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ARTICLE

Genetic analysis, *in silico* prediction, and family segregation in long QT syndrome

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The heritable cardiovascular disorder long QT syndrome (LQTS), characterized by prolongation of the QT interval on electrocardiogram, carries a high risk of sudden cardiac death. We sought to add new data to the existing knowledge of genetic mutations contributing to LQTS to both expand our understanding of its genetic basis and assess the value of genetic testing in clinical decision-making. Direct sequencing of the five major contributing genes, *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2*, was performed in a cohort of 115 non-related LQTS patients. Pathogenicity of the variants was analyzed using family segregation, allele frequency from public databases, conservation analysis, and Condel and Provean *in silico* predictors. Phenotype-genotype correlations were analyzed statistically. Sequencing identified 36 previously described and 18 novel mutations. In 51.3% of the index cases, mutations were found, mostly in *KCNQ1*, *KCNH2*, and *SCN5A*; 5.2% of cases had multiple mutations. Pathogenicity analysis revealed 39 mutations as likely pathogenic, 12 as VUS, and 3 as non-pathogenic. Clinical analysis revealed that 75.6% of patients with QTc $\geq$ 500 ms were genetically confirmed. Our results support the use of genetic testing of *KCNQ1*, *KCNH2*, and *SCN5A* as part of the diagnosis of LQTS and to help identify relatives at risk of SCD. Further, the genetic tools appear more valuable as disease severity increases. However, the identification of genetic variations in the clinical investigation of single patients using bioinformatic tools can produce erroneous conclusions regarding pathogenicity. Therefore segregation studies are key to determining causality.
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INTRODUCTION

Congenital long QT syndrome (LQTS), a cardiovascular disorder occurring in 1 in 2000 individuals[1] and is characterized on electrocardiogram by a QT interval prolongation that results from a repolarization abnormality within a structurally normal heart.[2,3] The LQTS phenotype ranges from asymptomatic to the presence of syncopal episodes, seizures, malignant ventricular tachyarrhythmias (usually *torsade de pointes*), ventricular fibrillation, and sudden cardiac death (SCD).[4,5]

To date, mutations in five genes - *KCNQ1* (KvLQT1 or Kv7.1, LQTS type 1)[6] and its associated β subunit *KCNE1* (mink, LQTS type 5);[7] *KCNH2* (hERG or Kv11.1, LQTS type 2)[8] and its associated β subunit *KCNE2* (mirp1, LQTS type 6);[9] and *SCN5A* ($Na_v1.5$, LQTS type 3)[10] - produce approximately 75% of LQTS cases.[11–16] Although 90% of LQTS mutations are localized in these five genes, pathogenic mutations have been reported for 11 other genes: *ANK2*, *KCNJ2*, *CACNA1C*, *CAV3*, *SCN4B*, *AKAP9*, *SNTA1*, *KCNJ5*, *CALM1*, *CALM2*,[17] and *RYR2*.[18]

Recent guidelines recommend genetic investigation of arrhythmias for clinical purposes.[19,20] Determining the genotype supports clinical diagnosis, improves treatment strategies, and may help identify risk of SCD.[20–22] In addition, positive genetic testing helps identify carrier family members who might also be at risk of SCD.[20] However, as genetic studies unravel more disease associations, the use of genetic information in clinical decision-making becomes increasingly complex, especially when discerning pathogenic mutations from neutral variants and variants of uncertain significance (VUS).[23–25]

To examine the spectrum of pathogenic mutations and to generate new data toward assessing the value of genetic testing in clinical decision-making, we performed a genetic screening of the five recommended major LQTS-related genes from 115 non-related patients clinically diagnosed with LQTS.

MATERIALS AND METHODS

Ethics and approvals

This study was approved by the Ethics Committee of Hospital Josep Trueta (Girona, Spain) and conforms to the principles outlined in the Declaration of Helsinki.

Sample population

This study enrolled 115 non-related Caucasian LQTS patients. Clinical data, including 12-lead ECG, personal history of syncope, and family history, were collected. The QTc value was obtained from the 12-lead ECG based on the Bazzett's formula. The diagnosis of LQTS was assessed by the re-evaluated Schwartz diagnostic criteria.[4,17] QTc $\geq$ 480 ms after exercise stress test was

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counted as 1 point.[17] Deafness was not reported in any of the patients analyzed.

All individuals signed a written informed consent to participate in the study. Informed consent of all patients was obtained in accordance with the Review Board guidelines of the Hospital Josep Trueta and Universitat of Girona (Girona, Spain).

Genetic analysis

Total genomic DNA was isolated from blood samples using the Puregene DNA purification Kit (Gentra System, Minneapolis, MI, USA). Exons and exon-intron boundaries of these genes were amplified (Verities PCR, Applied Biosystems, Austin, TX, USA). The PCR products were purified with Exosap-IT (USB Corporation, Cleveland, OH, USA) and directly sequenced in both directions (BigDye Terminator v3.1 Cycle Sequencing Kit, 3130XL Genetic Analyser, Applied Biosystems). Obtained DNA sequences were compared with the reference sequence of *KCNQ1* (NM_000218.2; MIM# 607542), *KCNH2* (NM_000238.3; MIM# 152427), *SCN5A* (NM_198056.2; MIM# 600163), *KCNE1* (NM_000219.3; MIM# 176261), and *KCNE2* (NM_172201.1; MIM# 603796) using the SeqScape v2.6 software (Applied Biosystems). Gene symbols were verified with HUGO Gene Nomenclature Committee (http://www.genenames.org/), and all GenBank reference sequences were obtained from the Consensus CDS Protein Set Database (http://www.ncbi.nlm.nih.gov/CCDS/).

