TITLE: Genetic diversity of*SCN5A* gene and its possible association with the concealed form of Brugada Syndrome development in Polish group of patients

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A short title: Novel molecular changes as the cause of BS.

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Abstract

Brugada Syndrome (BS) is an inherited channelopathy associated with a high incidence of sudden cardiac death. The article presents the discovery of new genetic variants at *SCN5A* gene which might be associated with the development of concealed form of Brugada Syndrome. The study involved a group of 59 patients (37 men) with suspected concealed form of Brugada Syndrome. Pharmacological provocation with intravenous ajmaline administration was performed. Six patients with positive test result were subjected to molecular analysis of *SCN5A* gene with MSSCP method. Additionally, MSSCP genotyping was performed for samples obtained from the family members with Brugada Syndrome, despite they had negative ajmaline challenge test results.Genetic examinations of the *SCN5A* gene at 6 positive patients showed 6 known polymorphisms, 8 new single nucleotide point (SNP) variants located at exons and 12 new single nucleotide point variants at introns. Among detected in exons SNP, two of them were synonymous SNPs (no change in the coded amino acids), whereas 3 represented non-synonymous SNPs and affected the protein sequences.

Key words: Brugada syndrome, genetic variants, *SCN5A* gene, MSSCP

INTRODUCTION

About 5-10% of sudden cardiac death (SCD) cases are caused mainly by electrical heart diseases [1]. In the recent years, a special attention has been paid to one of them, the Brugada Syndrome (BS). The diagnosis of BS is based on ECG criteria as well as on the clinical picture. Typical BS ECG changes occur as a result of ion function disorders in the heart, which are caused by genetic mutations and lead to improper course mainly of repolarization processes in cardiomyocytes. Electrocardiographic features of the syndrome are dynamic and ECG curve is periodically normal – typical BS characteristics disappear, which makes it difficult to diagnose BS. Concealed form of BS causes underestimation of this disease occurrence frequency and a great number of people remain undiagnosed. Specific pharmacological provocation tests with class I medicine are critical in reveling concealed ECG features of BS.

Genetic background of the BS

The first gene to be linked to BS is the *SCN5A*, the gene that encodes the α-subunit of the cardiac sodium channel gene [2]. Almost 400 mutations at the *SCN5A* genehave been indentified at the syndrome patients since 2001 [3, 4]. Numerous detected mutations have been studied at the functional level [5]. The mutations at the *SCN5A* geneoccur in approximately 18% to 30% of BrugadaSyndrome cases. A higher incidence of *SCN5A* mutations has been reported in familial rather than in sporadic cases [6].

Another gene loci on chromosome 3, which is close to but distinct from *SCN5A*, have recently been linked to the syndrome (3p22-p24) [6] and GPD-1L [8]. Those mutations resulted in the loss of function of the cardiac sodium channel. Another genes associated with BS were reported in the last years and shown to encode the α1 and β subunits of the L-type cardiac calcium channel [9].

The *SCN5A* gene remains the main gene linked to BS. Of note, negative *SCN5A* results generally do not rule out causal gene mutations. Current, knowledge of a specific mutation may not provide guidance in formulating a diagnosis or determining a prognosis. Mutation screening of the *SCN5A* gene in patients with BS may only support a clinical overt or suspicious diagnosis.

In recent years the genotyping of *SCN5A* gene were more correlated to the prognostic value than to the diagnosis of the BS itself. Some of the *SCN5A* mutations were related to a worse clinical course[10], and other with the better [11] prognosis of the BS patients.

MATERIALS AND METHODS

Patient populations

The study involved a group of 59 Polish patients (37 men) with suspected concealed BS based on specific ECG and/or clinical criteria:

- complete and incomplete right bundle branch block (RBBB) in ECG,
- suspected but non-diagnostic ECG (type 2 and 3),
- history of sudden cardiac arrest (SCA),
- unexplained syncopes,
- sudden cardiac death (SCD) amongst family members under 45,
- family history of BS.

The protocol of the study has been approved by the Commission for Bioethics. Written informed consent was obtained from all of the patients.

The ajmaline challenge test

All patients were performed withpharmacological provocation with intravenous ajmaline administration dosed 1mg/kg body weight for 5 min, in safe conditions during 12-lead 24-hour Holter ECG monitoring.

In patients with positive test result, molecular tests of the *SCN5A* gene were performed.

Molecular tests were performed in the family members of patients with BS, even if the pharmacological provocation test was negative in these individuals.

Occurrence of type 1 electrocardiographic patterns (cove-shaped ST elevation in right precordial leadswith J wave or ST elevation of ≥2mm (mV) at its peak followed by a negative T wave withlittle or no isoelectric interval in more than one right precordial leads V1-V3) or conversion of types 2 or 3 to the diagnostic type 1 pattern after ajmaline administration was considered as a positive test result [3]. Occurrence of type 2 or 3 ST segment elevation was considered as negative test result.

DNA analysis

The genetic analysis was conducted in collaboration with Kucharczyk TE/BioVectis Company (Warsaw, Poland). Genomic DNA were analysed in 7 patients with positive result of ajmaline challenge test (one patient with positive result of ajmaline challenge test did not agree to be genotyped), and in 1 family member of patients with negative result of ajmaline challenge test. Genomic DNA were extracted from peripheral blood leucocytes (100 µl of frozen blood was used). Isolation was performed according to the manufacturer's protocol (A&A Biotechnology, Poland). Regions most likely to contain genetic mutations at 28 exons of the *SCN5A* gene were covered by 41 PCR amplicons, covering 28 exons and partial intron

sequences, as previously described [12]. Several pairs of primers were synthesized to PCR amplify exons 12, 17 and 28 due to their large sizes, named as a 12a, 12b, 17a etc. PCR primers were designed to cover the full coding sequence (exons), as well as, partial fragments of flanking non-coding fragments (introns). The PCR products were separated on agarose gel to examine their specificity and to normalise the DNA concentration. Next, 328 PCR products were screened by multitemperature single strand conformation polymorphism (MSSCP) [13] method for the presence of a single point mutation or a polymorphism. The MSSCP conditions were individually optimized for each PCR product. MSSCP was performed on 7 to 10%T polyacrylamide gels, 3.3%C at 0.75× TBE buffer. For some regions glycerol was added to polyacrylamide gel up to 5% w/v concentration. MSSCP analysis was performed using DNA*Pointer* System in 0.5× TBE buffer. Temperature profile of electrophoresis was 35–15–5°C. Electrophoresis was performed with 40 W of electrical power. Before applying samples onto the gel, 10 min of preelectrophoresis (40 W at 35°C) was performed. At the beginning samples were maintain for 10 min at 100 V for concentration. Subsequently, MSSCP separation was made. The PCR products that have altered MSSCP mobility were followed by Sanger method. 20 ng DNA of PCR products were used as a matrix for sequencing reaction. Both strands were sequenced at PCR products that revealed a genetic alternation. Genetic alterations were identified using the BLAST (Basic Local Alignment Search Tool) program and its BLASTN version as well as UCSC (University of California Santa Cruz) Genome Bioinformatics and NCBI (National Center for Biotechnology Information) databases of single nucleotide polymorphisms (SNPs).

