



Mutational analysis of *SCN5A* gene in long QT syndrome



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ABSTRACT

The *SCN5A* gene encodes for the *I_{Na}* channel implicated in long QT syndrome type-3 (LQTS-type-3). Clinical symptoms of this type are lethal as most patients had a sudden death during sleep. Screening of *SCN5A* in South Indian cohort by PCR-SSCP analyses revealed five polymorphisms – A29A (exon-2), H558R (exon-12), E1061E and S1074R (exon-17) and IVS25 + 65G>A (exon-25) respectively. In-silico and statistical analyses were performed on all the polymorphisms.

Exon-2 of *SCN5A* gene revealed A282G polymorphism (rs6599230), resulting in alanine for alanine (A29A) silent substitution in the N-terminus of *SCN5A* protein. Exon-12 showed A1868G polymorphism (H558R – rs1805124) and its 'AA' genotype and 'A' allele frequency were found to be higher in LQTS patients pointing towards its role in LQTS etiology.

Two polymorphisms A3378G (E1061E) and the novel C3417A (S1074R) were identified as compound heterozygotes/genetic compounds in exon-17 of *SCN5A* located in the DIIS6–DIIS1 domain of the *SCN5A* transmembrane protein. IVS25 + 65G>A was identified in intron-25 of *SCN5A*. The 'G' allele was identified as the risk allele.

Variations were identified in in-silico analyses which revealed that these genetic compounds may lead to downstream signaling variations causing aberrations in sodium channel functions leading to prolonged QTc. The compound heterozygotes of *SCN5A* gene polymorphisms revealed a significant association which may be deleterious/lethal leading to an aberrant sodium ion channel causing prolonged QTc.

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1. Introduction

Na^v channels of the heart are important for the orderly progression of action potentials from the sinoatrial node, through the atria, across the atrioventricular node, along the specialized conduction system of the ventricles (His–Purkinje system), and ultimately throughout the myocardium to stimulate rhythmic contraction (George, 2005). These

channels open and inactivate rapidly during depolarization, but also re-open during the plateau and repolarization phases, carrying 'persistent' or 'late' inward current (late *I_{Na}*). Late *I_{Na}* has been demonstrated in cardiac ventricular specimens of various mammalian species including humans, and its role has been documented in the generation of normal as well as altered action potential (AP) durations (Maltsev et al., 1998; Berecki et al., 2006).

The human cardiac Na⁺ channel, encoded by the *SCN5A* gene, is primarily responsible for the initiation and propagation of cardiac action potentials. Mutations in *SCN5A* have long been known to cause a variety of cardiac rhythm disorders, including type 3 (LQT3) of the congenital long-QT syndrome (cLQTS) (Jiang et al., 1994; Wang et al., 1995). LQT3 arises from a delayed repolarization of the ventricular myocytes, due to a shift in the delicate balance between inward and outward currents during the plateau of the AP, resulting in an increased propensity for ventricular tachyarrhythmias and sudden death (Bennett et al., 1995; Moss et al., 1995). For instance, an increase of Na⁺ influx during

Abbreviations: LQTS, Long QT syndrome; cLQTS, Congenital long QT syndrome; FDRs, First degree relatives; LQT3, Long QT syndrome type-3; *SCN5A*, Sodium channel, voltage-gated, type V, alpha subunit.

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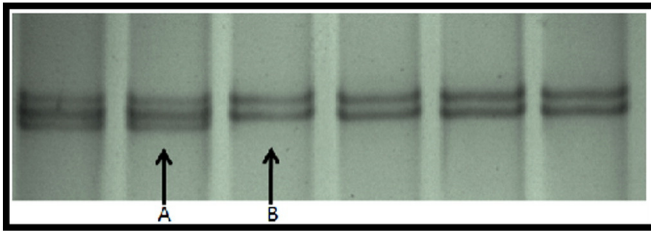


Fig. 1. SSCP gel picture of exon 2 of SCN5A gene.

depolarized membrane potentials is proposed to impair membrane repolarization, and prolong APs (Berecki et al., 2006).

The NaV1.5 channel of the heart is a multisubunit protein complex composed of a single large α -subunit, SCN5A (sodium channel, voltage-gated, type V, alpha subunit), along with one or more auxiliary β -subunits, fine-tuned for the frequency and waveform of APs (Catterall 2000; Ruff, 1998). The majority of LQT3-related mutations lead to gain-of-function due to a late inward *I*Na (Wedekind et al., 2001; Rivolta et al., 2002; Smits et al., 2005).

Approximately 10% of LQTS cases are due to mutations in SCN5A gene with the triggering factors associated with arrhythmic events being different among the genetic subsets of LQTS. It is also observed that SCN5A mutations often produce distinct clinical features including bradycardia, and patients often tend to experience cardiac events during sleep or rest (George, 2005).

Screening of all the 28 exons (split as 34 fragments) of SCN5A was warranted in view of the ethnic and geographic diversity, hence mutation screening was carried out by PCR based SSCP analysis on 46 LQTS, 69 first degree relatives (FDRs) and 150 control DNA samples.

2. Methodology

2.1. Study subjects

Blood samples were collected for molecular analyses from confirmed 46 LQTS probands and 69 first degree relatives from Care Hospitals, Hyderabad, Sri Jayadeva Institute of Cardiovascular Science and Research, Bangalore, Institute of Maternal and Child Health, Calicut Medical College, Calicut and Krishna Institute of Medical Sciences, Hyderabad. The QTc of the LQTS patients and their FDRs was confirmed by electrocardiogram. This study has been approved by the Institutional Ethics Committee, Dept. of Genetics, Osmania University, Hyderabad and informed written consent was obtained from the probands and their family members. Blood samples from 150 controls (75 M: 75 F), without any history of cardiovascular or systemic conditions, were collected from Osmania General Hospital, Hyderabad for comparative analysis.

Table 1

Genotype and allele frequency distribution of exon 2 A282G polymorphism (A29A) of SCN5A gene (rs6599230).

Genotype	Controls n (%)	LQTS n (%)	FDR n (%)	Allele	Controls	LQTS	FDRs
GG	29 (19)	7 (16)	21 (30)	G	0.6	0.58	0.65
GA	121 (81)	39 (84)	48 (70)	A	0.4	0.42	0.35

Table 2

Odds risk estimates of exon 2 A282G polymorphism (A29A) of SCN5A gene (rs6599230).

	Genotype	OR (95% CI)	p value
LQTS vs Ctrl	GG	1.00	0.56
	GA	1.30 (0.53–3.21)	
	G vs A	1.08 (0.61–1.9)	
FDRs vs Ctrl	GG	1.00	0.074
	GA	0.55 (0.28–1.05)	
	G vs A	0.8 (0.45–1.4)	
LQTS vs FDRs	GG	1.00	0.48
	GA	1.35 (0.58–3.17)	
	G vs A	0.74 (0.42–1.3)	

p < 0.05.

