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Immunohistochemical Marker for Na⁺ CP Type V α (C-20) and Heterozygous Nonsense *SCN5A* Mutation W822X in a Sudden Cardiac Death Induced by Mild Anaphylactic Reaction

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Abstract: A sudden death likely due to mild anaphylactic reaction in a young man is described. Autoptic, histologic, immunohistochemical, and laboratory findings were strongly consistent with the diagnosis of a mild anaphylactic reaction. Genetic molecular analysis, performed on formalin-fixed, paraffin-embedded tissues, showed a mutation described as W822X in a family with electrocardiographic pattern typical of Brugada Syndrome. It results in a nonsense mutation generating a truncated form of the channel protein. The mutation is due to a point substitution of a guanine with an adenine residue (G2466A). Immunohistochemistry and laser scanning confocal microscopy on sections from heart formalin-fixed, paraffin-embedded tissues led us to confirm the cellular localization of the Na⁺ CP type V α (C-20) at the intercalated disks of ventricular myocytes and nearly 50% reduction in Na⁺ channels expression in ventricular myocytes when compared with control cases. We suggest that the anaphylactic reaction that occurred in the young man could serve as a trigger mechanism, responsible for his sudden death with a *SCN5A* mutation associated with the Brugada syndrome.

Key Words: Na⁺ CP type V α (C-20), confocal laser scanning microscopy, immunohistochemistry, sodium channel, channelopathies, W822X, sudden cardiac death

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The *SCN5A* has been identified as the gene involved in heritable forms of Brugada syndrome (BrS); this channel produces a depolarizing inward sodium current I_{Na} and then inactivates within milliseconds¹. Mutations in *SCN5A* leading to the BrS phenotype are associated

with a loss of channel function and a reduction of the Na⁺ current.^{2–6} BrS is associated with life-threatening ventricular arrhythmias and with high incidence of sudden death, but no morphologic changes is usually observed in subjects with structurally normal hearts. BrS is characterized by a specific electrocardiographic (ECG) pattern of right bundle branch block and ST segment elevation in leads V_{1–3} with a normal QT interval, and no structural heart disease.⁷ The syndrome is inherited as an autosomal dominant trait. Even when the genetic abnormality is present, the ECG pattern may be normal, but they can be unmasked by fever, vagotonic agents, sodium channel blockers, other medications, and even external factors may act as trigger for ventricular arrhythmias in patients with this disorder.

In this study, we investigated and screened for *SCN5A* mutations in a previously healthy 24-year-old man who suddenly collapsed and died; postmortem findings were strongly consistent with a cardiac fibrillation due to mild anaphylactic reaction occurring just before death. The decedent's family (parents and the sister) was also clinically investigated.

CASE REPORT

Clinical and Autoptical Evaluation

The man was 24-year old who suddenly collapsed after having eaten peanuts 30 minutes before. Bystanders started cardiopulmonary resuscitation, while his friends called the emergency service. Upon the arrival of the rescue team, the man was already unconscious and pulseless. The blood pressure was undetectable. ECG monitoring revealed ventricular fibrillation; cardiopulmonary resuscitation was unsuccessful. History taken from family members revealed that the patient had been apparently in good health. He had no history of allergies; he had never complained of palpitations nor had he ever experienced loss of consciousness. There was no history of sudden death in the family and the man was not taking any medications.

Autopsy was performed 24 hours after the death. External examination was unremarkable. The heart was normal in size and weight; the coronary vessels and main branches were normal. Macroscopically, the lungs appeared edematous, heavy, and foamy. The examination of other organs was unremarkable.