Functional analysis of SCN5A variants

An *in silico*analysis was performed to evaluate the putative functional impact of the three identified variations (S321Y, S519F and K974D). We used the Polymorphism phenotyping-2 (PolyPhen-2) server [14], which integrates sequence-based and structure-based features to predict amino acid substitution effects using a naïve Bayes classifier. An amino acid change was classified as “probably damaging” if its probability score was greater than 0.85 or as “possibly damaging” if the score was between 0.85 and 0.55. To assess the influence of putative unstructured regions we used DISOPRED3 [15] software along with DOMPRED [16] to predict possible domain boundaries and disordered binding regions. Finally, we used Phyre [17] for structural feature predictions, mainly transmembrane regions and secondary structure using three different algorithms.

RESULTS

Patient demographics

Study inclusion criteria were met by 59 patients (22 women and 37 men) (Table 1). Average age of the group was 31.6 ± 12.2 years, from 16 to 62 years. Average age for women was 29.68 ± 10.9 years while for men 32.8 ± 12.9 years. The majority of patients (72.8%) was under 40.

Echocardiography in all the included patients revealed no significant organic heart disease.

Clinical characteristic of the group with positive result of pharmacological provocation test

Pharmacological provocation test was carried out on the whole study group. No significant undesirable effects were observed. None of the patients met the criteria of discontinuation prior the scheduled conclusion of the study.

Positive test result - type 1 ST segment elevation (Fig.1a) - which was considered as diagnostic for BS was obtained in 7 individuals (11.86%). The other 52 patients (88.14%) had negative provocation test result (Fig. 1b).

The group of 7 patients with type 1 ST segment elevation diagnostic for BS following ajmaline administration consisted of 6 men (85.7%) and 1 woman (14.3%). Average age of this group was 36.5 ±15.2, from 16 to 52 years. The group of patients with negative test result included 31 men (59.6%) and 21 women (40.4%). Average age of this group was 30.9 ±11.7, from 18 to 62 years. No statistically significant corelation between gender, age or body mass and ajmaline test was observed.

As regards the group of 7 individuals with positive provocation test result, 2 patients had history of SCA (men), among which in 1 person the diagnosed SCA mechanism was ventricular fibrillation. The SCA mechanism in the second individual remains unknown. Both patients were implanted a cardioverter – defibrillator. Within the group of the other 5 patients, initially considered as asymptomatic, 16 month following the provocation test syncopes occurred in 1 person (woman), what was an indication for implanting a cardioverter-defibrillator. The other 4 individuals have remained asymptomatic during the observation period lasting from 39 to 60 months.

Results of MSSCP analysis and DNA sequencing of the SCN5A sodium gene

Genetic examinations of *SCN5A* gene showed 6 known polymorphisms: rs6599230 (A›G, A29A), rs41312393 (A›G, intron), rs1805126 (T›C, A1818G), rs7429945 (A›G, exon, non-translated region), rs41315485 (T›C, exon, non-translated region) and rs7430407 (A›G,

E1061E). Three of them were noted at regions of coding proteins, two at non-coding regions and one at intron. Numerous new genetic variants were detected at: non-translated regions (8 SNP), at introns (12 SNP), in the protein coding regions (5 SNP) – 2 DNA sequence variants caused no change in the coded amino acid, whereas 3 altered the coded amino acid.

An example of MSSCP analysis of 2 amplicones, no 2 and 7, of the *SCN5A* gene for 8 particular patients is presented in (Figure 2). On the other hand, Figure 2 shows an example of patients derived-ampliconessequence analysis, which were compared with reference sequences (Figure 3). All detected polymorphisms were further analyzed in context of their localization and its impact on aa SCN5A protein sequence.

Known polymorphisms

The rs6599230 polymorphism at exon 2 of the *SCN5A* gene was found in 2 patients related with each other. It involved an alteration of nucleotides in 38614716 position (A›G) of reference sequence, however, detected variant did not alter the aa in protein sequence (A29A), thus had no impact on protein functionThe patients with this variant were a man (father) and a woman (daughter), both asymptomatic. The pharmacological provocation test was positive in the man and negative in the woman.

On the other hand,in exon 17b of the *SCN5A* gene, a known rs7430407 polymorphism was identified in 1 person. It involved a nucleotide alteration in 38562471 position (A›G) of reference sequence. The patient with this variant was a man with asymptomatic BS diagnosed based on pharmacological provocation. This genetic alteration caused no amino acid changes in protein sequence (E1061E).

Genotyping of exon 24 of the *SCN5A* gene revealed a known rs41312393 polymorphism in 3 individuals. It involved an alteration in nucleotides in 38538672 position (A›G) of reference sequence and was located at intron. The 3 persons were asymptomatic – 2 men with positive pharmacological provocation test and a woman related to one of the men (daughter) with negative result.

At exon 28c of the *SCN5A* gene a known 1805126 polymorphism was identified in 4 patients. This genetic change involved a nucleotide alteration in 38532410 position (T›C) of reference sequence and caused no change in the amino acid sequence in the coded protein (D1818D). Clinically, they were 2 asymptomatic individuals related to each other (father and son); one had negative result of pharmacological provocation (father) whereas the second patient had type 1 change in ST segment typical of BS. The other 2 patients were not related; one was a man with symptomatic BS and with history of SCA while the second was a man with asymptomatic BS.

Further, analysis of exon 28f of the *SCN5A* gene revealed the presence of known rs7429945 polymorphism, that was detected in 7 patients. It involved a nucleotide alteration in 38531693 position (A›G) of reference sequence. This genetic change occurred in the non-translation part of the exon in 6365 mRNA position. The described genetic change was present in almost every patient. Its presence was not observed only in a man with symptomatic BS and history of SCA.

Another known polymorphism is rs41315485 identified in 6 patients at exon 28k of the *SCN5A* gene. It involved an alteration in nucleotides in 38530279 position (T›C) of reference sequence, in 7779 mRNA position and was located in the non-translation region. The polymorphism was not observed only in 2 individuals from the analyzed group. They were men (brothers) – one with symptomatic and the other with asymptomatic BS.

New genetic variants in non-translation regionsat exons

At exon 1 of the *SCN5A* gene a new polymorphism that involved an alteration in nucleotides in 38631119 position (G›A) of reference sequence in 49 mRNA position was observed. The change was connected with the region transcribed on mRNA but is not translated as a protein. The person with this genetic variant was a man with diagnosed symptomatic BS (with history of SCA). The polymorphism was not observed in other patients.

