Mutational screening of SCN5A linked disorders in Polish patients and their family members

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Abstract. Mutations in SCN5A lead to a broad spectrum of phenotypes, including the Long QT syndrome, Brugada syndrome, Idiopathic ventricular fibrillation (IVF), Sudden infant death syndrome (SIDS) (probably regarded as a form of LQT3), Sudden unexplained nocturnal death syndrome (SUNDS) and isolated progressive cardiac conduction defect (ICCD) (Lev-Lenègre disease). Brugada Syndrome (BS) is a form of idiopathic ventricular fibrillation characterized by the right bundle-branch block pattern and ST elevation (STE) in the right precordial leads of the ECG. Mutations of the cardiac sodium channel SCN5A cause the disorder, and an implantable cardioverter-defibrillator is often recommended for affected individuals. In this study sequences of the coding region of the SCN5A gene were analysed in patients with the LQT3, Brugada Syndrome and other arrhythmogenic disorders. Different mSSCP patterns are described with no disease-related SSCP conformers in any sample. Direct sequencing of the SCN5A gene confirmed the absence of mutations. This suggests that the analysed region of the SCN5A gene is not commonly involved in the pathogenesis of the Brugada Syndrome and associated disorders.

Key words: Brugada Syndrome, long QT Syndrome, mutation, mSSCP analysis, SCN5A gene, sodium channel.

Introduction

Mutations in SCN5A result in multiple arrhythmic syndromes, including LQT3 (Long QT Syndrome type 3), IVF/BS (idiopathic ventricular fibrillation/ Brugada Syndrome), an inherited cardiac conduction defect (ICCD), SUNDS (Sudden Unexplained Nocturnal Death Syndrome) and SIDS (Sudden Infant Death Syndrome), constituting a spectrum of disease entities termed Na⁺ channelopathies (Moric et al. 2003). The Brugada syndrome is the mirror image of LQT3 and both are known to share the same position or locus on a specific chromosome in the diploid cells (3p24–p21). These diseases are allelic disorders, if not the same disease with variable penetrance and variable modifiers world-wide. Interestingly, death occurs during sleep in all of these disorders, suggesting a common mechanism (Schwartz et al. 2000; Splawski et al. 2000; Moric et al. 2003).

The loss of function in the cardiac sodium channels, either by reducing expression levels or by increasing its inactivation kinetics, is the main pathological origin of the Brugada syndrome, whereas the mutation of LQT3 is associated with a gain in the function mechanism with a slow and constant entry of Na⁺ in phase 2 (prolonged inactivated), which in turn increases the duration of the ST segment leading to a prolonged QTc at the expense of ST, altogether resulting in the late appearance of the T-wave (Balser 1999; Deschenes et al. 2000). Gellens et al. (1992) demonstrated that SCN5A is the gene encoding the α-subunits of the sodium
channels that are associated with initiating cardiac action potentials. George et al. (1995) mapped the SCN5A gene to 3p21, thus making it an important candidate gene for the long QT syndrome-3 (LQT3).

The SCN5A gene consists of 28 exons spanning 80 kb and encodes a complete ion channel (without complexing with a beta subunit) with 2016 amino acids (Gellens et al. 1992; Vatta et al. 2000). SCN5A is highly expressed in the human myocardium but not in the skeletal muscles, liver or uterus (Wang et al. 1995a,b). It was recently reported by Hartmann et al. (1999) to be expressed in the brain. The SCN5A gene expressed in the heart plays a vital role in the generation and propagation of the cardiac impulse in working cardiac myocytes and cells of the His-Purkinje system (Grant 2001).

The SCN5A gene encodes a protein with a predicted topology of four domains, DI through DIV. The homologous domains are joined by the intra-cytoplasmic inter-domain loops IDI/II to IDIII/IV. The first loop, IDI/II has multiple consensus sequences for protein kinase A-dependent phosphorylation. IDIII and IDIV are among the most variable regions of the α-subunit. IDIII/IV is short and greatly conserved among sodium channel isoforms (Grant 2001).