2.2. Molecular analyses

Genomic DNA was isolated from peripheral blood samples by standard protocols in 150 controls, 46 probands and their (69) first degree relatives (FDRs). Primers used for SCN5A were described by Syrris et al. (2001). The whole coding region of SCN5A was amplified using 34 PCR fragments. Exon 1 was excluded as it corresponds to the 5' UTR part of the gene. Fragments were amplified on Eppendorf Thermal Cycler Gradient in the presence of 1U Taq DNA polymerase, 0.2mM deoxyribonucleotide, 1.5mM MgCl₂, 100ng forward and reverse primers and genomic DNA. Polymerase Chain Reaction-Single Strand Conformational Polymorphism (PCR-SSCP) analyses were carried out using standard protocols by Orita et al. (1989) and products were checked on native PAGE gel followed by silver staining to identify variations. The samples showing variations were sent for commercial sequencing.

2.3. Statistical analysis

Fisher's exact test and odds risk estimate were computed for possible genotype association. Interactive SNPs were analyzed by means of logistic regression (OR) to determine the significance of risk genotypes at 95% confidence interval (CI) followed by haplotype frequency computation by the EM algorithm using SNPstat software (Sole et al., 2006). Linkage disequilibrium was inferred by Haploview software (Barrett et al., 2005).

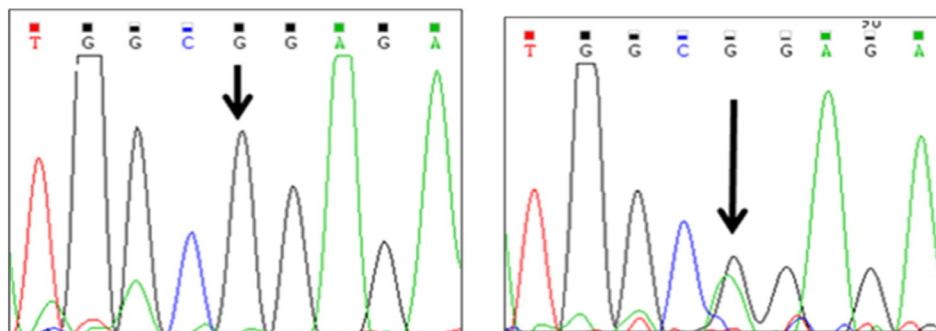


Fig. 2. Electropherogram showing homozygous 'GG' and 'AG'.

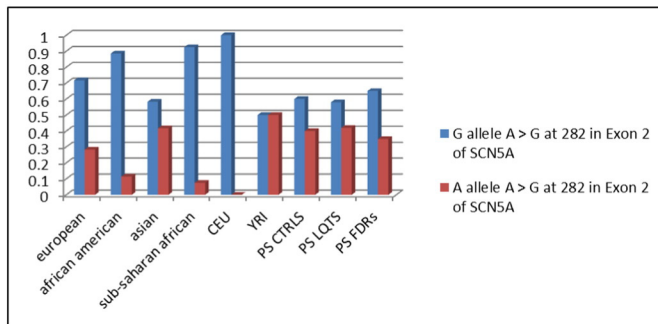


Fig. 3. Hapmap of exon 2 A282G polymorphism (A29A) of SCN5A gene (rs6599230). PS—present study; CTRLS—controls.

2.4. In-silico analysis

The splice binding sites were predicted by Rainbow splicing: *EBI Databases ASD alternative splicing* (www.ebi.ac.uk/asd-srv/wb.cgi) and <http://sfmap.technion.ac.il/> (Paz et al., 2010). Secondary mRNA structure predictions were carried out by Genebee Online tool genebee.msu.edu/services/rna2_reduced.html and <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi> (Lorenz et al., 2011).

2.5. Hapmap

The hapmap comparing allelic frequency of different populations has been done.

3. Results

Mutational screening of SCN5A gene revealed variations in exon 2 (A29A), 1st fragment of exon 12 (H558R), 2nd fragment of exon 17 (E1061E; S1074R) and exon 25 (IVS25+65G>A) respectively.

3.1. Exon 2 of SCN5A gene

3.1.1. Molecular analysis

SSCP analysis of this exon revealed two types of band pattern variations (A, B) in controls, patients and family members (Fig. 1). To confirm the underlying variation of each band pattern, the samples were commercially sequenced (ABI 3730XL capillary DNA Sequencer). Electropherograms are given in Fig. 2 and the genotypes were identified as GG and GA respectively. Sequencing revealed A282G polymorphism (rs6599230), resulting in a synonymous substitution of alanine (A29A), with band pattern 'A' representing heterozygous genotype 'GA' and band pattern 'B' homozygous for 'GG'.

3.1.2. Statistical analysis

The heterozygous 'GA' genotype was found to be predominant in controls, LQTS patients and FDRs. The genotype 'GA' was higher in LQTS (84%) compared to controls (81%) and FDRs (70%) while 'GG' genotype was found to be higher in FDRs (30%) than controls (19%) and patients (16%). Allelic frequency revealed slightly higher 'A' allele frequency in LQTS (0.42) compared to FDRs (0.35) and controls (0.4)

Table 3

Clinical characteristics of LQTS patients exhibiting genotypes of exon 2 A282G polymorphism (A29A) of SCN5A gene (rs6599230).

SCN5A A29A	cLQTS (%)	Females (%)	Deafness (%)	F/h sudden death (%)	Consanguinity (%)	Syncope (%)
GG	100	50	–	25	12.5	75
GA	74	58	11	34	29	55

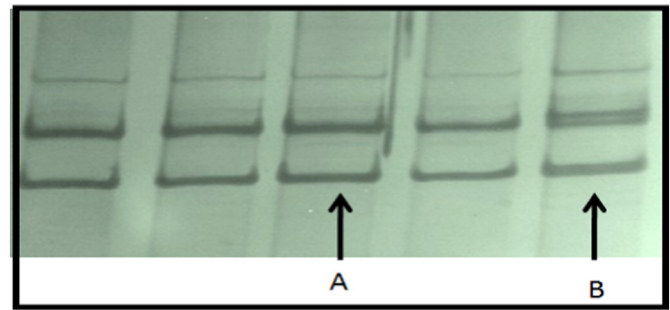


Fig. 4. SSCP band pattern variation in exon 12A of SCN5A gene.

(Table 1). The odds risk estimates were calculated at 95% CI by comparing genotypes of LQTS and FDRs with controls in addition to calculating odds ratio for FDRs and LQTS. All the possible combinations did not reveal any significant association reflecting the need for large sample size (Table 2).