Another new DNA sequence changes was observed in 2 patients who were related with each other. It was connected with the change in nucleotides in 38614815 position (G›C) of reference sequence found at exon 2. The genetic variant was present in 182 mRNA position and was related to a non-translation mRNA part. The patients with this polymorphism (a woman and a man) were asymptomatic; pharmacological provocation test was positive in the man and negative in the woman.

A new DNA sequence changewas also observed at exon 28g of the *SCN5A* gene in 5 patients. It involved an alteration in nucleotides in 38531355 position (G›A) of reference sequence in 6703 mRNA position. The genetic alteration was present in a non-translation part of the exon. The 5 individuals included 2 men with symptomatic BS and history of SCA. Moreover, the group included the brother and the father of the patient with history of SCA, one with negative and the second with a positive result of pharmacological provocation test. The last person with this polymorphism was a man with asymptomatic BS.

Genotyping of exon 28i of the *SCN5A* gene revealed 4 new polymorphismslocalized in a non-translation part of the exon. These changes were found in all the examined persons, among which 4 patients had all the 4 genetic variants, 3 patients had two new sequence

changes: in 38530974 (C›T) and 38531102 (C›T) position of reference sequence, while polymorphism in 38530974 position (C›T) was observed in all the patients.

Further, analysis of exon 28l of the *SCN5A* gene showed a new sequence variant in 38529996 position (C›G) of reference sequence in 8062 mRNA position. The change was found only in 1 person. Clinically, the person with this polymorphism was a man with asymptomatic BS.

New polymorphisms in protein coding regions that cause no alteration in the coded amino acid

As regards the group of 8 examined patients, 2 unknown genetic variants were observed in one patient at exon 28c of the *SCN5A* gene in protein coding regions; they caused no change in the coded amino acid thus we should consider them as a polymorphic change. The two novel polymorphic variants were detected in positions: 38532614 (C›T, F1750F) and 38532617 (C›T, L1749L). In both cases the changed nucleotide is in the 3 codon position, which may influence the fact that it causes no alteration in the amino acid sequence. These genetic variants were observed in a patient with diagnosed BS and history of SCA who required implantation of cardioverter-defibrillator.

New sequence changes in the protein coding regions that alter the coded amino acid

During genetic analysis of the *SCN5A* gene presence of 3 unknown genetic variants that altered the coded amino acid were found in 5 patients.

The first variantcontained change in nucleotides in 38589682 position (C›A) of reference sequence and was observed in 1 patient with negative result of pharmacological provocation. This genetic change was observed in exon 8 of the *SCN5A* gene. It altered serine amino acids into tyrosine in 321 position of the coded protein (S321Y). This variant was found in none of the other patients.

Another genetic variant detected in one patient involved a change in nucleotides in 38585541 position (C›T) of reference sequence which altered serine amino acids into phenylalanine in 519 protein position (S519F). New variant was found at exon 12 of the analyzed gene. The change was observed in a patient with asymptomatic BS and its presence was confirmed neither in 2 family members of the patient nor in the other examined patients.

The last new sequence variant, which according to the UCSC GenomiBioinformatic database is found in the protein coding region, was observed in 4 patients. The polymorphism was observed at exon 17 of the *SCN5A* gene and involved a change in amino acids in 38562732 position (G›T) of reference sequence. This genetic change altered lysine amino acids into aspartic acid in 974 position of the coded protein (K974D). BS was diagnosed in 2

of the persons while the other 2 individuals were family members of patients with negative results of pharmacological provocation. Schematic representation of detected changes is illustrated on Figure 4. To evaluate the possible influence of new missense mutations on channel function, bioinformatics analysis has been conducted.

In silico functional analysis of SCN5A variants

The three variants identified are located at the cytoplasmic region of the SCN5A-encoded protein (Figure 5A). Confirmed disease associated genetic variants can be found in close proximity to each one of the new variants, as well as sites of aminoacid modifications (e.g. arginine methylation site at 513 and 526 or a glicosylation site at 318). This would hint that the observed variants are located in important regions for protein function. To further explore their possible functional impact we employed a well known bioinformatics algorythm Poly-Phen2. The tool indicated a high possibility of damage caused by mutating K974D with prediction score close to 1 (highest possible) (Figure 5B). For the two other mutations possible damage was also reported, but with lesser probability. We sought to confirm these predictions with more structural insights. We used three different software tools to establish whether these variations would occur in unstructured and putative domain regions. Indeed S519F is located in a large domain of unknown function (DUF3451, PFAM: PF11933), which is also predicted as an unstructured/disorderd region by all three bioinformatics methods (Figure 5C). This would suggest a possible protein-binding interaction in this region which could be hampered by this variant (especially since serine contains a hydroxylic polar group and phenyloalanine is hydrophobic and aromatic). On the other hand S321Y is also located in Transmembrane ion channel family domain (Ion_trans, PF) which is predicted to be structured. The same goes with K974D, located just at the beginning of the Sodium ion transport-associated domain (Na_trans_assoc, PF:). Again, this is a structured region but very close to predicted unstructured binding region (945-956).

New point mutations found at introns

In the regions of the *SCN5A* gene, which according to the UCSC Genomic Bioinformatic database are at introns, 12 new point mutations were found.

MSSCP analysis of exon 4 of the *SCN5A* gene detected 4 new mutations within the intron. The first was a mutation in 38603806 position of reference sequence and involved a type A insertion. The second mutation was an alteration in nucleotides in 38603801 position (T›A) of reference sequence. The two said genetic changes were confirmed in 5 patients, among which 3 were asymptomatic and 2 were symptomatic (1 with history of SCA and 1 with syncopes). Another genetic change detected at this exon in 2 other individuals was a

change in nucleotides in 38604076 position (G›T) of reference sequence. The first patient had positive provocation result and history of SCA whereas the second patient was asymptomatic and also had positive provocation result. The last sequence change at this exon was found only in one patient in 38604075 position (G›T) of reference sequence. The patient was asymptomatic with negative result of ajmaline test.

MSSCP analysis showed presence of 2 new genetic variants at exon 6 of the *SCN5A* gene within the intron in 7 patients out of 8. The first involved a change in nucleotides in 38595390 position (C›G) of reference sequence. The second mutation was a change in 38595384 position (C›G) of reference sequence. These DNA variants were observed in all the examined patients apart from one individual who was asymptomatic and had negative result of pharmacological provocation test. At exon 7 of the *SCN5A* gene a DNA sequence variant within the intron (pos. 38591480, C›G) was identified in 4 patients out of 6 with positive ajmaline provocation test. Moreover, 3 patients from this group had symptomatic BS and had an implanted cardioverter-defibrillator either due to history of SCA or unexplained syncopes. Concurrently, this polymoprhism was confirmed in neither of the patients with negative result of provocation test. The results of MSSCP genotyping of 41 amplicones represented SCN5A gene are summarized in Table 2. Additionally, Table 3 contains the list of intronic alterations and exchanges in non-coding regions along with short stretches of sequences alignments (WT/MT).