It is believed that each of the four domains bears a structure with six trans-membrane spanning regions (S1-S6) and pore domains found between S5 and S6. The S1 to S6 segments are hydrophobic and have an α-helical conformation of sufficient length to cross the membrane. The fourth trans-membrane segment S4 is the voltage sensor. The loop between S5 and S6 is thought to curve back into the membrane to form the pore or P-loop. The amino and carboxy-termini ends are located intracellularly (Figure 1).

LQT3 accounts for approximately 5-10% of the genotyped LQT families (Catterall 1993; Wang et al. 1996). Molecular biological analysis can document SCN5A gene mutations in 10-20% of patients with BS (Priori et al. 2002).

To date, mutational analyses have revealed about 105 distinct mutations in SCN5A, out of which more than 30 mutations are associated with LQT3 and 48 mutations are associated with BS, whereas the rest of the mutations are affiliated with the remaining sodium channel disorders (Moric et. al. 2003). Missense mutations were most common (72%), followed by deletions (10%), splice-site mutations (8%), frameshift mutations (5%), and nonsense (4%). Most mutations resided in the intracellular (52%) and transmembrane (30%) domains, others were found in pore (12%) and in extracellular segments (6%) (Herbert et al. unpublished).

The major aim of this study was to screen for novel gene mutations linked with the SCN5A gene and possible genetic heterogeneity as to identify patients at the risk of sudden cardiac death among 66 subjects: 45 patients with various arrhythmogenic disorders and their healthy family members.

![Figure 1. Predicted organization of SCN5A. A – Genomic structure of SCN5A; exons and introns are indicated by open boxes and horizontal lines; size of each exon is given below as bp. B – Schematic representation of a voltage-gated Na+ channel α-subunit composed of four domain (DI- DIV), each of six membrane-spanning alpha helices (S1–S6) and β-subunit.](image-url)
In this study the sequence analysis of exons 2 through 28 (27 exons) of the α-subunit were studied, except for exon 1 which was excluded as it corresponds to the 5’ UTR part of the gene. Mutations in this coding region could cause variable arrhythmogenic disorders including LQTS and BS.

**Materials and Methods**

**Genomic DNA extraction**

Sixty-six subjects were screened: patients from different families (n = 45), with the diagnosis based on clinical features, such as changes in ECG and echocardiography, and their healthy family members (n = 21) formed a group of individuals who were studied in detail (sequencing) (Table 1).

Genomic DNA was extracted from 5 mL samples of peripheral blood according to the standard protocol using Genomic DNA Prep Plus (A&A BIOTECHNOLOGY). The quality of extracted DNA samples was assessed by electrophoresis on 0.75% agarose gel, ethidium bromide staining and visualization on an UV transilluminator.

**Polymerase chain reaction (PCR)**

Forty-one oligonucleotide primer pairs used to amplify exons 2 to 28, as proposed by Wang et al. (1996), were synthesized by IDT, Inc., Gdansk. The PCR amplification was carried out in a total volume of 25 μL containing: Tfl buffer (200 mM Tris HCl pH 8.4, 500 mM KCl), Tfl PCR Enhancer, 25 mM magnesium chloride, 10 mM dNTP mix, 10 pmol each of the forward and reverse primers, 1 Unit of Tfl polymerase, 1-2 μg genomic DNA. PCR reactions were performed as follows: a) Samples were denatured at 94°C – 3 minutes – one cycle, followed by 35 cycles at: 94°C – 1 minute, 64°C – 1 minute, 72°C – 1 minute, and finally incubated at 72°C for 10 minutes b) touchdown PCR: 11 cycles with variable annealing temperature ranged between 70°C-60°C – 1 minute, followed by 30 cycles with annealing at 60°C c) touchdown PCR: 13 cycles with variable annealing temperature ranging between 70°C-58°C – 1 minute followed by 30 cycles with annealing at 60°C using a Perkin Elmer GeneAmp PCR System 9600. Products were identified by electrophoresis on 6-10% PAA gel with TAE buffer at 120-150 V for 45-60 minutes, and silver stained. Electrophoregrams were analysed with the GelScan v.1.45 software (Kucharczyk). Product sizes were computed based on the electrophoregrams.