3.1.3. In-silico analysis

In the mRNA secondary structure of the variant, there was a loss of four stems and loops when compared to wild type. The thermodynamic stability is also affected due to a decrease in the free energy from -61.3 kcal/mol to -63.3 kcal/mol. These variations may alter the binding of splicing proteins thus affecting the splicing mechanism.

The A29A substitution is present in the N-terminus of SCN5A transmembrane ion channel. Though being a synonymous substitution, the codon usage was reduced in variant codon GCG (0.11) in contrast to wild type codon GCA (0.23). This indicates a decrease in usage of the codon which may lead to aberrant translated protein.

3.1.4. Hapmap

The allelic frequency was compared across the populations using the hapmap data. The 'G' allele frequency was found to be predominant in all the reported populations. However, the 'A' allele frequency was found to be higher in the present study of South Indian population and Asian population when compared to other populations. This indicates the ethnic diversity of the Asian population which needs to be confirmed by large cohort studies (Fig. 3).

3.1.5. Genotype-based clinical characteristics

Table 3 describes the clinical features of LQTS patients expressing the two genotypes of SCN5A A29A polymorphism. It was observed that 100% of patients harboring 'GG' genotype were cLQTS type. More than 50% of these patients were females increasing their risk to LQTS. Syncope was higher in 'GG' genotypic patients (75%) than 'GA' genotype (55%). Interestingly females (58%), deafness (11%), family history of sudden death (34%) and consanguinity (29%) were also found to be higher in LQTS patients with 'GA' genotype emphasizing the role of 'A' allele in the etiology of this disorder. Presence of 'A' allele and familial consanguinity may possibly cause higher number of sudden deaths in families due to cLQTS. The 'A' allele may cause variations in SCN5A gene expression leading to sodium channel dysfunction and LQTS.

3.2. Exon 12 (1st fragment of the 12th exon) of SCN5A gene

3.2.1. Molecular analysis

Two types of band patterns were observed on PCR-based SSCP analysis of exon 12A of SCN5A in controls, patients and FDRs (Fig. 4). Commercial sequencing of the band pattern variations revealed A1868G transition (rs1805124) and the two band patterns identified were homozygous 'AA' and 'GG' genotypes (Fig. 5). The A1868G polymorphism leads to a missense substitution of histidine to arginine at the 558th codon (H558R).

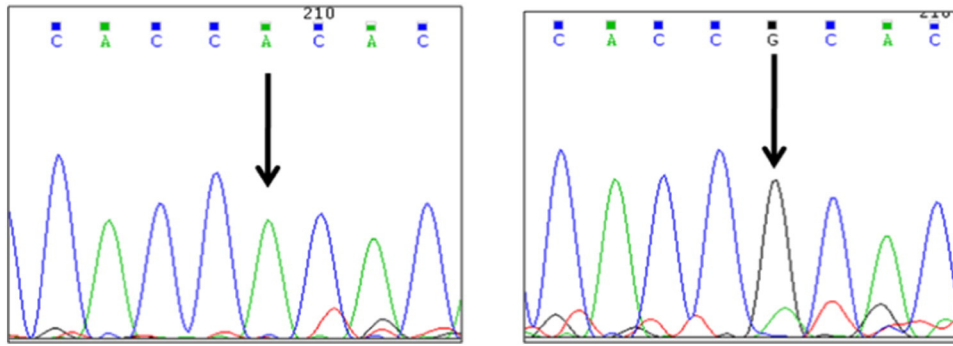


Fig. 5. Electropherograms exhibiting 'AA' and 'GG' genotypes of exon 12 A1868G polymorphism (H558R) of *SCN5A* gene (rs1805124).

3.2.2. Statistical analysis

Genotype frequency of 'GG' was higher in controls (68%) and FDRs (71%) compared to LQTS patients (47%) whereas 'AA' genotype was higher in LQTS patients (53%) than controls (32%) and FDRs (29%). Correspondingly, the 'A' allele frequency was found to be almost two-fold higher in LQTS patients (0.53) when compared to FDRs (0.29) and controls (0.32) (Table 4). This highlights the significance of 'A' allele in LQTS patients.

Table 5 gives the odds risk estimates which revealed the 'AA' genotype to be the risk genotype with OR of 2.43 (95% CI—1.23–4.79, $p=0.01$) when LQTS group was compared to controls. When LQTS group was compared to FDRs, the genotype 'AA' showed a 2.8 fold risk (95% CI—1.28–6.13, $p=0.0092$). Correspondingly, the 'A' allele was identified as the risk allele when LQTS was compared to controls (OR—2.3 (95% CI—1.3–4.2) and $p=0.0029$) and LQTS was compared to FDRs (OR—2.7 (95% CI—1.54–4.94) and $p=0.0007$). Thus, 'A' allele may act as the risk allele and the encoded protein may lead to dysfunction of sodium channel.

3.2.3. In-silico analysis

It revealed the addition of a stem and loop in the mRNA secondary structure of the variant. A decrease in free energy was observed on comparing the wild type to the variant mRNA (−71.67 kcal/mol to −73.54 kcal/mol) leading to a thermodynamically unstable mRNA thus ending in functional alterations.

Variations were identified in binding sites of three spliceosome complex proteins i.e., 9G8, SRp55 and NOVA1. Surprisingly, the binding site

for NOVA1 was disrupted in the variant sequence. 9G8 and SRp55 are exonic splicing enhancers and variations in their binding sites may lead to aberrant splicing and hence may have an effect on gene regulation.

This polymorphism was found to substitute histidine for arginine at the 558th codon of *SCN5A* transmembrane protein. The H558R polymorphism is located in DIS6–DIIS1 domain of *SCN5A* and a change from an aromatic amino acid may cause changes in the protein folding and conformation leading to an aberrant ion channel.

3.2.4. Hapmap

The population diversity indicates a higher 'G' allele frequency in all populations except in the South Indian LQTS patients of the present study wherein a higher 'A' allele frequency was observed. A similar pattern was observed in the controls and FDRs with the 'G' allele frequency being higher than the 'A' allele (Fig. 6).

3.2.5. Genotype-based clinical characteristics

Correlation of genotypes of H558R polymorphism with the clinical features indicates that the distribution of cLQTS patients was almost equal in both the genotypes with a female preponderance. Interestingly, patients harboring 'GG' genotype were of higher percentage in cLQTS type (86%), females (76%), patients with deafness (14%), family history of sudden deaths (48%) and consanguinity (29%) (Table 6). Although, the effect of 'GG' genotype is quite evident on the clinical features, the influence of 'AA' genotype also needs to be considered since it is an

Table 4

Genotype and allele frequency distribution of exon 12 A1868G polymorphism (H558R) of *SCN5A* gene (rs1805124).

