA was extracted from peripheral blood using the EZ1 Bio-Robot extractor (Qiagen, Venlo, the Netherlands). Molecular analysis was conducted on SCN5A coding sequence, including flanking intronic regions, by polymerase chain reaction amplification. Mutation screening was performed using Denaturing High Performance Liquid Chromatography (Transgenic, Omaha, NE, USA) and automated direct sequencing (ABI 3730, Applied Biosystems Inc., Foster City, CA, USA). Primer sequences are available on request. SCN5A NCBI reference sequences are NM_198056 (nucleotides are numbered starting from the ATG) and NP_932173.

### Constructs preparation

The c.3258_3261del4 and c.3878T>C (p.F1293S) SCN5A mutants were prepared by site-directed mutagenesis using Quick Change® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and plasmid pcDNA3.1-SCN5A as template, which contains the hH1 clone as previously described.15 SCN5A cDNA was amplified using Pfu+ DNA polymerase (Stratagene) and the following oligonucleotides: 5’-GCCCTCCGATTCCAG-3’ (sense) and 5’-CTGGAATCCGGGCGGGGCGCCACCGGA-CACAGGC-3’ (antisense) for c.3258_3261 del4, and 5’-GCAACACCTGGGCTCTCCGAGATGGGCCCCCA-3’ and 5’-TGGGCCCCCATCTCGGCAGAGCCAGGTGGTTCG-3’ for p.F1293S. The mutated c.3258_3261del4 and p.F1293S clones were sequenced to ensure the presence of the mutations and the absence of spurious variations.

### Transient expression of wild-type and mutant channels

Transient transfection of wild-type (WT) and mutant SCN5A cDNA was performed as previously described.14 To characterize WT and mutant SCN5A separately, HEK293 (human embryonic kidney) cells were transfected with 5 µg of pcDNA3.1 containing WT SCN5A cDNA, p.F1293S or c.3258_3261del4 using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). To mimic the heterozygote situation, 2.5 µg of p.F1293S and 2.5 µg of c.3258_3261del4 were co-transfected.

### Patch-clamp experiments and data analysis

Patch-clamp experiments were performed according to the previously described methods.16 Whole-cell recordings were carried out at 37°C, and cells were perfused with 2 µM nifedipine, 0.5 µM verapamil, 0.5 µM diltiazem, 0.1 µM lidocaine and 4 µM quinidine. Data was recorded using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and filtered at 2 kHz. The holding potential was 0 mV. 50 ms depolarizing voltage steps were applied from -110 to 110 mV in 10 mV increments, and the test potentials were 0 mV in all cases. The experiments were performed at room temperature (21°C) and the patch-clamp setup was shielded from external magnetic fields.

### Table 1. Electrocardiogram intervals duration (ms). In parenthesis reference values.

|          | PR       | QRS      | QTc      |
|----------|----------|----------|----------|
| Proband  | 180-200  | 100 (90-110) | 395 (<440) |
| Mother   | 160 (120-200) | 110 (70-110) | 430 (<460) |
| Father   | 150 (120-200) | 108 (70-110) | 440 (<450) |

Figure 1. Twelve-lead surface electrocardiogram (ECG). (A) Proband, first ECG showing sinus rhythm with focal right bundle branch block; (B) Proband, second ECG revealing sinus bradycardia with typical type I Brugada syndrome (BrS) pattern in V1 and V2. In both panels the electrodes were placed in the same position. (C) Mother ECG and (D) Father ECG, showing sinus rhythm and no type I BrS pattern.
22°C. Step-pulse voltage protocols were generated with Axopatch 200B using Pclamp 9 software (Molecular Devices, Sunnyvale, CA, USA). The pipette solution contained 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES (in mM, pH 7.35 with CsOH). Bath solution contained 145 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, and 10 glucose (in mM, pH=7.35 with NaOH).

Conductance \( G(V) \) was calculated by the equation:

\[
G(V) = \frac{I}{(V_m - E_{rev})}
\]

where \( I \) is the peak currents, \( E_{rev} \) is the measured reversal potential, and \( V_m \) is the membrane potential.