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Routine microscopic histopathologic studies were performed on all organ samples using hematoxylin-eosin staining. Immunohistochemistry was performed on cardiac tissue samples using polyclonal antibodies anti-Na⁺ CP type V α (C-20) (Santa Cruz, CA). We used 4- μ m thick paraffin sections mounted on slides covered with 3, aminopropyl-triethoxysilane (Fluka, Buchs, Switzerland). Pretreatment was necessary to facilitate antigen retrieval, and to increase membrane permeability to antibodies. That was accomplished by boiling slides in 0.1M citric acid buffer for 14 minutes. The primary antibody was applied in a 1:500 ratio for 120 minutes at 20°C. The detection system used was the LSAB+ kit (Dako, Copenhagen, Denmark), a refined avidin-biotin technique, in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. The sections were covered with the TdT enzyme, diluted in a ratio of 30% in reaction buffer (Apotag Plus Peroxidase In Situ Apoptosis Detection Kit, Chemicon) and incubated for 60 minutes at 38°C. The positive reaction was visualised by 3,3 diaminobenzidine peroxidation, according to standard methods. Immunohistochemical techniques were used to estimate mast cell population,⁸ using the anti-tryptase antibody as mast cell specific marker on 5- μ m thick paraffin sections. Enzyme pretreatment with proteinase K (0.01%; 37°C) was necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies. The primary anti-tryptase antibody (DAKO) was applied in a 1:100 ratio and incubated overnight at 4°C. The positive reaction was visualized by 3-diamino 9-ethyl carbazole (Sigma). The sections were counterstained with Mayer's hematoxylin, dehydrated, cover slips applied, and the slides observed in a Leica DM5000B optical microscope (Leica, Cambridge, UK). Cardiac myocytes were studied using a confocal imaging system microscope^{9,10} (CLSM SPE Leica).

Laboratory Analysis

Toxicologic analysis of blood and urine specimens for therapeutic and nontherapeutic drugs was performed using gas chromatography-mass spectrometry. Serum concentration of mast cell β -tryptase in femoral blood was also performed by means of an immunoenzymatic technique. At the dissecting table, 10cm³ of femoral blood was extracted and immediately frozen at -20°C. The tryptase was dosed using an immunoenzymatic technique (UniCAP TRYPTASE Fluoroenzymeimmunoassay Pharmacia, AB Uppsala, Sweden). Tryptase level $\leq 40 \mu\text{g/L}$ was considered to indicate that mast cell had not occurred near the time of death. The lower limit of sensitivity for the assay was 10 $\mu\text{g/L}$.

Molecular Genetics

A genetic screen was performed on sections from formalin-fixed and paraffin-embedded samples. Each sample consisted of three 30- μ m sections. After paraffin removal, DNA extraction was accomplished using the Nucleon HT Hard Tissues Extraction Kit (Amersham Biosciences) and following the manufacturers' instructions. The kit is designed specifically for difficult samples such as formalin-fixed, paraffin-embedded tissues. Extracted DNA was resuspended in DNAse free water, and quantified spectrophotometrically (A260/A280).

Double Polymerase Chain Reaction

A modified double polymerase chain reaction (PCR) protocol was used.^{11,12} Two microgram samples of DNA extracted from each sample were used to carry out PCR reactions using Phusion High-Fidelity PCR Kit (Finnzymes).

Reactions were performed in a MyCycler Thermal Cycler (Bio-Rad) with an initial step of denaturation (98°C, 30 s), followed by 35 cycles of denaturation (98°C, 10 s), annealing (47 to 52°C, depending from the primers used, 30 s), and extension (72°C, 1 min), with a subsequent final extension step (72°C, 10 min). Primers used for this study are indicated below. In particular, SCN5A exons have been amplified using published intron-primers.¹³ After the first PCR, 5 μL of the first amplified reaction were used to carry out a second PCR protocol, using the same primers and the same cycle steps. After the second PCR reactions, the PCR products were evaluated by electrophoresis on 2.5% high resolution agarose gel. To better estimate the molecular weight of the PCR products, 2 types of molecular weight ladders were used, a 100bp (Amersham Biosciences) and another a 50 bp one (Sigma).