Genotype	Controls n (%)	LQTS n (%)	FDRs n (%)	Allele	Controls	LQTS	FDRs
AA	48 (32)	25 (53)	20 (29)	G	0.68	0.47	0.71
GG	102 (68)	21 (47)	49 (71)	A	0.32	0.53	0.29

Table 5

Odds risk estimates of exon 12 A1868G polymorphism (H558R) of *SCN5A* gene (rs1805124).

	Genotype	OR (95% CI)	p value
LQTS vs Ctrl	GG	1.00	0.01
	AA	2.43 (1.23–4.79)	
	G vs A	2.3 (1.3–4.2)	
FDRs vs Ctrl	GG	1.00	0.65
	AA	0.87 (0.47–1.62)	
	G vs A	0.86 (0.47–1.5)	
LQTS vs FDRs	GG	1.00	0.0092
	AA	2.80 (1.28–6.13)	
	G vs A	2.7 (1.54–4.94)	

$p < 0.05$.

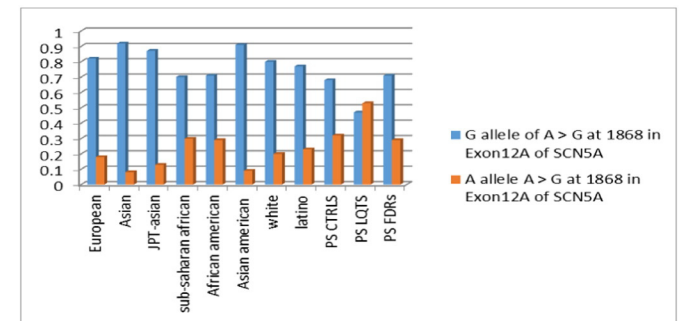


Fig. 6. Hapmap of exon 12 A1868G polymorphism (H558R) of *SCN5A* gene (rs1805124). PS—present study; CTRLS—controls.

Table 6

Clinical characteristics of LQTS patients exhibiting genotypes of exon 12 A1868G polymorphism (H558R) of *SCN5A* gene (rs1805124).

<i>SCN5A</i> H558R	cLQTS (%)	Females (%)	Deafness (%)	F/h sudden death (%)	Consanguinity (%)	Syncope (%)
AA	80	52	–	24	24	60
GG	86	76	14	48	29	57

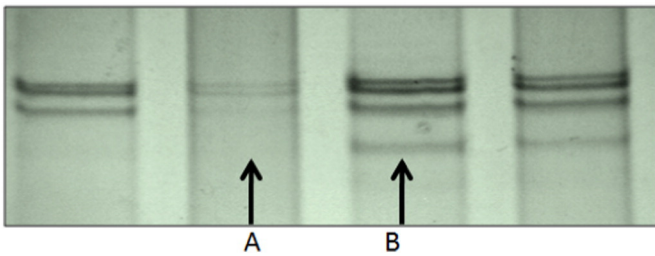


Fig. 7. SSCP band pattern variation in exon 17 of *SCN5A* gene.

established risk allele in the present study. Interestingly, subjects harboring 'AA' genotype were observed to have higher incidence of syncope (60%) highlighting its importance as the risk genotype which may have expressed in homozygous state due to the consanguinity.

3.3. Exon 17 (2nd fragment of the 17th exon) of *SCN5A* gene

3.3.1. Molecular analysis

The PCR-SSCP analysis of exon 17 (17B – 2nd fragment) of *SCN5A* gene revealed band patterns 'A' and 'B' which on sequencing revealed two SNPs as compound heterozygotes/genetic compounds (Fig. 7):

- A. A transition A3378G – neutral substitution E1061E (rs7430407)
- B. A novel transversion of C3417A – missense substitution S1074R [submitted to dbSNP (ss770677264)].

3.3.1.1. A3378G (E1061E – rs7430407). Band patterns 'A' and 'B' revealed 'GG' and 'AG' genotypes respectively on commercial sequencing in controls, LQTS and FDRs (Fig. 8).

3.3.2. Statistical analysis

Table 7 shows that 'GA' genotype was predominant in controls, LQTS patients and FDRs compared to 'GG' genotype. Interestingly, the 'GA' genotype was of high frequency in LQTS (62%) and FDRs (72%) than controls (53%). Correspondingly, the 'A' allele frequency was higher in LQTS (0.31) and FDRs (0.36) when compared to controls (0.27) indicating its role in LQTS etiology.

The odds risk estimates presented in Table 8 revealed a significant association of 'GA' genotype with 2.30 fold risk (95% CI–1.24–4.27, $p = 0.0066$) when FDRs were compared to controls. Higher 'A' allele frequency in LQTS and FDRs indicates its possible role in this disorder.

Table 7

Genotype and allele frequency distribution of exon 17 A3378G polymorphism (E1061E) of *SCN5A* gene (rs7430407).

Genotype	Controls n (%)	LQTS n (%)	FDR n (%)	Allele	Controls	LQTS	FDR
GG	70 (47)	17 (38)	19 (28)	G	0.73	0.69	0.64
GA	80 (53)	29 (62)	50 (72)	A	0.27	0.31	0.36

Table 8

Odds risk estimates of exon 17 A3378G polymorphism (E1061E) of *SCN5A* gene (rs7430407).

	Genotype	OR (95% CI)	p value
LQTS vs Ctrl	GG	1.00	0.29
	GA	1.44 (0.73–2.85)	
	G vs A	1.21 (0.65–2.24)	0.53
FDRs vs Ctrl	GG	1.00	0.0066
	GA	2.30 (1.24–4.27)	
	G vs A	1.52 (0.83–2.7)	0.17
LQTS vs FDRs	GG	1.00	0.06
	GA	0.23 (0.10–1.51)	
	G vs A	1.25 (0.69–2.25)	0.45

$p < 0.05$.

3.3.3. In-silico analysis

mRNA secondary structure analysis showed that the A3378G substitution leads to complete transformation of the mRNA. The free energy also changed from -48.57 kcal/mol in wild type to -66.13 kcal/mol in the variant leading to thermodynamic instability affecting the downstream signaling.

The A3378G (E1061E) substitution had affected the binding sites for five spliceosome proteins of which three proteins were exonic splicing enhancers (ESE) (9G8, Tra2alpha and Tra2beta) and the other two were exonic splicing silencers (ESS) (hnRNPA2B1 and hnRNPH1). New binding sites were created for 9G8 and hnRNPA2B1 whereas Tra2beta binding site had been disrupted. Variations in ESE and ESS may affect the splicing and spliceosome complex.