**mSSCP analysis (multitemperature SSCP)**

For the purpose of the analysis 10 μL aliquots of the amplified samples were mixed with 10 μL of formamide loading dye (95% formamide, 20 mM EDTANa₂, 0.05% xylene cyanol, 0.05% bromophenol blue). Samples were subsequently denatured by heating at 95°C for 10 minutes, and placed on ice to avoid renaturation. Then the samples were loaded on 8% non-denaturing PAA gel (PAA acrylamide : bis acrylamide 37.5:1) in TBE buffer (0.89 M tris-base, 0.89 M boric acid, 20 mM EDTA pH 8.0), containing 5% glycerol. Horizontal electrophoresis was performed at 200 V at the increasing temperature gradient, of 4°C, 10°C and 25°C, for 2.5 h.

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<th>Table 1. Study population characteristics</th>
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<td><strong>Probands</strong></td>
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HCM = hypertrophic cardiomyopathy, LQT = long QT syndrome, IVF = idiopathic ventricular fibrillation, BS = Brugada Syndrome, ARVC = arrhythmogenic right ventricular cardiomyopathy, pts = patients, LVEF = left ventricular ejection fraction, IVS = interventricular septum, ECG = electrocardiogram, MRI = magnetic resonance imaging.
DNA sequencing

PCR products were analysed by direct PCR sequencing, using the dRhodamine-labelled nucleotide incorporation. The reaction mix in the total volume of 20 µL contained: 8 µL Terminator Ready Reaction Mix (Perkin Elmer), 10 pmols of the primer (reverse or forward), 10 µL of the PCR product purified using a Microcon-100 column. Samples were amplified using the following sequencing protocol: 96°C – 10 sec, 50°C – 5 sec, 60°C – 4 minutes; 25 cycles were performed. Nonincorporated labelled nucleotides were removed by means of purification on a Spin Column (Perkin Elmer). An automatic ABI PRISM 377 Sequencer was used for the recording, evaluation, and documentation of DNA sequences.

Results

A total number of 66 individuals were ascertained and phenotyped, including 45 patients with various arrhythmogenic disorders and their healthy family members. Sixty-six genomic DNA samples, extracted from the whole blood, were amplified with 41 different pairs of oligonucleotide primers proposed by Wang et al. (1996).

The screening for mutations was based on the mSSCP typing. Figure 2 shows an example of the analysis of exon 28 in 15 DNA samples. Typical results of the analyses for six out of the 22 analysed exons are shown as denstometric curves presented above the original electrophoregrams. Each of the amplified fragments displayed a characteristic, distinct SSCP pattern of single-strand and double-strand bands (Figure 3).

The results of the analysis, presented in Figure 3, revealed the presence of conformation variants in the amplified sequences of exons 3, 5, 13, 18, 20 and 28(VIII). In particular, the presence of more than two bands migrating as single strands seemed to indicate the sequence heterozygosity in exons 5, 13, 18 (four bands) and 28(VIII) (three bands). The virtually identical migration patterns (with more than two single-stranded bands) were obtained for suspected exons in all the samples, patients as well as the non-affected family members (Figure 2).

As presented in Figures 2 and 3, in exons 3; 5; 13; 18; 20; 28(VIII) of the SCN5A the existence of more than one type of the polymorphic band pattern were observed; i.e. for exon 3 – two types (776 bp + 576 bp; 1058 bp + 926 bp), for exon 5 – four types (1154 bp + 1034 bp; 1737 bp + 1591 bp + 874 bp + 677 bp; 1418 bp + 998 bp; 1442 bp + 1132 bp), exon 13 – three types (564 bp + 508 bp; 888 bp + 830 bp + 707 bp + 661 bp; 919 bp + 713 bp), exon 18 – three (895 bp + 803 bp; 678 bp + 628 bp; 1605 bp + 1500 bp + 750 bp + 612 bp), exon 20 – two (756 bp + 604 bp; 859 bp + 735 bp), exon 28(VIII) – three types (1248 bp + 1123 bp; 1248 bp + 1149 bp + 1026bp; 1149 bp + 1034 bp), respectively. It may indicate the existence of polymorphism types among these domains (exons) and these need to be confirmed by sequencing analysis.