List of primers used in this study:

β Actin Forward: 5'-AAACTGGAACGGTGAAGGTG-3'
 β Actin Reverse: 5'-TCAAGTTGGGGACAAAAAG-3'
 SCN5A Exon 9 Forward: 5'-GGGAGACAAGTCCA GCCCAGCAA-3'
 SCN5A Exon 9 Reverse: 5'-AGCCCCACACTTGCTG TCCCTTG-3'
 SCN5A Exon 12 Forward: 5'-GCCAGTGGCTCAAAA GACAGGCT-3'
 SCN5A Exon 12 Reverse: 5'-CCTGGGCACTGGTC CGGCGCA-3'
 SCN5A Exon 16 Forward: 5'-GAGCCAGAGACCTTC ACAAGGTCCCCCT-3'
 SCN5A Exon 16 Reverse: 5'-CCCTTGCCACTTACCA CAAG-3'
 SCN5A Exon 22 Forward: 5'-AGTGGGGAGCTGTTC CCATCCT-3'
 SCN5A Exon 22 Reverse: 5'-GGACCGCCTCCAC TCC-3'
 SCN5A Exon 23 Forward: 5'-TTGAAAAGGAAATG TGCTCTGGG-3'
 SCN5A Exon 23 Reverse: 5'-AACATCATGGGTGAT GGCCAT-3'

DNA Purification From Agarose Gels

After electrophoresis on 2.5% agarose gels, the PCR products of the expected molecular weight were cut from the gel and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), following manufacturers' instructions. The DNA obtained was eluted in DEPC -treated water, spectrophotometrically quantified and used for incubation with appropriate restriction enzymes.

Restriction Fragment Length Polymorphism Analysis

Amplified fragments, cut and purified from the gel, were incubated with appropriate restriction enzymes to verify the presence of a mutation that resulted in either the loss or in the gain of a restriction site in its sequence. The enzymes used in this work were: Hae II (Promega) MspAII (Promega), Van 91I (Roche), RCA I (Roche), BstOI (Promega). Restriction reactions were carried out following the manufacturers' instructions. For each reaction, 1 microgram of DNA was digested with the appropriate restriction enzyme. Control experiments were performed to ensure enzyme specificity, and to avoid over-digestion of the bands. Control experiments were performed using DNA extracted from healthy volunteers and from human cell lines not bearing mutations on the gene of interest.

1 Electrophoresis on Agarose Gel

3 The restriction products were electrophoresed on 2.5%
5 agarose gel. The molecular weight of the cutting products was
7 compared with the molecular weight of the uncut DNA
9 fragment, and with negative controls.

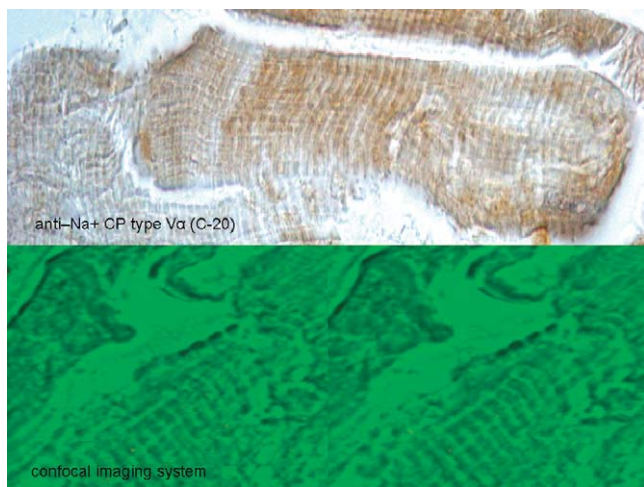
7 Family's Clinical and Molecular Genetics Evaluation

9 The sister and parents of the deceased underwent detailed
11 clinical examinations including baseline 12 leads ECG, but they
13 refused the genetic screening.

13 RESULTS

15 Histologic, Immunohistochemical, and Confocal Laser Microscopy Results

17 Histologic examination revealed generalized stasis.
19 Areas of focal pulmonary edema mixed with areas of
21 acute pulmonary emphysema were observed. Examina-
23 tion of cardiomyocytes showed eosinophilic cross-bands
25 ranging from segments of hypercontracted or coagulated
27 sarcomeres, to total disruption of myofibrils or at least
29 extreme granulation. Myocardial contraction band necrosis
31 was variably distributed in multiple foci. The
33 number of foci and myocytes with nonhemorrhagic
35 contraction band necrosis was quantitated. There were
37 219 and 272 in the anterior and 594 and 461/100 mm² in
39 the posterior left ventricle, 19 and 36 in the interven-
41 tricular septum, and 6 and 22 in the right ventricle. The
43 cellular localization of the Na⁺ CP type V α (C-20) was
45 concentrated in the intercalated disks of ventricular
47 myocytes (Fig. 1). Our results confirm the specific
49 localization of this subtype of sodium channel to the
51 intercalated disk. Heterozygote expression in hearts