3.3.3.1. Codon usage. The A3378G substitution was found to cause a synonymous substitution of glutamic acid at the 1061th codon. The substitution E1061E was located in the DIIS6–DIIS1 domain of the *SCN5A* transmembrane protein. Codon usage revealed an increase of variant codon GAA usage (0.58) than the wild type codon GAG (0.42) which may lead to prolonged depolarization and QTc due to the formation of aberrant transmembrane domain.

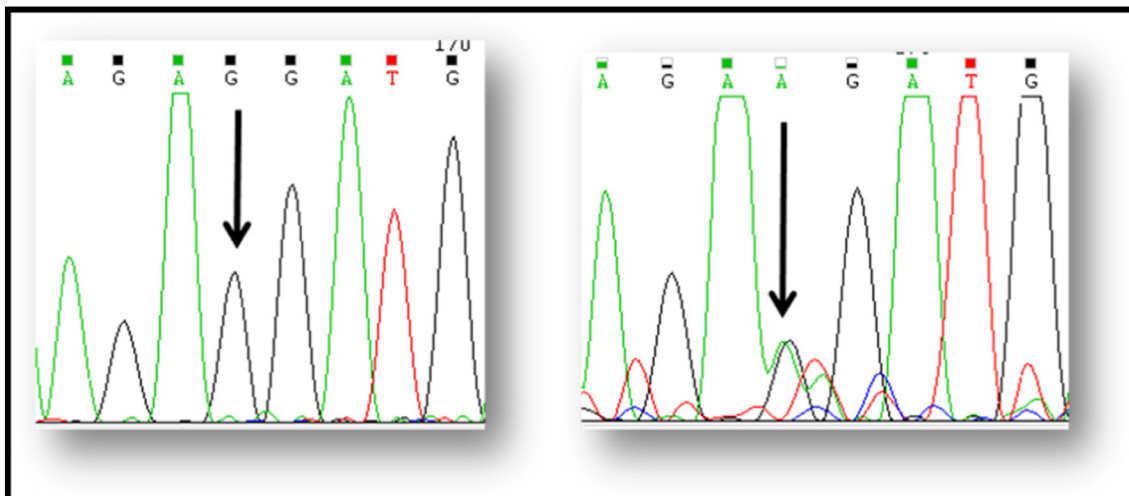


Fig. 8. Electropherograms exhibiting 'GG' and 'GA' genotypes of exon 17 A3378G polymorphism (E1061E) of *SCN5A* gene (rs7430407).

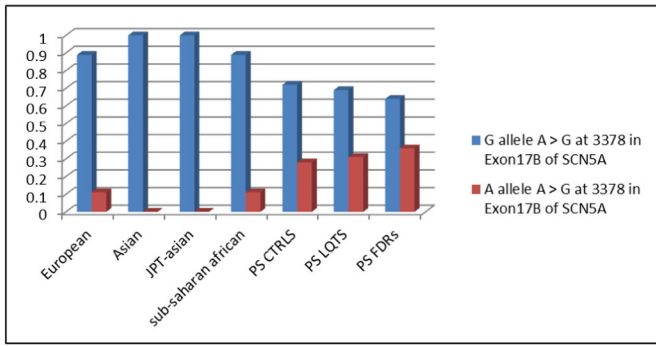


Fig. 9. Hapmap of exon 17 A3378G polymorphism (E1061E) of SCN5A gene (rs7430407). PS—present study; CTRLS—controls.

Table 9
Clinical characteristics of LQTS patients exhibiting genotypes of Exon 17B A3378G polymorphism (E1061E) of SCN5A gene (rs7430407).

SCN5A E1061E	cLQTS (%)	Females (%)	Deafness (%)	F/h sudden death (%)	Consanguinity (%)	Syncope (%)
GG	83	62	6	31	31	52
GA	88	53	12	35	24	71

Table 10
Genotype and allele frequency distribution of exon 17 C3417A polymorphism (S1074R) of SCN5A gene.

Genotype	Controls n (%)	LQTS n (%)	FDR n (%)	Allele	Controls	LQTS	FDR
CC	70 (47)	17 (38)	19 (28)	C	0.73	0.69	0.64
CA	80 (53)	29 (62)	50 (72)	A	0.27	0.31	0.36

Table 11
Odds risk estimates of exon 17 C3417A polymorphism (S1074R) of SCN5A gene.

	Genotype	OR (95% CI)	p value
LQTS vs CtrlS	CC	1.00	0.29
	CA	1.44 (0.73–2.85)	
	C vs A	1.21 (0.65–2.24)	
FDRs vs CtrlS	CC	1.00	0.0066
	CA	2.30 (1.24–4.27)	
	C vs A	1.52 (0.83–2.7)	
LQTS vs FDRs	CC	1.00	0.06
	CA	0.23 (0.10–1.51)	
	C vs A	1.25 (0.69–2.25)	

p < 0.05.

3.3.4. Hapmap

On comparing the allelic frequency of the A3378G (E1061E) substitution, the ‘G’ allele frequency was observed to be higher across all the

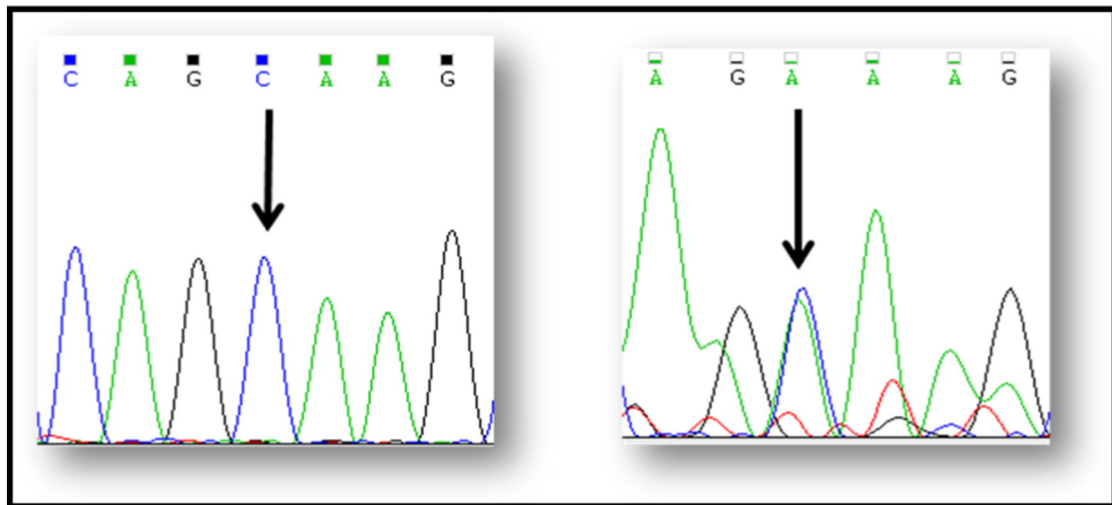


Fig. 10. Electropherograms exhibiting ‘CC’ and ‘CA’ genotypes of exon 17 C3417A polymorphism (S1074R) of SCN5A gene.