DISCUSSION

The major gene related with BS is the *SCN5A* gene. Despite the great development in molecular studies, it is estimated that mutations in the *SCN5A* gene cause only about 18-30% of BS cases [18]. These mutations are more common in familial cases of the disease rather than sporadic ones [19]. Negative results of genetic studies do not exclude causal gene mutations. Neither diagnosis nor prognosis of BS can be based on genetic test results. In the presented work a molecular analysis of the whole the *SCN5A* gene was carried out with respect to patients with positive provocation test (apart from 1 person who failed to give their informed consent) as well as their family members (1st degree of kinship) who gave their informed consent. Due to both low predicted BS incidence in the Polish population (lack of accurate data) and a considerably low percentage of the known genetic changes being the underlying cause of the disease (18-30% as above), the work was limited only to analyzing the occurrence of the known mutations. The molecular study of the 28 exons and short exon/intron fragments of *SCN5* gene was carried out including also the alterations in the

sequence of the few non-coding regions of the gene (introns). In this study, the new genetic variants were found both at exons and introns. It is a commonly accepted fact that the effects of DNA sequence change depend on their location in the gene. However, all too often, it is assumed that only genetic alterations in the coding sequences, i.e. at exons, have an impact on the clinical course of the disease. Recent studies and findings have shown that intronic mutations may play a major role in the splicing process, alter its course, lead to coding sequence abnormalities and consequently influence the structure and function of the encoded proteins. Numerous data reported in scientific papers show that both intronic and exonic alterations may result in an aberrant splicing process, leading to the formation of abnormal proteins, what in turn affects the severity of the disease symptoms. These mutations/polymorphisms at introns leading to the disturbances of the splicing process are described in the disorders of cardiovascular system [20].

In the course of DNA analysis of the *SCN5A* sodium gene the following 6 known polymorphisms were identified: rs6599230, rs41312393, rs1805126, rs7429945, rs41315485 and rs7430407. In this group 3 polymorphisms were observed in the protein coding regions, 2 – in the non-translation regions and one at the intron. Neither of them had been associated with BS before. Also, 8 new genetic variants were found at exons in the non-translation regions, 12 at introns, 2 in the protein coding regions that cause no change in the coded amino acid and. None of 3 point mutations (S321Y, S519F and K974D) in the protein coding regions that alter the coded amino acid has been associated previously with BS [5]. According to Zimmer et al., as well as bioinformaticanalysiswe are able to localize their positions in protein sequence. S321Y is localized in the intracellular loop III, S519F in the intracellular loop IV, whereas K974D is localized in the C-terminal intracellular fragment of SCN5a protein. According to the bioinformatic results, K974D aa alteration is recognized as a highly damaging for protein function (prediction score amounted about 1, the highest possible). Two other aa changes were also reported as possible damage, however their probability score amounted from 0.85 to 0.55.

As mentioned previously, also intronic changes could affect protein function. We performed some basic bioinformatic analysis of detected changes, however we obtained contradictory data. Due to the large number of detected polymorphisms in introns, we decided to perform more detailed analysis, including in vitro studies.

The majority of detected polymorphisms and genetic changes found in the study had never been reported as mutations leading to development of BS. The lack of data in the literature and the lack of a population control for this part of the *SCN5A* gene made it

impossible to state clearly whether the BS syndrome were significantly associated with mentioned changes. It is also noteworthy, that several genes are associated with BS syndrome, thus further genetic study are needed. However, at least new polymorphisms/mutations that were found in our patients of a specific phenotype are worth considering.

Special attention ought to be paid to genetic changes present only in symptomatic patients, e.g. with history of SCA. These genetic changes include:

- a new polymorphism which involves an alteration in nucleotides in 38631119 position (G›A) of reference sequence in 49 mRNA position and developed in a man with history of SCA and BS diagnosed on the basis of provocation test result; it was found in none of the other patients;
- a new genetic variant at exon 28 which involves an alteration in nucleotides in 38531355 position (G›A) of reference sequence in 6703 mRNA position and developed in 5 patients including 2 men with positive provocation test results and history of SCA as in other individuals (i.e., the brother and the father on one of these men);
- two new genetic variants at exon 28c in the protein-coding regions with no alteration in the coded amino acid (C›T, I1749I; C›T, F1750F) both developed in a man with history of SCA and positive provocation test result;
- a new polymorphism at exon 7 which involves an alteration in nucleotides in 38591480 position (C›G) of reference sequence and developed in 4 patients out of 6 individuals with positive ajmaline provocation test; 3 of these patients had symptomatic BS following implantation of cardioverter-defibrillator either due to history of SCA or unexplained syncopes; concurrently the mutation was confirmed in none of the patients with negative provocation test result;

Considering new data on the role of genetic changes not only in BS diagnostics but also in prognosis for diagnosed patients [21, 22], further studies aimed at determining the role of the identified genetic disorders seem to be extremely interesting.

CONCLUSIONS

New genetic variants/polymorphism in the *SCN5A* gene are present in patients with concealed form of Brugada Syndrome, yet their role in pathogenesis requires further studies.

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TABLES

Inclusion criteria	Number of induded patients
RBBB in ECG (complete and incomplete)	35 patients (59.32%) - RBBB complete – 8 patients (13.6%) - RBBB incomplete – 27 patients (45.76%)
History of SCA ,	7 patients (11.8%)
Unexplainedsyncopes,	31 patients (52.5%)
SCD amongst family members under 45,	5 patients (8.5%)
Family history of Brugada syndrome,	4 patients (6.8%)
Suspected but non-diagnostic ECG (type 2 and 3).	16 patients (27.11%) - type 2 – 4 patients (6.78%) - type 3 – 12 patients (20.33%)

Tab. 1. Distribution of the examined population depending on inclusion criteria.

	Major clinical data	Genetic alterations in DNA sequences of *SCN5*amplicones (reference sequence ref|NT_022517.17|Hs3_22673)
Patient 1	Asymptomatic Negative pharmacological provocation test Father of the patient with diagnosed BS	amplicone 4: Pos. 38603806, insertion A, intron Pos. 38603801 homozygote A, intron amplicone 8: Pos. 38589643 insertion A, intron Pos. 38589682 C›A, amino acid pos. 321, S>Y amplicone 17a: Pos. 38562732 G›T, amino acid pos. 974, K>D amplicone 28c: Pos. 385924060 T›C, exon, amino acid pos. 1818 D>D, rs1805126 amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28g : Pos. 38531355 heterozygote G›A, mRNA position 6703, near 3'UTR, non-translated region amplicone 28i: Pos. 38530974 C›T, exon, mRNA position 7084, near 3'UTR, non-tranlated region Pos. 38531102 C›T, exon, mRNA position 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485
Patient 2	History of sudden cardiac arrest Positive pharmacological	amplicone 4: Pos. 38603806, insertion A, intron Pos. 38603801 homozygote T›A, intron amplicone 6: Pos. 38595390 G›C, intron