The normalized peak conductance was plotted against membrane potentials. Steady-state inactivation was estimated by a pre-pulse protocol (500 ms from a holding potential of -140 mV; shown in the inset). The normalized peak currents were plotted as a function of membrane potentials. Steady-state activation and inactivation were fitted with the Boltzmann equation:

\[
y = \frac{1 + \exp((V_h - V_m)/k)}{1}
\]

where \( y \) represents variables; \( V_h \), midpoint; \( k \), slope factor; \( V_m \), membrane potential.

The biophysical characteristics of sodium channels were analyzed using Clampfit (Axon Instruments, Sunnyvale, CA, USA) and Igor software (WaveMetrics, Lake Oswego, OR, USA). Data were presented as mean±SD. Comparisons among data were made using one-way ANOVA followed by Tukey test with \( P \leq 0.05 \) considered as statistically significant.

**Results**

### Genetic analysis

SCN5A molecular analysis identified in the proband two variants compatible with BrS (Figure 2): the first was a novel heterozygous four nucleotides deletion in exon 18 (c.3258_3261del4, from now on named Del4), leading to frameshift and generation of a premature stop codon in the predicted protein after 56 amino acids, within the cytoplasmic linker between domains DII and DIII (p.E1087PfsX57). The second was a c.3878T>C heterozygous nucleotide substitution in exon 22, leading to the aminoacidic change p.F1293S (from now named FS). This variant, located in the extracellular linker between S2-S3 in domain DIII, had been previously reported both in a BrS patient\(^{17}\) and in apparently healthy individuals with a frequency of 1/295 among Caucasians from the US,\(^{18}\) therefore its pathogenicity was unclear. The analysis by the computer-based algorithms PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) suggested that the FS substitution might be tolerated.

We subsequently performed a targeted mutation analysis on the proband’s parents to determine the configuration of the c.3258_3261del4 and c.3878T>C alleles, due to the different putative functional effect on the sodium channel subunits. Indeed, molecular analysis revealed that the two changes identified in the index case were in a trans configuration, with the FS missense mutation inherited from the father and the Del4 frameshift deletion from the mother, consistently with the proband being a compound heterozygote (Figure 2).

### In vitro electrophysiology

In order to determine how the two variants could affect cardiac sodium currents, *in vitro* electrophysiology studies were performed. We generated two plasmids carrying Del4 or FS variants by site-directed mutagenesis and the biophysical characteristics of the mutants were studied and compared to WT channels using whole-cell patch-clamp methods.

Figure 3A shows representative macroscopic current traces obtained from HEK293 cells transiently transfected with SCN5A WT, FS, Del4 or co-transfection of FS+Del4 to mimic the proband’s compound heterozygous condition. Figure 3B shows their current-voltage relationships, suggesting that current density was significantly attenuated in the cells trans-

![Figure 3A](image1.png)  
**Figure 3A.** Representative macroscopic current traces obtained from HEK293 cells transiently transfected with SCN5A WT, FS, Del4 or co-transfection of FS+Del4 to mimic the proband’s compound heterozygous condition.

![Figure 3B](image2.png)  
**Figure 3B.** Current-voltage relationships of HEK293 cells transiently transfected with SCN5A WT, FS, Del4 or co-transfection of FS+Del4 to mimic the proband’s compound heterozygous condition.

| WT | FS | Del4 | FS+Del4 |
|----|----|------|---------|
| Current density (pA/pF) | -259.7±7.0 | -150.7±5.9* | -1.26±0.22** | -64.1±2.5* |
| Activation | Vh (mV) | -40.6±0.6 | -41.0±0.3 | NA | -31.6±0.3** |
| | k | 7.6±0.5 | 7.1±0.2 | NA | 7.7±0.3 |
| Fast-inactivation | Vh (mV) | -91.4±0.1 | -84.3±0.2** | NA | -84.4±0.5** |
| | k | 7.8±0.1 | 7.1±0.2 | NA | 6.6±0.4 |

Data were represented as mean±SD. The numbers in parenthesis represent number of patches. WT, wild-type; FS, p.F1293S; A, slope factor; Vh, midpoint; NA, not applicable; *P<0.05 and **P<0.01 vs SCN5A WT; ^P<0.01 vs FS.
fected with FS. On the other hand, no significant current was obtained from cells transfected with Del4 alone (Table 2). Accordingly, co-expression of FS and Del4 showed further decreased current compared to FS alone (from 58% to 24% compared to WT).