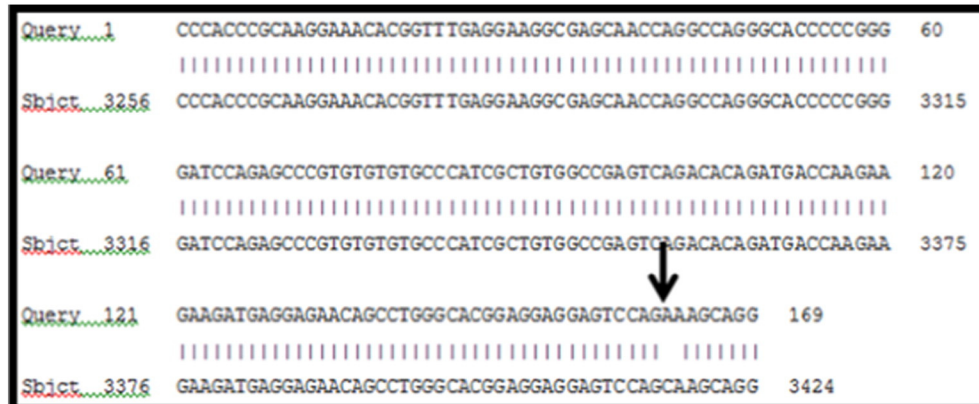


Fig. 11. NCBI BLAST of exon 17 C3417A polymorphism (S1074R) of SCN5A gene.

Table 12

Clinical characteristics of LQTS patients exhibiting genotypes of exon 17 C3417A polymorphism (S1074R) of *SCN5A* gene.

SCN5A S1074R	cLQTS (%)	Females (%)	Deafness (%)	F/h sudden death (%)	Consanguinity (%)	Syncope (%)
CC	83	62	6	31	31	52
CA	88	53	12	35	24	71

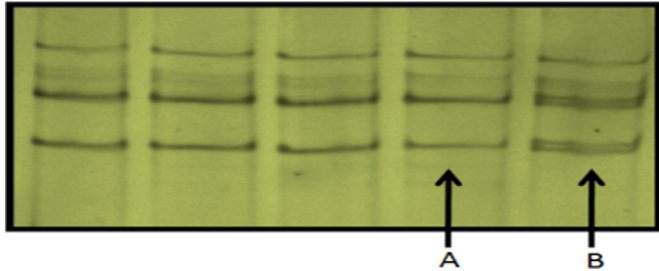


Fig. 12. SSCP band pattern variation in exon 25 IVS25 + 65G>A polymorphism of *SCN5A* gene.

populations. It was interesting to note that although the 'A' allele was absent in Asian and Japanese populations, it was fixed in the South Indian cohort highlighting the uniqueness of the gene pool (Fig. 9).

3.3.5. Genotype-based clinical characteristics

Table 9 shows the clinical features of the LQTS patients exhibiting the A3378G (E1061E) genotypes. The patients with 'GA' genotype were found to have higher percentage of cLQTS (88%), deafness (12%), family history of sudden death (35%) and syncope (71%) implying it as the risk genotype. In both the genotypes, the frequency of cLQTS and females were found to be high corresponding to the epidemiological data. Higher percentage of SCDs in families and manifestation as syncope in probands may be attributed to the 'A' allele.

3.3.5.1. Novel C3417A (S1074R – ss770677264). The novel transversion identified in exon 17B was C3417A substitution. Fig. 10 shows the electropherogram with 'CC' and 'CA' genotypes for band patterns 'A' and 'B' respectively. This transversion was leading to the substitution of serine with arginine at the 1074th codon of *SCN5A*. Fig. 11 shows the NCBI BLAST of exon 17 C3417A (S1074R) polymorphism of *SCN5A* indicating the position of the polymorphism.

3.3.6. Statistical analysis

Genotypic and allelic frequency presented in Table 10 revealed higher frequency of 'CA' genotype and 'C' allele in all the groups. An

Table 13

Genotype and allele frequency distribution of exon 25 IVS25 + 65G>A polymorphism of *SCN5A* gene.

Genotype	Controls n (%)	LQTS n (%)	FDR n (%)	Allele	Controls	LQTS	FDR
GG	113(75)	42 (91)	64 (93)	G	0.88	0.96	0.96
GA	37 (25)	4 (9)	5 (7)	A	0.12	0.04	0.04

Table 14

Odds risk estimates of exon 25 IVS25 + 65G>A polymorphism of *SCN5A* gene.

	Genotype	OR (95% CI)	p value
LQTS vs Ctrls	GG	1.00	0.014
	GA	0.30 (0.10–0.89)	
	A vs G	3.27 (1.01–10.5)	
FDRs vs Ctrls	GG	1.00	0.0011
	GA	0.24 (0.09–0.64)	
	A vs G	3.27 (1.01–10.5)	
LQTS vs FDRs	GG	1.00	0.75
	GA	1.25 (0.32–4.92)	
	A vs G	1.0 (0.2–4.11)	

p < 0.05.

interesting observation was that the 'CA' genotype was higher in LQTS (62%) and FDRs (72%) compared to controls (53%). The 'A' allele frequency was found to be higher in LQTS (0.31) and FDRs (0.36) than controls (0.27) indicating the role of this allele in LQTS.

Table 11 shows the odds risk estimates with significant association of 'CA' genotype (OR–2.30 (95% CI–1.24–4.27) and p–0.0066) when FDRs were compared to controls further strengthening the association in LQTS.

3.3.7. In-silico analysis

mRNA secondary structure indicated a change in free energy from –48.57 kcal/mol in wild type to –66.13 kcal/mol in the variant. This may lead to thermodynamic instability of the mRNA causing splicing variations. Changes in binding site of spliceosome proteins viz. Tra2beta and hnRNPA1 respectively were revealed. Variation in scores of exonic splicing enhancer Tra2beta and binding site of exonic splicing silencer hnRNPA1 may affect the splicing mechanism causing variation in *SCN5A* protein.

C3417A polymorphism of *SCN5A* caused a missense substitution leading to a change of polar, uncharged and hydrophilic serine to polar, basic and hydrophilic arginine at the 1074th codon. Such a change may cause alterations in the *SCN5A* protein folding due to difference in properties. It was found to be located in DIIS6–DIIS1 domain of *SCN5A* transmembrane structure.