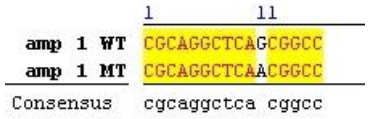
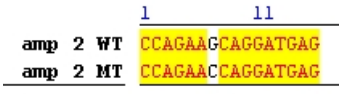
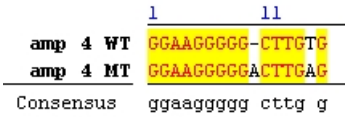
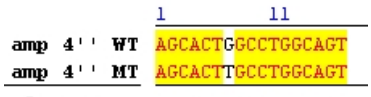
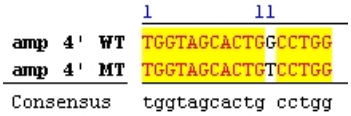
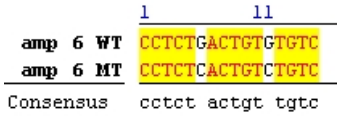
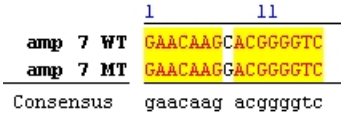
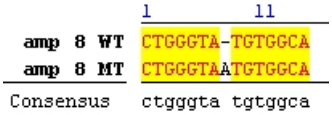
	provocation test Diagnosed BS	Pos. 38595384 homozygote G›C, intron amplicone 7: Pos. 38591480 C›G, intron amplicone 28g: Pos. 38531355 G›A, exon, mRNA position 6703, near 3'UTR, non-translated region amplicone 28i: Pos. 38530974 G›A, exon, mRNA position 7084, near 3'UTR, non-translated region
Patient 3	History of sudden cardiac arrest Positive pharmacological provocation test Diagnosed BS	amplicone 1: Pos. 38631119 G›A, mRNA pos 49, non-translated region amplicone 4: Pos. 38604076 G›T, intron amplicone 6: Pos. 38595390 homozygote C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 7: Pos. 38591480 C›G, intron amplicone 17a: Pos. 38562732 G›T, amino acid pos. 974, K>D amplicone 28c: Pos. 38532617 C›T, exon, amino acid pos. 1749, I>I Pos. 38532614 C›T, exon, amino acid position 1750, F>F Pos. 38592406T›C, exon, amino acid pos. 1818, D>D, rs1805126 amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28g : Pos. 38531355 heterozygote G/A, mRNA position 6703, near 3'UTR, non-translated region amplicone 28i: Pos. 38530974 C›T, exon, mRNA position 7084, near 3'UTR, non-translated region Pos. 38531102 C›T, exon, mRNA position 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485
Patient 4	Asymptomatic Positive pharmacological provocation test Brother of the patient with diagnosed BS	amplicone 4: Pos. 38604076 G›T, intron amplicone 6: Pos. 38595390 homozygote C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 12a: Pos. 38585541 C›T exon, amino acid pos. 519, S>F Pos. 38585647 homozygote A›T, intron amplicone 22:Pos 38544050, heterozygote C/T, intron amplicone 24: Pos 38547178 heterozygote A/G, intron, polymorphism rs41312393 amplicone 28c: Pos. 38592406 T›C, exon, amino acid pos. 1818, D>D, rs1805126 amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28g: Pos. 38531355 G›A, exon, mRNA position 6703, near

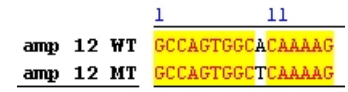
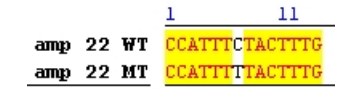
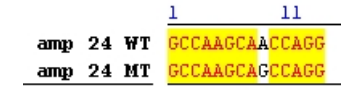
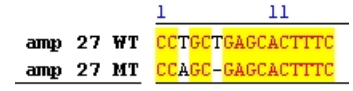
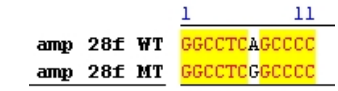
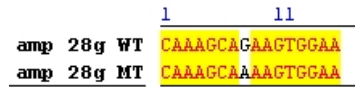
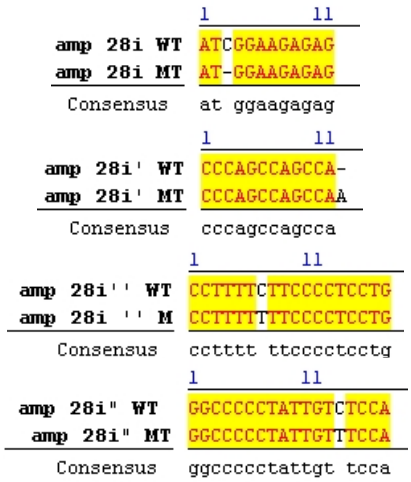
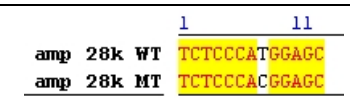
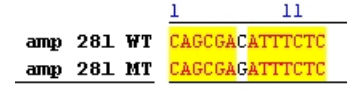
		3'UTR, non-translated region amplicone 28i: Pos. 38530974 C›T, exon, mRNA position 7084, near 3'UTR, non-translated region Pos. 38531102 C›T, exon, mRNA position 6956, near 3'UTR, non-translated region
Patient 5	Asymptomatic Positive pharmacological provocation test Father of the patient with diagnosed BS	amplicone 2: Pos. 38614716 A›G, exon, amino acid pos. 29, A>A, polymorphism rs6599230 Pos. 38614815, G›C, exon, mRNA pos.182, non-translated region amplicone 4: Pos. 38604075 homozygote G›T, intron amplicone 6: Pos. 38595390 homozygote C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 17a: Pos. 38562732 G›T, amino acid pos. 974, K>D amplicone 17b: Pos. 38562471 homozygote A›G, amino acid pos. 1061, E›E, polimorphism rs7430407 amplicone 24: Pos 38547178 heterozygote A›G, intron, polymorphism rs41312393 amplicone 27: Pos. 38536074 deletion T, intron Pos. 38536077 homozygote T›A, intron amplicone 28c: Pos. 38592406 T›C, exon, amino acid pos. 1818, D>D, rs1805126 amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28g: Pos. 38531355 G›A, exon, mRNA position 6703, near 3'UTR, non-translated region amplicone 28i: Pos. 38530853 deletion C, exon, mRNA pos. 7205, near 3'UTR, non-translated region Pos. 38530856 insertion A, exon, mRNA pos. 7202, near 3'UTR, non-translated region Pos. 38530974 C›T, exon, mRNA pos. 7084, near 3'UTR, non-translated region Pos. 38531102 C›T, exon, mRNA pos. 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485
Patient 6	Unexplained syncopes Positive pharmacological provocation test Diagnosed BS	amplicone 4: Pos. 38603806, insertion A, intron Pos. 38603801 homozygote T›A, intron amplicone 6: Pos. 38595390 C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 7: Pos. 38591480 C›G, intron amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945