55 **FIGURE 1.** Immunohistochemistry on cardiac tissue samples
57 using polyclonal antibodies anti-Na⁺ CP type V α (C-20);
59 positive reaction at the intercalated disks (100 $\times$). Confocal
laser scanning microscopy confirms the specific localization of
the subtype of sodium channel to the intercalated disk
(orange punctuate positiveness).

61 samples showed a nearly 50% reduction in Na⁺ channels
63 expression in ventricular myocytes when compared with
65 control cases (Fig. 2).

67 A large number of degranulating mast cells with
69 tryptase-positive material external to the cell membrane
71 was observed. The halo of tryptase positiveness around
73 the mast cells revealed evidence of mast cell degranulation
(Fig. 3).

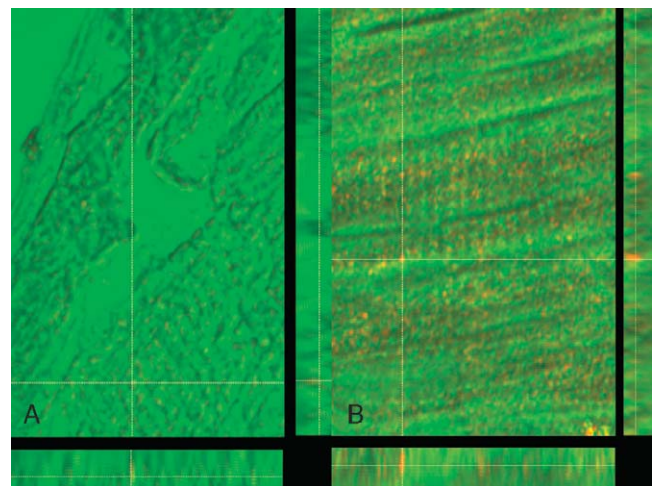
69 Laboratory Analysis

71 Toxicologic analysis resulted negative. The concen-
73 tration of mast cell α -tryptase detected serum was 111 μ g/
L (postmortem normal value ≤ 40 μ g/L).

75 Molecular Genetics

77 Multiple tissue blocks were sampled. Recent litera-
79 ture reports suggest that, formalin exposure should result
81 in extensive nucleic acids fragmentation, and that
83 extraction protocols developed for paraffin-embedded
85 tissues should result in the recovery of short-length
87 fragments (typically ranging from 200 to 600 bp), which
89 should be adequate for restriction fragment length
polymorphism analyses of exons in the *SCN5A* gene.
The double PCR approach has been chosen for its
extreme sensitivity in analyzing difficult samples, as
reported in previous studies.^{11,12} Control standard PCR
reactions, with a single amplification for as long as 45
cycles, failed to amplify the target fragment in all but one
of the samples, confirming the goodness of the choice for
a double PCR assay (not shown).

91 From DNA samples positive for β -actin fragment
93 expression, we then performed double PCR reactions on a
95 selected number of exons of the *SCN5A* gene.¹⁴⁻¹⁶ The
97 primer sequences, listed in methods, were intronic
99 primers, previously published by Wang and collabora-
101 tors.^{13,17} Their use has been validated in other subsequent



115 **FIGURE 2.** Heterozygote expression in hearts samples showed
117 a nearly 50% reduction in Na⁺ channels expression in
ventricular myocytes (A) when compared with control cases
(B) (orange punctuate positiveness).