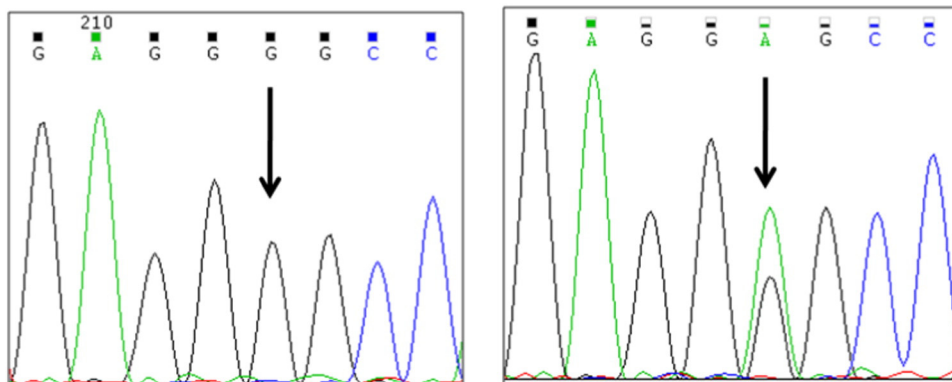


Fig. 13. Electropherograms exhibiting 'GG' and 'GA' genotypes of exon 25 IVS25 + 65G>A polymorphism of *SCN5A* gene.

Table 15
Clinical characteristics of LQTS patients exhibiting genotypes of SCN5A gene IVS25 + 65G>A polymorphism.

SCN5A IVS25 + 65G>A	cLQTS (%)	Females (%)	Deafness (%)	F/h sudden death (%)	Consanguinity (%)	Syncope (%)
GG	83	67	5	33	31	60
GA	75	–	–	50	–	75

3.3.8. Genotype-based clinical characteristics

The clinical features of the LQTS patients exhibiting the C3417A (S1074R) genotypes are given in Table 12. LQTS patients with 'CA' genotype were observed to be higher in cLQTS (88%), deafness (12%), family history of sudden death (35%) and syncope (71%) implying it as the risk genotype. Higher percentage of females (62%) and consanguinity (31%) were observed in 'CC' genotype. Hence, 'A' allele may be the risk allele in the manifestation of LQTS.

3.4. Exon 25 of SCN5A gene (IVS25 + 65G>A)

3.4.1. Molecular analysis

Two types of band patterns (A and B) were observed on screening exon 25 of SCN5A gene by PCR-based SSCP in controls, LQTS and FDRs (Fig. 12). Two types of band patterns 'A' and 'B' revealed 'GG' and 'GA' genotypes respectively of IVS25 + 65G>A polymorphism on commercial sequencing (Fig. 13).

3.4.2. Statistical analysis

'GG' genotype was found to predominate in LQTS and FDRs. It was observed to be higher in LQTS (91%) and FDRs (93%) when compared to controls (75%). Correspondingly, the 'G' allele was also higher in LQTS (0.96) and FDRs (0.96) than controls (0.88) pointing towards the role of this allele in the development of this fatal disorder (Table 13).

The odds risk estimates show the 'GA' heterozygote as the protective genotype with an OR of 0.3 (95% CI–0.10–0.89 and $p=0.014$) when LQTS was compared to controls. When FDRs were compared to controls an OR 0.24 (95% CI–0.09–0.64 and $p=0.0011$) was observed. The 'G' allele was identified as the risk allele in the case of LQTS and FDRs with a three-fold risk (OR–3.27 (95% CI–1.01–10.5) and $p=0.046$) (Table 14).

3.4.3. In-silico analysis

The effect of intronic variation on mRNA secondary structure caused a free energy change in the variant (–77.75 kcal/mol) compared to wild type (–77.5 kcal/mol). Though the free energy change is negligible, the substitution leads to a complete change of secondary structure and affects the thermodynamic stability influencing downstream signaling.

The binding site of SC35 which is an exonic splicing enhancer was found to be affected apart from disruption of a binding site. Another spliceosome protein hnRNPH1, an exonic splicing silencer was also affected. Such variations in spliceosome protein binding sites may lead to aberrant splicing and abnormal transmembrane protein.

3.4.4. Genotype-based clinical characteristics

The 'GG' genotype observed in the LQTS cases was found to be present in 83% cLQTS, 67% females, 5% deaf, 33% with family history of sudden deaths, 31% of consanguineous and 60% of syncope cases thus, correlating its significance with clinical features (Table 15). Therefore, it may be inferred that the 'G' allele maybe playing a role as risk allele causing variations in SCN5A expression leading to ion channel anomalies, thus causing prolonged QTc. It is observed to affect higher number of females indicating the influence of natural selection in the preference of this gender. Parental consanguinity may contribute to accumulation of variations leading to SCDs and clinical manifestation as syncope in probands.

3.5. Haplotype and linkage disequilibrium analysis

Haplotype frequency for various allelic combinations between the five polymorphisms of SCN5A gene was computed for their possible association with long QT syndrome. The frequency of GGGCG haplotype in controls (0.43) and GAGCG haplotype in LQTS (0.34) was reported. There was a significant association of GAGCG, AGAAG, AGGCG, AAGCG, and GAGCA haplotypes ($p < 0.0001$) with LQTS. The increased significance of association of these haplotypes with long QT syndrome implicates the functional role of compound haplotypes in disease susceptibility (Table 16).

A pair-wise comparison of the five polymorphisms, depicting LD measures is represented in Fig. 14. Significant D' values were observed for the polymorphisms with IVS25 + 65G>A polymorphism with A29A, E1061E and S1074R ($D' = 0.99$) indicating a strong/tight linkage disequilibrium between these SNPs whereas moderate LD was found for H558R and E1061E ($D' = 0.66$) and H558R and S1074R ($D' = 0.66$) polymorphisms.

4. Discussion

The SCN5A mutations have a dominant phenotype at the molecular level in cLQTS. Most mutant cardiac NaV channels associated with LQTS exhibit a characteristic impairment of inactivation, leading to persistent inward Na⁺ current causing prolonged membrane depolarizations (George, 2005). Screening of SCN5A in South Indian cohort of LQTS, FDRs and controls revealed five polymorphisms – A29A (exon-2), H558R (exon-12), E1061E and S1074R (exon-17) and IVS25 + 65G>A (exon-25). Other polymorphisms like D1819D, IVS9-3C>A, G298S, G514C, P1008S, G1406R and D1595N identified in other ethnic populations, were not reported in the present study, strengthening the genetic

Table 16
Haplotype frequencies and odds risk estimates of the five polymorphisms of SCN5A gene.