		amplicone 28i: Pos. 38530853 deletion C, exon, mRNA pos. 7205, near 3'UTR, non-translated region Pos. 38530856 insertion A, exon, mRNA pos. 7202, near 3'UTR, non-translated region Pos. 38530974 C›T, exon, mRNA pos. 7084, near 3'UTR, non-translated region Pos. 38531102 C›T, exon, mRNA pos. 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485
Patient 7	Unexplained syncopes Positive pharmacological provocation test Diagnosed BS	amplicone 2: Pos. 38614716, A›G, amino acid pos. 29, A>A, polymorphism rs6599230 Pos. 38614815, G›C, exon, mRNA pos.182, non-translated region amplicone 4: Pos. 38603806, insertion A, intron Pos. 38603801 homozygote T›A, intron amplicone 6: Pos. 38595390 C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 17a: Pos. 38562732 G›T, amino acid pos. 974, K>D amplicone 24: Pos 38547178 heterozygote A/G, intron, polymorphism rs41312393 amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28i: Pos. 38530853 deletion C, exon, mRNA pos. 7205, near 3'UTR, non-translated region Pos. 38530856 insertion A, exon, mRNA pos. 7202, near 3'UTR, non-translated region Pos. 38530974 C›T, exon, mRNA pos. 7084, near 3'UTR, non-translated region Pos. 38531102 C›T, exon, mRNA pos. 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485
Patient 8	Asymptomatic Positive pharmacological provocation test	amplicone 4: Pos. 38603806, insertion A, intron Pos. 38603801 homozygote T›A, intron amplicone 6: Pos. 38595390 C›G, intron Pos. 38595384 homozygote C›G, intron amplicone 7: Pos. 38591480 C›G, intron amplicone 28f: Pos. 38531693 A›G, exon, mRNA position 6365, near 3'UTR, non-translated region, rs7429945 amplicone 28i: Pos. 38530853 deletion C, exon, mRNA pos. 7205, near 3'UTR, non-translated region Pos. 38530856 insertion A, exon, mRNA pos. 7202, near 3'UTR, non-translated region Pos. 38530974 C›T, exon, mRNA pos. 7084, near 3'UTR, non-translated region

		Pos. 38531102 C›T, exon, mRNA pos. 6956, near 3'UTR, non-translated region amplicone 28k: Pos. 38530279 T›C, exon, mRNA pos. 7779, 3'UTR, non-translated region, polimorphism rs41315485 amplicone 28l: Pos. 38529996 homozygote C›G, exon, mRNA pos. 8062, near 3'UTR, non-translated region

1 **Table 2.** The results of MSSCP genotyping of 41 amplicones represented SCN5A gene.

Amplicone	Position	Detected polymorphism (WT›MT)	Localization	Partial alignment (WT›MT)
1	Pos. 38631119	G›A	Exon, non-translated region	
2	Pos. 38614815	G›C	Exon, non-translated region	
4	Pos. 38603806 Pos. 38603801	insertion A T›A	intron	
4	Pos. 38604075	G›T	intron	
4	Pos. 38604076	G›T	intron	
6	Pos. 38595390 Pos. 38595384	G›C G/C	intron	
7	Pos. 38591480	C›G	intron	
8	Pos. 38589643	insertion A	intron	

12	Pos. 38585647	A›T	intron	
22	Pos. 38544050	C›T	intron	
24	Pos. 38538672	A›G	intron	
27	Pos. 38536074 Pos. 38536077	deletion T T›A	intron	
28f	Pos. 38531693	A›G	exon, near 3'UTR, non-translated region	
28g	Pos. 38531355	G›A	exon, near 3'UTR, non-translated region	
28i	Pos. 38530853 Pos. 38530856 Pos. 38530974 Pos. 38531102	deletion C insertion A C›T C›T	exon, near 3'UTR, non-translated region	
28k	Pos. 38530279	T›C	3' UTR, non-translated region	
28l	Pos. 38529996	C›G	exon, near 3'UTR, non-translated region	

1 **Table 3.** The list of intronic alterations and exchanges in non-coding regions along with short
2 stretches of sequences alignments (WT/MT).

3

FIGURES

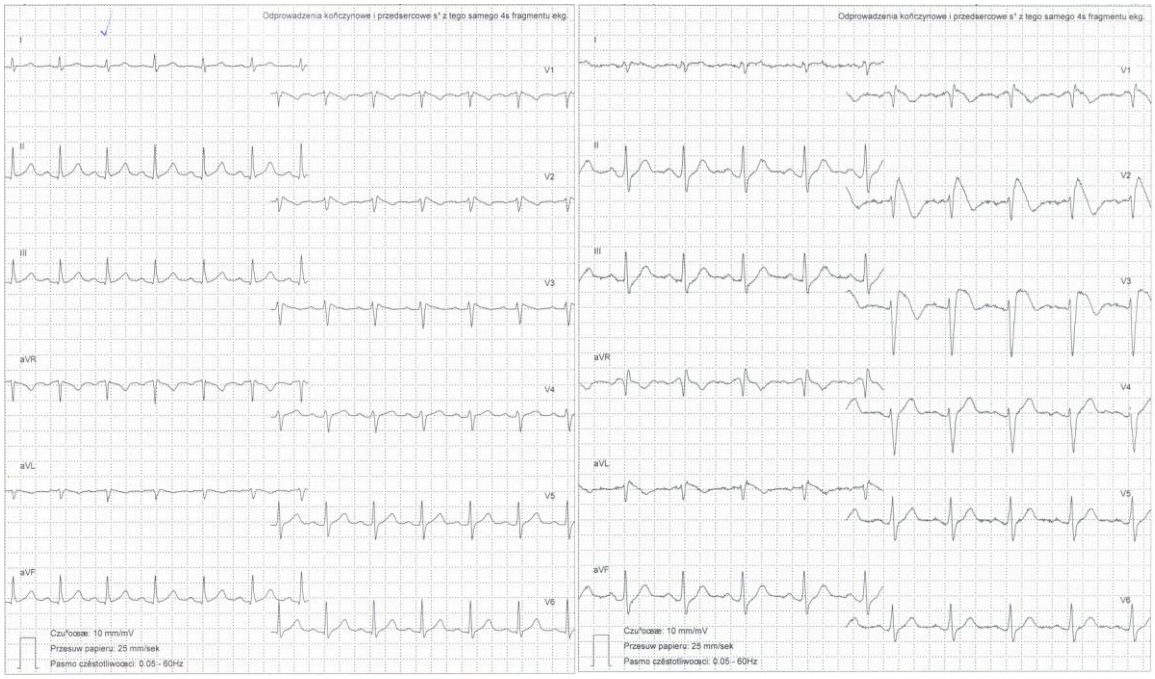


Figure 1a. 12-lead ECG from a patient with positive test result (before and after test). The configuration of the ST-segment elevation in leads V1 to V3 is a coved type.