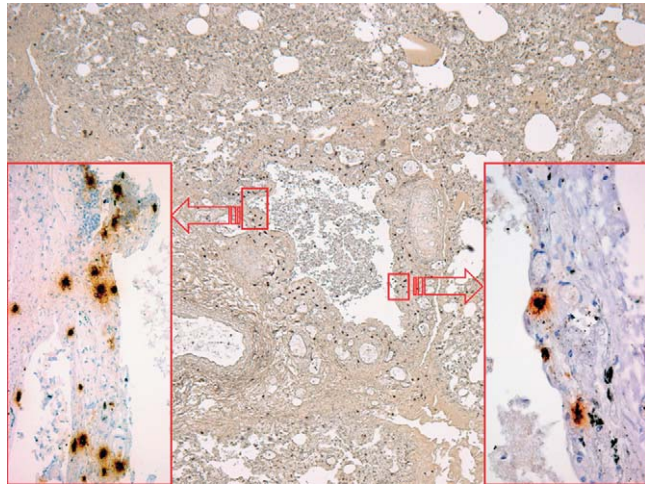


FIGURE 3. Lung: degranulating mast cells with tryptase-positive material outside the cells was documented (Ab anti-tryptase, $\times 10$). A halo (golden reaction) of tryptase positivity around the mast cells (inserts) reveals evidence of mast cell degranulation (Ab anti-tryptase, $\times 100$).

studies. Exon-targeted PCR disclosed that sequences from exons 9, 12, 16, 22, and 23 had successfully been amplified from the samples, and these were used for further restriction fragment length polymorphism analyses.

We found that between the 5 exons investigated, the mutated genotype was present in exon 16 (Fig. 4). The PCR product of exon 16 is a 400-bp band, which was successfully amplified in the investigated subject and in the control genome coming from cultured human cells, negative for this mutation. The PCR product coming from healthy control specimen (lane 2) was cut from the restriction enzyme, showing the band of 400 bp and the 350-bp band indicative of the cut at position 26 of the exon sequence. The DNA fragment coming from the investigated subjects resulted uncut from the restriction enzyme contrary to the control specimen. Lack of this restriction site is a feature carried by the mutation described as W822X,¹⁸ which results in a nonsense mutation generating a truncated form of the channel protein. The mutation is due to a point substitution of a guanine with an adenine residue (G2466A). As a control, an overnight digestion reaction, as well as an overdigest with modified enzyme-substrate were performed (not shown), resulting in the absence of restriction products indicative of enzyme cutting.

Family Evaluation

An ECG in the asymptomatic 20-year-old sister showed changes consistent with a type 2 BrS (Fig. 5). To confirm BrS ECG, she underwent a flecainide test, which was positive. A cardioverter-defibrillator was immediately implanted. So far her clinical course has been uneventful. Both of the parents had normal appearing ECG, but they decline provocation testing.