The results of the visual inspection of the mSSCP bands were confirmed by the comparative analysis performed using GelScan v.1.45 software (Kucharczyk). Migration positions of the single stranded products were computed based on the results of mSSCP electrophoresis, in comparison with the molecular DNA size standard (plasmid DNA pBR322 digested with endonuclease HaeIII).

In addition to the mSSCP analysis, the DNA samples from all the investigated patients were analysed by nucleotide sequence determination. Direct sequencing of both DNA strands (i.e. using either the forward or the reverse primers) revealed the absence of mutations or polymorphisms in exons 3, 5, 13, 18, 20, and 28 of the SCN5A gene.
Figure 3. Pictorial view of electrophoregrams and densitometric curves showing different band patterns obtained from mSSCP analysis of exons: 3 (lane 1), 5 (lane 5), 13 (lane 5), 18 (lane 16), 20 (lane 15), 28(VIII) (lane 7).
The sequences were consistent with the sequence from the EMBL database www.ncbi.nlm.nih.gov (GenBank GI 37622905 – GI 37622906).

**Discussion**

The mSSCP analysis of six SCN5A exons suggested the presence of the heterozygous conformation variants in the amplified sequences of exons 3, 5, 13, 18, 20, and 28. The presence of four or three single strand bands in the SSCP patterns may indicate the presence of heterozygosity in the corresponding genomic segments (Figures 2,3). However, direct sequencing of the samples presented in Figures 2 and 3 did not reveal any heterozygous positions in the suspected exons, neither using forward nor reverse sequencing primers. Most importantly, the virtually identical migration patterns (with more than two single-stranded bands) obtained in the affected as well as in the non-affected individuals, indicated that – had the conformation variants reflected real nucleotide changes – they should have been regarded as common polymorphisms rather than disease-causing mutations.

The genomic organisation and sequence of the SCN5A gene were first reported by Wang et al. (1996). In that study a total of 41 primer pairs were used to determine the sequence of 28 exons by direct sequencing. Several studies have since used those primers for mutational analysis of some SCN5A exons by SSCP. However, a number of PCR fragments (approximately > 350 bp) are too large for effective SSCP analysis and contain long intronic sequences of unknown composition (Syrris et al. 2001). For example, some of the exons range from 53 (exon 24) to 3257 (exon 28) bp.

The primers as designed by Wang et al. (1996) were also used in this study and it may have contributed to some of our problems in the interpretation of the mSSCP results. Slight differences do exist between the primers of Wang et al. (1996) and those of Syrris et al. (2001), and these differences in fragment sizes of respective genes and their exons could sometimes be misleading with the specifics of mSSCP analysis.

Additionally, in an mSSCP analysis, the amplified product can be mixed in a 1 : 1 ratio with denaturing buffer, of which, if the dilution profile is not changed, the polymorphism is identical. However, if the temperature profile of mSSCP differs from the original, then obviously an additional mutation could be present, because there is no existing universal theoretical model of mSSCP analysis. Probably, due to a double polymorphism within a “long” PCR product, missense mutation in an SSCP gel could be missed. The direct sequence analysis of 27 exons of SCN5A gene was carried out in this study because of the complexity of the resolution profiles generated by the mSSCP technique.

The failure to identify any SCN5A mutation (or polymorphism) among the analysed individuals with various arrhythmogenic disorders is not surprising. This may be so because of the false negative results probably being caused by the relatively low sensitivity of SSCP analysis for mutation screening (the presence of transient conformers) compared to more advanced techniques, such as denaturing high performance liquid chromatography (DHPLC) (Jongbloed et al. 2002).

However, the SSCP methodology (the most widely used screening method) for mutation detection can detect between 88 and 90% of mutations (Condie et al. 1993) and therefore one cannot entirely exclude the presence of SCN5A mutations within this gene in these individuals. Though PCR and SSCP are sensitive for the detection of point mutations or small deletions, both methods could possibly miss large gene deletions. Furthermore, promoter mutations resulting in aberrant gene expression cannot be discounted in patients testing negative for mutations (Condie et al. 1993).