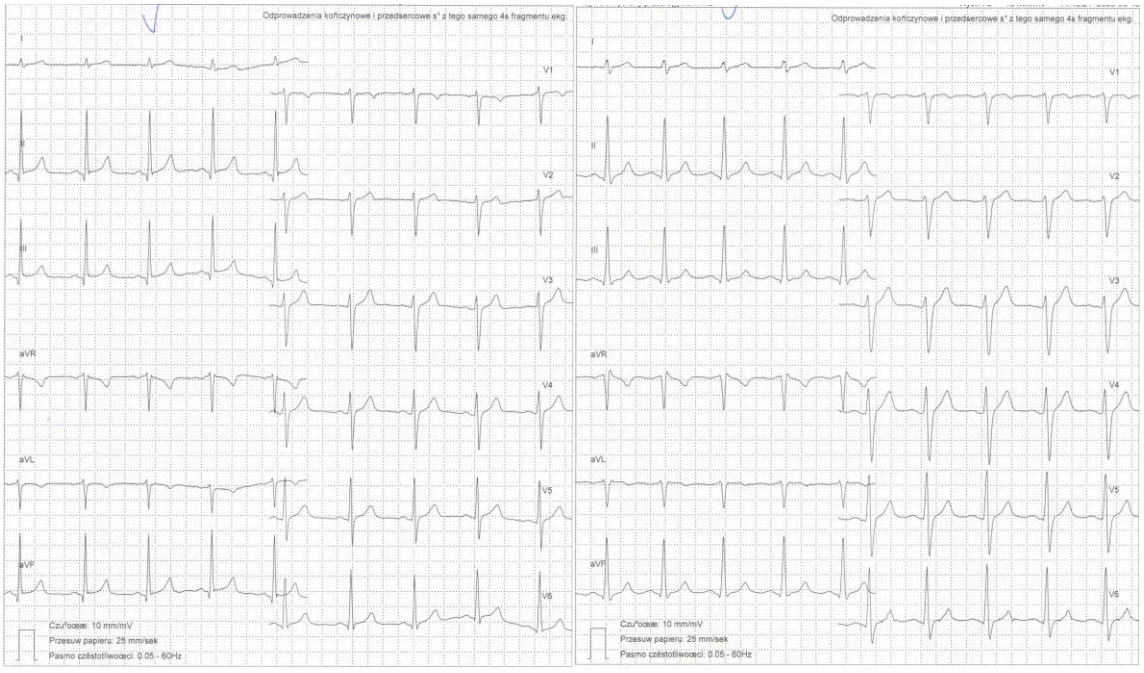


Figure 1b. 12-lead ECG from a patient with negative test result (before and after test).

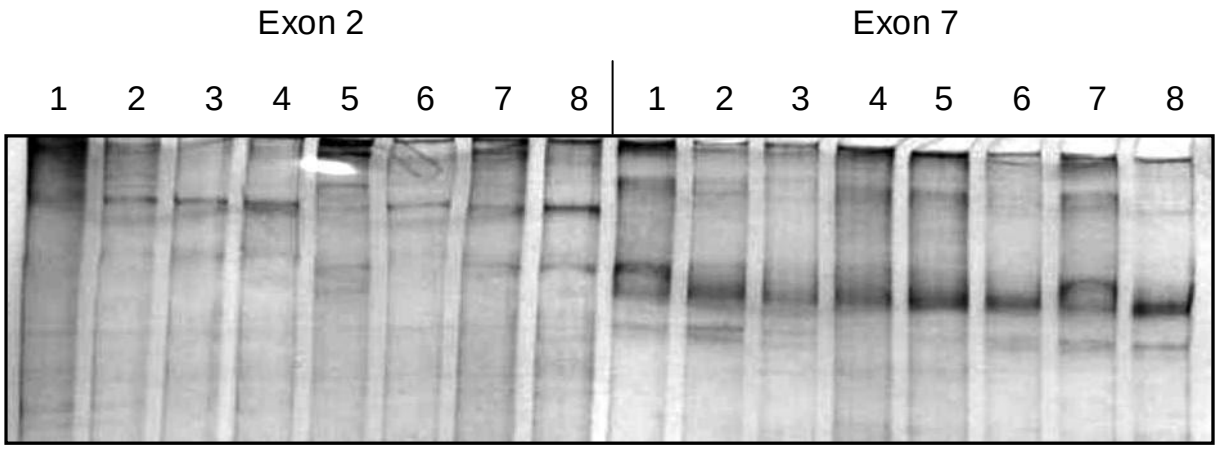


Figure2. MSSCP separation of exon 2 and exon 7 PCR products. Note that sample no. 5 and 7 at exon 2 and sample no. 1 at exon 7 have distinct electrophoretic profiles suggesting the presence of minor genetic variants.