The normalized conductance of WT, FS and co-expression of FS+Del4 was then plotted against the membrane potentials (Figure 3C). Steady-state inactivation was examined by a pre-pulse protocol and normalized currents were plotted as a function of membrane potentials. Data were fitted with the Boltzmann’s equation (see Materials and Methods section, for data analysis): co-expression of FS+Del4 was found to shift the voltage-dependency of steady activation towards more positive potentials by about 10 mV (P<0.01 vs WT or FS), but FS alone had no significant effect (P=0.12 vs WT) (Table 2). Furthermore, FS alone and FS+Del4 were found to shift towards more positive potentials the voltage-dependency of the steady-state inactivation by about 7 mV (P<0.01 vs WT). In addition, FS rendered the conduction-voltage relation steeper than the WT (slope factor, 5.1±0.2 vs 7.6±0.5, P<0.05) (Table 2). There was no further difference in steady state activation and inactivation. A magnified illustration in the inset indicates the window currents, reflecting the current densities of WT and mutant channels: the co-expression of Del4 shifted the activation curve of FS towards more positive values, which might contribute to the further decreased current density by Del4.

Discussion

Although BrS is considered a rare disease, more than 300 mutations in the SCN5A gene have been identified to date, generally transmitted in a dominant fashion.1 However, the incomplete penetrance and variable expressivity in BrS suggests that compound heterozygosis in each of the BrS-causing genes or digenic inheritance with double heterozygote BrS subjects may be determined and modulate the clinical phenotype.

The present study reports a BrS patient carrying two heterozygous SCN5A gene variants in a trans configuration. The first mutation (c.3258_3261del4) is a novel frameshift deletion in exon 18, creating a premature stop codon in the cytoplasmic linker between domains DII and DIII, which is compatible with loss of function of the sodium channel. The second change (p.F1293S) remains controversial, because it had been previously identified in a BrS patient and in apparently healthy individuals.8 Although computer-based algorithms PolyPhen-2 and SIFT indicated this substitution may be tolerated, its localization in the extracellular linker between S2-S3 in domain DIII, close to the voltage sensor segment, and the aminoacid conservation among different species suggests a possible pathogenic role.

Clinically, the three year-old proband, who is compound heterozygote for the aforementioned SCN5A changes, presented with heart murmur and displayed transient BrS type I ECG pattern with right bundle branch block and inverted T waves. The patient did not show other symptoms, although Holter monitoring recorded an isolated episode of self-terminating ventricular tachycardia. The parents, each carrier of either change, were completely asymptomatic and failed to display a BrS ECG phenotype. The mother carried the most severe mutation (Del4), while the father harbored the FS substitution, leading to a lesser decrease of sodium current. Gender is known to play a role in the variable expressivity of BrS. In fact, despite equal genetic transmission of the mutation, the clinical phenotype is 8 to 10 times more prevalent in males than in females.9 We may speculate that the mother, carrying the frameshift deletion mutation, did not present with symptoms because she was protected by the female sex and the father carried a relatively mild mutation.

The effects of these variants were studied by heterologous expression of the mutated channels in HEK293 cells. Both mutations significantly reduced the amplitude of sodium current compared with WT and were thus expected to generate the substrate for the BrS phenotype. Accordingly, loss of Na+ current by BrS mutations has been linked to three primary mechanisms: (i) truncation of the Na+ channel α-subunit, which yields a non-functional channel6 (ii) alteration in channel gating, such as changes in activation, inactivation, or reactivation kinetics21 and (iii) altered trafficking of the channels through the endoplasmic reticulum/Golgi complex to the plasma membrane.22 A common denominator of these mutations is a reduced cardiac Na+ current leaving the transient outward K+ current (Ito) unopposed in phase 1 and loss of the action potential dome in the right ventricular epicardium. In the present study, Del4 mutant sodium channels exhibit complete loss of function, while FS showed reduced current and a shift towards more positive potentials of the voltage-dependency of the steady-state inactivation, which might also cause loss of channel function. Co-expression of FS+Del4 mutants halved current compared to FS alone (58% to 24% with respect