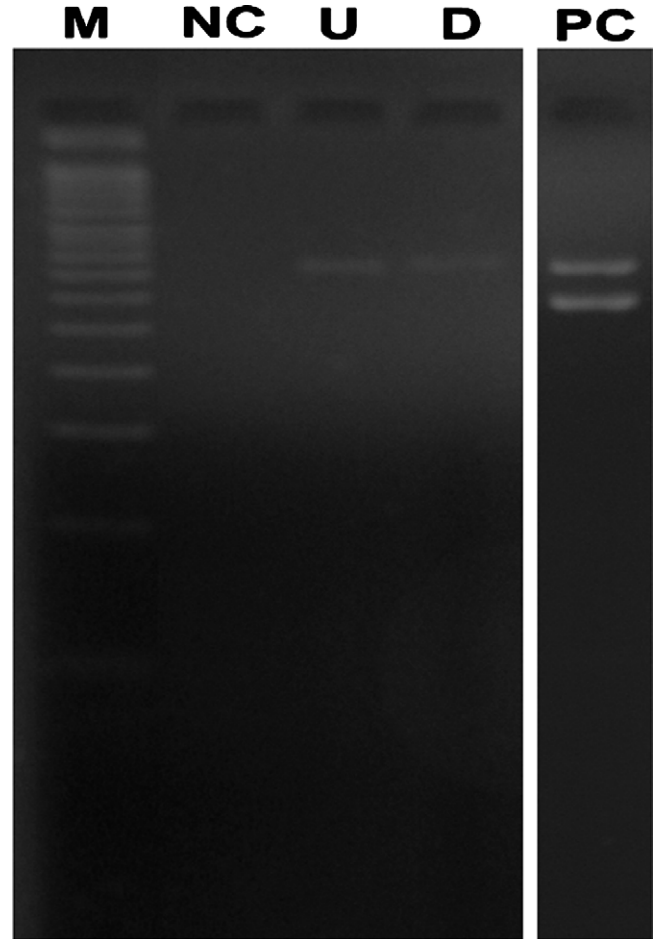


FIGURE 4. Restriction pattern of exon 16 of *SCN5A* gene in investigated patient and in control DNA from cultured human cells. M: 50-bp ladder; NC: No polymerase negative control; U: Undigested fragment of exon 16; D: Restriction pattern of exon 16 amplified from the investigated subject, showing that the restriction enzyme did not perform the expected cut; PC: restriction products of exon 16, amplified from a human cell line wild type for the mutation, showing the presence of both the unrestricted band (400 bp) and the cleavage product at about 350 bp.

DISCUSSION

A fatal ventricular arrhythmia likely due to mild anaphylactic reaction was responsible for the sudden death in the young man carrying a nonsense mutation described as W822X, typical of BrS. Blood β -tryptase level (111 $\mu\text{g/L}$) and β -tryptase immunohistochemical investigation provided paramount information in assessing the cause of death.

Brugada and Brugada¹ were the first to report a distinct clinical and ECG syndrome consisting of right bundle branch block with a normal QT interval and persistent ST segment elevation in precordial leads V₁ to V₂-V₃, occurring in individuals without electrolyte disturbances, ischemia or structural heart disease, but associated nonetheless with a high rate of sudden

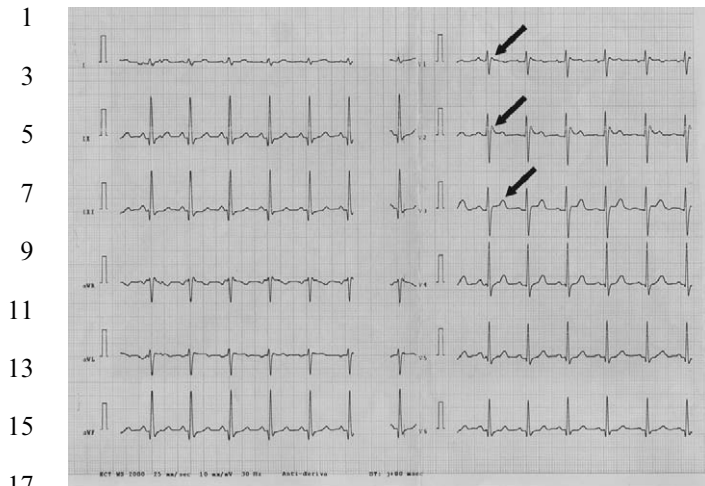


FIGURE 5. Sister's ECG: saddleback appearance with a high take-off ST-segment elevation of ≥ 2 mm followed by a trough displaying ≥ 1 mm ST elevation followed by a positive T wave (type 2). ECG indicates electrocardiogram.

unexpected death. Since the initial report, significant progress has been made in understanding the pathophysiologic aspects of this disease. However, the precise pathophysiology remains to be fully elucidated. Different mutations in the cardiac sodium channel gene *SCN5A* have been reported; most cause a loss of cardiac sodium channel function.¹⁹ According to current theory, loss of function caused by reduced sodium currents results in pronounced early repolarization of the right ventricular epicardium because this is where potassium current “*I_{to}*” (transient outward current), is predominantly localized. The result is marked abbreviation of the action potential duration in the epicardium, but without any remarkable effect on action potential duration in the endocardium. The result is a transmural voltage gradient that manifests itself as an ST segment elevation in the right precordial leads of the ECG, and as a transmural dispersion of repolarization duration and refraction. The latter, can serve as a substrate for the development of ventricular arrhythmias via a phase 2 reentry mechanism.²⁰ One feature of BrS is that ECG changes vary over time. Several reports have described ECG in patients with BrS that change dynamically, both in terms of configuration as well as degree of ST segment elevation in precordial leads.²¹ The typical ECG pattern is modulated by exercise or pharmacologic interventions that interact with autonomic nervous activities.^{22–23} Autonomic dysfunction manifested by increased parasympathetic tone and decreased sympathetic tone has been demonstrated to be a specific property of symptomatic BrS. This is supported by evidence that the level of ST segment elevation in this disorder is increased by high vagal tone, β -adrenergic blockade, and α -adrenergic stimulation, but reduced by β -adrenergic stimulation and α -adrenergic blockade.^{4,24–26} Although the mechanism responsible for