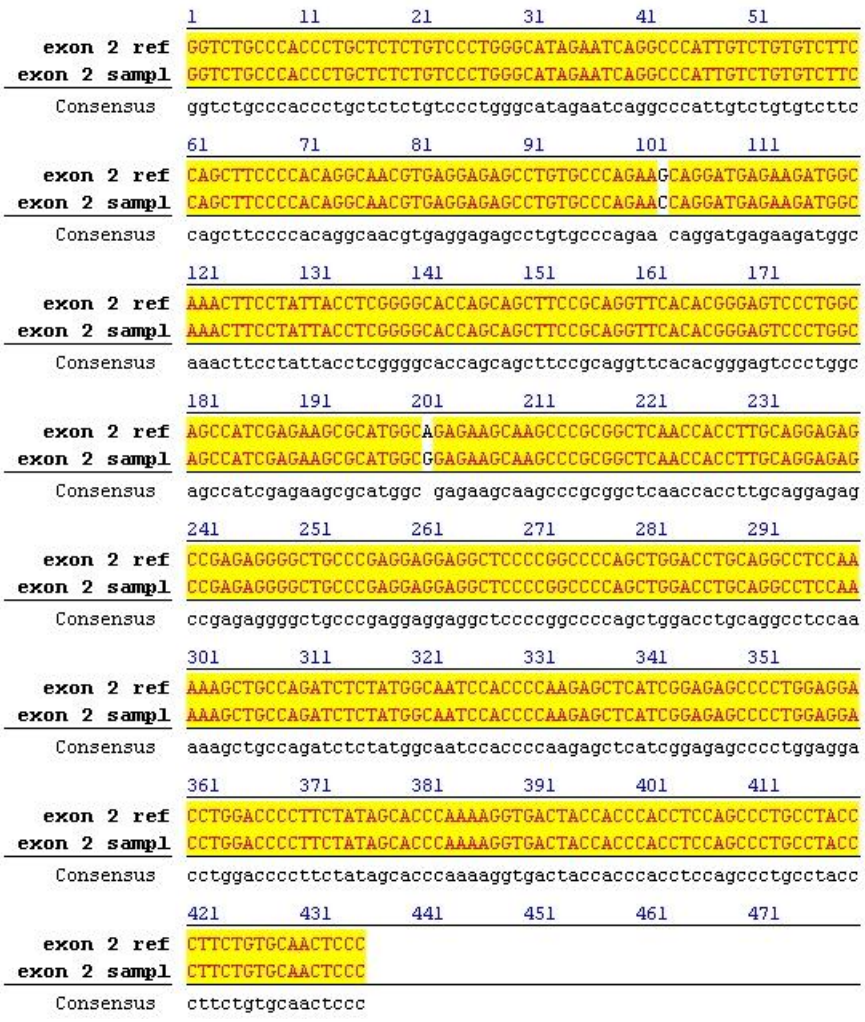


Figure 3. Sequence alignment of *SCN5* exon 2 reference (WT) and MT amplicone detected in sample 5 (pos. 38614716 heterozygote A›G, exon, amino acid pos. 29, polymorphism rs6599230; pos. 38614815 heterozygote G›C, exon, pos. 182 in mRNA, non-translating region). White color shows changed nucleotides.

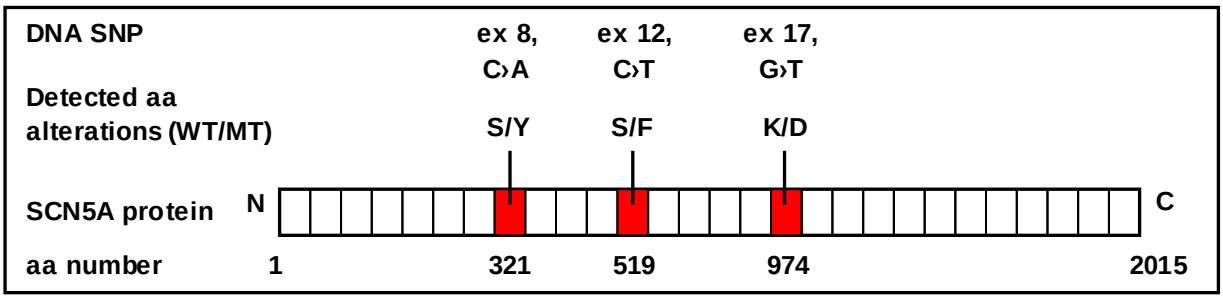


Figure 4. Schematic illustration of Nav1.5, showing the location of the novel putative amino acid changes.

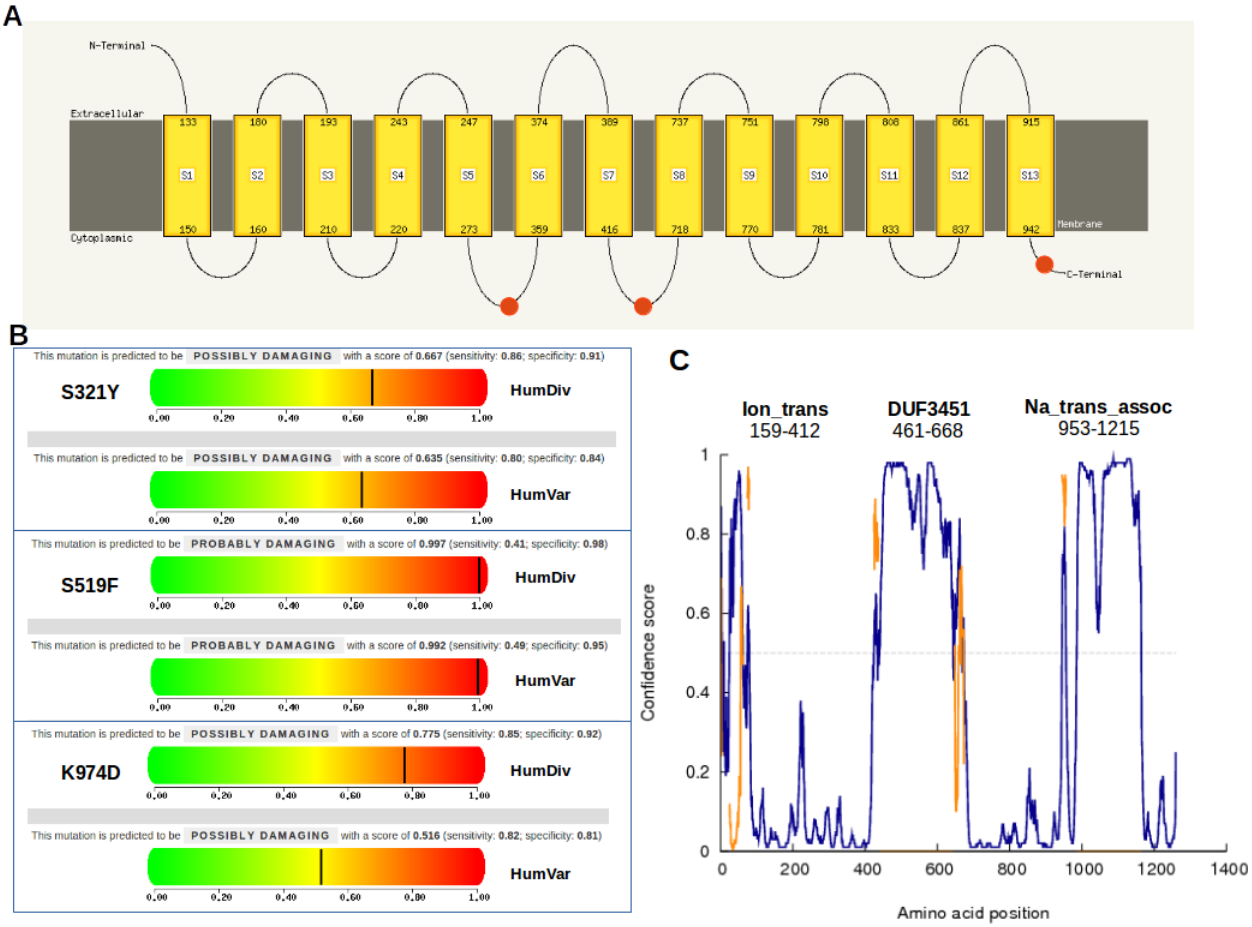


Figure 5.A. Prediction of transmembrane, extracellular in cytoplasmic regions in SCN5a protein. Location of the three new variants is depicted as orange dots. B. Prediction of functional effects of non-synonymous mutations done in Poly-Phen2 software. All three variants have two scores from HumDiv (genomic oriented) and HumVar (diagnostic oriented). C. Prediction of intrinsically disordered regions in SCN5a (1-1400AA) done by DISOPRED3. Domain organisation shown with respect to disorederd regions. Orange color marks putative binding sites. High confidencescoreindicatedbetterchance of unstructured fragment.