Haplotype	Haplotype frequency		OR (95% CI)	p
	Controls	LQTS		
GGGCG	0.43	0.04	1.00	–
GAGCG	–	0.34	124,662,872,049.23 (124,662,872,045.13–124,662,872,053.32)	<0.0001
AGAAG	0.10	–	67,375,283,145.30 (40,036,686,944.86–113,381,728,741.97)	<0.0001
AGGCG	0.13	0.23	12,373,079,257.30 (6,071,115,461.05–25,216,632,971.27)	<0.0001
AAAAA	–	–	–	–
AAGCG	–	0.14	129,022,760,452.53 (129,022,760,451.82–129,022,760,453.24)	<0.0001
AAAAG	–	–	–	–
GAGCA	–	0.04	137,082,703,288.18 (137,082,703,287.92–137,082,703,288.44)	<0.0001

$p < 0.05$.

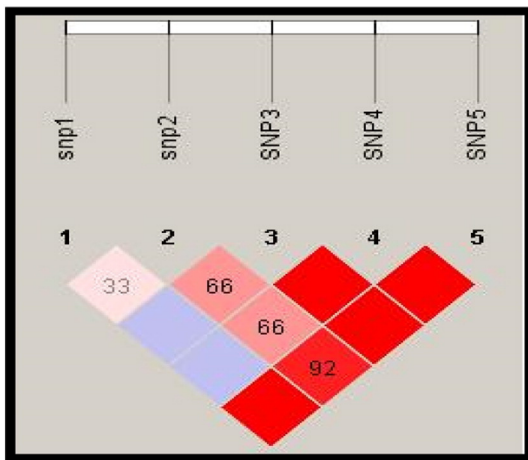


Fig. 14. Pairwise linkage disequilibrium analysis of *SCN5A* polymorphisms. SNP-1—A29A; SNP-2—H558R; SNP-3—E1061E; SNP-4—S1074R; SNP-5—IVS25 + 65G > A.

diversity of this population. Further, it is interesting to observe the presence of only two genotypes in all the five SNPs of *SCN5A* emphasizing the unique genetic composition of Indian population.

The clinical heterogeneity associated with *SCN5A* mutations is partly explained by corresponding differences in the degree and characteristics of channel dysfunction and associated specific clinical features (George, 2005).

A282G polymorphism (rs6599230), resulting in alanine for alanine (A29A) silent substitution, was identified in exon-2 of *SCN5A* gene. Of the two genotypes identified, 'GA' genotype and 'A' allele frequency were found to be higher in LQTS patients than controls. Though odds risk estimates did not reveal a significant association, the role of 'A' allele in the diseased phenotype cannot be overruled. More number of females, deaf cases and percentage of consanguinity was observed in 'GA' genotype associated with LQTS. The substitution led to alterations in the mRNA secondary structure with subsequent influence on downstream signaling. Although this polymorphism resulted in a silent substitution at the 29th codon in the N-terminus of *SCN5A* protein, the codon usage revealed an increase in usage which may play a role in etiology of LQTS. The allelic frequency comparison with other populations revealed the frequency in South Indian population to be in concordance with Asian population.

Exon-12 revealed A1868G polymorphism (H558R — rs1805124) and its 'AA' genotype and 'A' allele frequency were found to be higher in LQTS patients compared to controls and FDRs pointing towards its role in LQTS etiology. The substitution also caused loss of a stem and loop of mRNA secondary structure and influenced exonic splicing enhancers which may lead to splicing variations and a truncated *SCN5A* transmembrane protein. This missense polymorphism is located in the DIS6–DIIS1 domain of *SCN5A*. A series of studies on *SCN5A* polymorphism H558R suggested that it could modulate the biophysical effects of a nearby mutation within the same gene in Chinese Han cardiac arrhythmic patients (Chen et al., 2004). This polymorphism has been suggested to be associated with nonfunctioning or poorly functioning sodium channels and a subsequently reduced conduction velocity (Husser et al., 2009). Therefore, it can be inferred that this polymorphism may lead to variations in *SCN5A* expression causing changes in protein folding and resulting in aberrant sodium ion channels.

Two polymorphisms A3378G (E1061E) and the novel C3417A (S1074R) were identified as compound heterozygotes/genetic compound in exon-17 of *SCN5A*. The heterozygotes AG/CA were identified as the risk genotypes when FDRs were compared to controls. Higher 'AG/CA' genotype and 'A' allele frequency (E1061E and S1074R) were found in LQTS and FDRs. 'CA' genotype was found to be higher in cLQTS and females, syncope, with an increased family history of sudden death, emphasizing its role in LQTS. The SNPs altered mRNA secondary

structure and influenced spliceosome protein binding sites which may lead to aberrant splicing. Though E1061E is a silent substitution, the codon usage was observed to be biased leading to protein structure variation and conformational changes. These polymorphisms were located in the DIS6–DIIS1 domain of the *SCN5A* transmembrane protein. The genetic compounds identified may lead to downstream signaling variations causing aberrations in sodium channel functions leading to prolonged QTc.

Screening of exon-25 led to the identification of IVS25 + 65G > A in intron-25 of *SCN5A*. 'G' allele was identified as the risk allele. Correlation with clinical features revealed higher cLQTS, females, deafness and consanguinity in patients of 'GG' genotype. This intronic polymorphism influenced the mRNA secondary structure and binding sites of two spliceosome proteins. It is increasingly recognized that mutations within the promoter and intronic sequences may have important effects on gene transcription and splice variants. Mutations in these regions can provide important insights in gene regulation and expression profiling (Koo et al., 2007). This intronic substitution may affect *SCN5A* gene expression which may alter downstream signaling leading to variations in sodium channel transmembrane.

Haplotype analysis revealed a significant association of GAGCG, AGAAG, AGGCG, AAGCG and GAGCA haplotypes with LQTS implicating the functional role of compound haplotypes in disease susceptibility. Hence, it can be concluded that compound heterozygotes of *SCN5A* gene polymorphisms may be deleterious/lethal, leading to an aberrant sodium ion channel causing prolonged QTc.

Screening of *SCN5A* gene revealed five polymorphisms including four reported and one novel in South Indian population. These studies need to be further confirmed by large cohort studies. The genotype-phenotype correlation was a preliminary attempt to unravel the complex etiopathogenesis of LQTS which needs to be carried forward to completely understand the etiology of this complex disorder.

Competing interest

There is no conflict of interest within the authors.

Consent

Informed written consent was obtained from the probands and their family members.

Ethics committee approval

The study has been approved by the Institutional Ethics Committee, Dept. of Genetics, Osmania University, India.

Author's contributions

S.F.Q. has carried out the molecular and statistical analysis described in this manuscript and has compiled the manuscript. **A.A.** has helped to carry out the in-silico analysis described in this manuscript. **A.V.** has interpreted the results described in this manuscript. The probands described in this manuscript have been diagnosed for LQT syndrome by **M.P.J.**, **C.V.**, **J.S.** and **H.R.** at their respective hospitals. **K.T.** has been critical in the review and compilation of the manuscript. As the corresponding author, the concept, design and compilation of this manuscript have been carried out by **P.N.**

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