ST elevation in BrS remains unclear, a relationship between ST segment elevation and occurrence of ventricular arrhythmias including VF has been reported by many investigators.^{22,27–28} The exact mechanism in triggering and termination of electrical storms remain unclear. Brugada and other investigators have stressed the functional electrical change and indicated the importance of increased adrenergic tone.¹ Any conditions or medications that increase outward currents or decrease inward currents may precipitate or unmask BrS.²⁹ Therefore, sodium-channel blockers including antiarrhythmics, tricyclics, and anesthetics can unmask the syndrome by decreasing the inward current, as can calcium-channel blockers, β -blockers, cocaine, and antidepressants. Vagotonic agents and hypokalemia have the same effect, but they work by enhancing the outward current. Fever and other conditions such as primary hyperparathyroidism, pneumonia, and focal myocarditis have also been described as potential triggers for life-threatening arrhythmia in BrS.

In our case, autopsy and laboratory findings were strongly consistent with the diagnosis of a mild anaphylactic reaction. Genetic molecular analysis showed a mutation described as W822X by Keller et al¹⁸ in a family with ECG pattern typical of BrS. It results in a nonsense mutation generating a truncated form of the channel protein. Keller et al demonstrated that this mutation resulted in a loss of Na⁺ channel function, showing that the transfected tsA201 cells failed to exhibit any Na⁺ currents, indicating that the truncated protein was in fact totally inactive. Alterations in sodium channel expression and function are known to have severe effects on excitability. In the heart, mutations in the gene encoding Nav1.5 cause inherited hyperexcitability syndromes, including long QT syndrome type III and BrS, which can lead to sudden cardiac death. Differential expression and localization of sodium channel subunits are likely to be important determinants of electric excitability of cardiac myocytes.³⁰ Anaphylaxis is a condition in which severe and prolonged hypotension can occur in response to chemical mediators such as histamine, prostaglandins, and tryptase that are released, at least in part, by sensitized tissue mast cells and blood born basophils. These chemical mediators promote peripheral vasodilatation and increased vascular permeability, resulting in sustained decreases in mean arterial pressure.³¹ The autonomic changes associated with anaphylactic hypotension have been studied. Potas et al³¹ in an experimental study on rats found that anaphylactic hypotension was associated with tachycardia and increased renal sympathetic activity. The magnitudes of tachycardia and sympathoexcitation were correlated with the magnitude of the hypotension. It is also well known that in anaphylactic reactions, the cardiovascular system is frequently involved with the occurrence of arterial hypotension, cardiovascular collapse, arrhythmias, or chest pain.³² These symptoms are partly due to vasodilatation and increased vascular permeability leading to hypovolemia, but partly also to the direct cardiotoxic

1 effect of mast cell mediators and hypoxemia following
bronchospasm and shock.³³ Mast cell mediators (hista-
mine, leukotrienes) have cardiotoxic effects, including
vasoconstriction, negative inotropy, and arrhythmogen-
esis.³³ Insect stings are reported to be associated with
atrial fibrillation³⁴; however, until now it has not been
clear if anaphylaxis acts as a vasoconstriction trigger or
has a direct arrhythmogenic action. Quercia et al³⁵
documented a case of ventricular fibrillation that
occurred after a hymenoptera sting. This supports the
hypothesis of an anaphylactic reaction as a trigger of
vasoconstriction as a cause of ventricular arrhythmia in
instance where the arrhythmia cannot be explained by
other causes such as coronary disease, a myocardiopathy
or a pharmacologic toxicity.³⁶

Is it reasonable to suggest that the anaphylactic
reaction occurred in the young man could serve as a
trigger mechanism, responsible for the sudden death? It
certainly seems possible and one wonders how many
times anaphylaxis has been the trigger for sudden death in
individuals with BrS.

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