Figure 3. Patch clamp current traces and current-voltage relationships of p.F1293S (FS), Del4, and FS+Del4 mutations in HEK293 cells. (A) Superimposed whole-cell current traces induced by a step-pulse protocol from a holding potential of -140 mV. (B) Current-voltage relationship of peak current densities. (C) Voltage-dependence of peak conductance and steady-state fast inactivation. The inset indicates the magnified illustration of the window currents within the dash square. The solid line represents wild type; the dot-dash line represents FS mutant and the dot line represents co-expression of FS and with Del4 mutants. The data were represented as mean±S.E.M.
to WT). This observation, which can be attributed to differences in the amount of FS DNA transfected (2.5 μg in co-transfection vs 5 μg in single transfection), may mimic the situation in the proband, harboring only a partially functional allele and a null one. In the case of the father, heterozygous carrier of FS mutation, probably the current will be higher than the 58% recorded in in vitro experiments, because of the presence of the WT allele. However, we chose to measure current of FS channels alone, in order to investigate current reduction caused by the mutation without interference of the WT SCN5A.

Only a brief and occasional episode of atypical ventricular tachycardia was documented in the proband during Holter monitoring and he has been asymptomatic ever since in a two years follow up. Indeed, the identification of new SCN5A changes in asymptomatic cases can lead to controversial interpretation, because of the difficulty in establishing correlations with the phenotype and the risk of malignant arrhythmic events. Asymptomatic individuals with a BrS ECG pattern generally have a better prognosis than their symptomatic counterparts, but this may not be always the case. Asymptomatic patients carrying functionally proven SCN5A mutations with loss-of-function properties should therefore be followed carefully to avoid potentially lethal events. In this case, since the proband missed any WT copy of the gene, and hence any fully functional sodium channel, a severe phenotype would have been expected. Of course, given the young age of the proband, a longer follow up will be necessary to verify these findings. However, the clinical consequence of SCN5A mutations may also be greatly influenced by unknown environmental factors and genetic modifiers.

The identification of double SCN5A mutants raises the possibility that many patients with a genetic SCN5A alteration may carry additional variants in the same or other genes, further complicating the genetic picture. Indeed, in spite of the low prevalence of SCN5A alterations, double mutants have been described in few cases to date, both in the same and in different alleles. Generally, in these cases the two variants consisted of a mutation associated with a rare polymorphism, which may have a modulator effect. However, another study reported an infant carrying two SCN5A mutations in trans, which co-expressed in vitro elicited a significant increase in late sodium current not present in either mutant alone, suggesting interaction between the mutant proteins. In addition, combination of different loss-of-function mutations in depolarizing channel genes has been described in infants with BrS-like phenotype. Only a wider genetic screening with high throughput technologies will therefore allow a better understanding of genotype/phenotype correlations and pathogenetic mechanisms, which in turn will improve patient clinical management.

### Conclusions

In summary, we found two heterozygous SCN5A gene mutations impairing the function of sodium channels in a BrS asymptomatic child and his parents. Genetic analysis confirmed therefore the diagnosis of BrS, ensuring him a proper follow-up, and prompted the identification of two asymptomatic individuals who might be at risk of potentially life-threatening arrhythmias. Although patch-clamp analysis revealed that each mutation produced a severe reduction of Na+ current, neither mutation alone determined ECG changes in the parents. Only the combined effect of the two variants resulted in a sufficient loss of sodium current recognizable at surface ECG, albeit not associated with a typical BrS clinical phenotype. Hence, our results suggest that the interactions between genetic mutations, environmental factors and phenotypic expression are increasingly complex, thus requiring collection and association of extensive genetic and clinical data.

### Study limitations

The results of our study show a clear loss of current by Del4 and FS, alone and in combination, compared with WT channels. However, caution should be exercised when translating in vitro data to clinical setting. Several factors may explain the discrepancy between the strong loss of function observed in in vitro expression systems and the minimal phenotypic expression observed in mutation carriers. HEK293 cells used in the present study expressed only the alpha subunit of the sodium channel, whereas cardiac myocytes have additional proteins that were not present in this in vitro model. In addition, the voltage-clamp protocol and the perfusion temperature used in the study were far different from the physiological conditions. Decreased current resulting from the co-expression of FS+Del4 might also be due to differences in transfection efficiency or to interactions between the two plasmids. Further clinical and genetic studies with longer follow-up periods will be required to evaluate the predictive value of SCN5A mutations in asymptomatic subjects.

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