Direct sequencing of both DNA (i.e. using either the forward or the reverse primers) revealed the absence of mutations or polymorphisms in exons 2 through 28 of the SCN5A gene. This suggests that the analysed region of the SCN5A gene is not a common target for mutations in the investigated subjects. The sequence concurs with sequences from GenBank GI 37622906.

The fact that no new mutations or polymorphisms were found in this study coincidentally confirms the results of the clinical diagnosis because a majority of our patients are linked with idiopathic VF. It has been estimated that BS accounts for 10-20% of patients with idiopathic VF (Priori et al. 2002). However, it is difficult for cardiologists to diagnose patients since there exist variants of the electrocardiogram with minimal ST segment elevation and even concealed forms that can only be detected by the administration of class I anti-arrhythmic drugs.
Studies by previous investigators have also confirmed the absence of mutations in some patients linked with LQT, BS, raising the possibility of genetic heterogeneity (Priori et al. 2000; Weiss et al. 2002). Since the \textit{SCN5A} gene is highly conservative (Wang et al. 1996), it could be so that it is conservative to the Polish patients and their family relatives used in this study. The fact that no \textit{SCN5A} mutations were detected in all the patients confirmed the genetic heterogeneity for this disorder.

There is increasing awareness of the role of common polymorphisms in altering gene function and in susceptibility to disease. Studies have linked gene polymorphisms to elevated risk for cystic fibrosis, the Alzheimer disease (Roses 1998; Saunders et al. 2000), and even heart disease (Roses 2000; Daley and Cargill 2001). In addition to their role in disease, polymorphisms are also believed to confer sensitivity to drug therapy (Roses 2000), as well as the proarrhythmic risk from drug therapy (Sesti et al. 2000; Splawski et al. 2002). Viswanathan et al. (2003) have reported the first case example in which a polymorphism in the same gene as a rare mutation alters the biophysical effect of the mutation on the channel protein.

Even if all the individuals analysed were affected with LQTS, mutations could be present in the remaining, not examined regions of the \textit{SCN5A} gene; alternatively, intronic mutations (splicing defects) or mutations in the promoter and regulatory regions could be responsible for the disease symptoms. The possibility of other genes being involved in the etiology of the long QT syndrome should not be neglected either: up to date, six candidate genes for LQTS were identified (Priori et al. 1999). This genetic heterogeneity makes genetic testing much more difficult than in case if a single gene were responsible for the disease. If no mutation was identified in \textit{SCN5A}, then \textit{ KCNQ1, KCNH2, KCNE1} and \textit{KCNE2} have to be screened (Wang et al. 1995a,b; Chouabe et al. 1997; Splawski et al. 1998; Abbott et al. 1999).

Even more importantly, among the 45 patients analysed in this study, only 8 were unequivocally diagnosed with the long QT syndrome and 2 with the Brugada Syndrome. The remaining 35 patients were affected with other arrhythmogenic disorders. This even further increases the number of candidate genes, which could be responsible for the clinical symptoms.

It may be concluded that the analysed region of the \textit{SCN5A} gene is not a common target for mutations in patients with various arrhythmogenic disorders (LQTS, BS, HCM, ARVC, IVF).

**Conclusions**

In this study, direct sequencing of exons 2 to 28 of the \textit{SCN5A} gene revealed the absence of mutations in a group of 45 patients with arrhythmogenic disorders, including 8 LQTS and 2 Brugada Syndrome patients. The analysed region of the \textit{SCN5A} gene is thus not a common target for mutations in patients with various arrhythmogenic disorders (LQTS, BS, HCM, ARVC, IVF). Studies conducted did not identify predominant new mutations or polymorphisms of the \textit{SCN5A} gene among the studied individuals, although the possibility of a disease-causing mutation with low penetrance cannot be ruled out. There are several recognised mutations causing BS, but in many cases a recognised mutation is not found. Such genetic heterogeneity may explain the heterogeneity of expression between the individuals analysed in this study. Future linkage studies in \textit{SCN5A}-negative families will determine if these families are linked to the \textit{SCN5A} locus, and may eventually identify additional loci responsible for the BS. The genetic findings of the patients used in this study coincidentally confirm the results of clinical diagnosis.

**REFERENCES**