Genetic variations were contrasted with HGMD Professional 2013.4 (http://www.biobase-international.com/), 1000 genomes project browser (http://browser.1000genomes.org), and dbSNP database v.137 (http://www.ncbi.nlm.nih.gov/projects/SNP). Exome Variant Server-NHLBI Exome Sequencing Project (ESP6500SI-V2, http://snp.gs.washington.edu/EVS/) and dbSNP database were used to find minor allele frequencies (MAFs) in all populations. UniProt database (http://www.uniprot.org/) was used to analyze the conservation of affected amino-acid regions between species. Mutation pathogenicity was predicted through two *in silico* programs: Condel (CONsensus DELeteriousness score of non-synonymous single-nucleotide variants),[26] which integrates the outputs of three tools: SIFT, Polyphen2, and Mutationassessor; and Provean v.1.1 (Protein Variation Effect Analyzer),[27] which is the first predictor that includes in-frame insertions/deletions.

Nomenclature is cited according to the HGVS guidelines (www.hgvs.org/mutnomen): 'c.' indicates a coding DNA sequence and 'p.' indicates a protein sequence. Sequence variants were checked using the Mutalyzer program (http://www.LOVD.nl/mutalyzer).

Sequence changes altering coding regions were defined as mutations, and only exonic mutations were included in the study. Mutations with an MAF in all populations $<1\%$ were considered as presuming disease alleles. Mutations with an MAF $\geq 1\%$ were excluded from the study. Novel mutations identified have been submitted to the LOVD v.3.0 (Leiden Open Variation Database, http://www.lovd.nl/3.0/home).

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). Mean values are expressed as mean ± SD. Statistical comparisons were performed using the unpaired Student's *t*-test and chi-squared test. Results were considered statistically significant when $P<0.05$.

RESULTS

In our cohort of 115 LQTS-affected non-related individuals (mean age of diagnosis 33.9 ± 19 years, 62.5% females), the mean QTc was 489.1 ± 56.5 ms. Syncope occurred in 57.1% of our patients, SCD had episodes occurred in 16.7%, and previous family history of LQTS and/or SCD was noted in 55.4% (Table 1).

Mutation analysis

We identified 59 index cases (51.3%) with a mutation in at least one of the 5 analyzed genes and 56 patients without mutations in these genes (48.7%) (Table 1). Genes most frequently found to have mutations were *KCNH2* (17.4% of total patients), *KCNQ1* (14.8%) and *SCN5A* (9.6%); and presence of more than one mutation in the same or multiple genes was identified in six index cases (5.2%) (Figure 1a). In total, 54 different mutations were found, 18 were novel, and 10 previously described mutations were identified in >1 patient (Supplementary Tables S1 and S2).

Pathogenicity of novel mutations. None of the 18 novel mutations were present in the general population. Alignment analysis revealed that only *KCNH2* p.(Gly238Arg) was not conserved between species (Supplementary Figure S1).

Segregation analysis from the eight available families carrying novel mutations (Table 2) revealed that two families segregated with the clinical diagnosis, five families showed incomplete penetrance (IP),

Table 1 Clinical data from non-related LQTS patients

	Number of patients	*Mean age (years)*	*Gender (female)*	*Syncope (yes)*	*Previous SCD (yes)*	*Family history (yes)*	*Mean QTc (ms)*
Total	115	33.9 ± 19 (109)	62.5% (112)	57.1% (105)	16.7% (102)	55.4% (101)	489.1 ± 56.5 (97)
Non-carriers	56	38.9 ± 19.1[a] (51)	58.5% (53)	59.2% (49)	16.3% (49)	56.3% (48)	470.8 ± 48.1[a] (44)
Carriers							
Total	59	29.6 ± 17.9 (58)	66.1% (59)	55.4% (56)	17.0% (53)	54.7% (53)	504.2 ± 58.8 (53)
KCNQ1 mutation	17	24.5 ± 15.0 (14)	70.6% (17)	64.3% (14)	14.3% (14)	73.3% (15)	514.4 ± 59.1 (14)
KCNH2 mutation	20	29.4 ± 14.6 (18)	70% (20)	70% (20)	16.7% (18)	52.9% (17)	500 ± 60.7 (18)
SCN5A mutation	11	39.0 ± 19.1 (9)	63.6% (11)	27.3%[b] (11)	20% (10)	40% (10)	492.2 ± 60.1 (9)
KCNE1 mutation	3	29.3 ± 30.9 (3)	33.3% (3)	66.7% (3)	0.0% (3)	0% (3)	470.7 ± 17.9 (3)
KCNE2 mutation	2	35.0 ± 1.41 (2)	50% (2)	50% (2)	50% (2)	50% (2)	467.5 ± 38.9 (2)
Single mutation	53	29.8 ± 17.0 (52)	66.0% (53)	58.0% (50)	17.0% (47)	53.2% (47)	503.4 ± 62.4 (46)
Multiple mutations	6	28.2 ± 26.5 (6)	66.7% (6)	33.3% (6)	16.7% (6)	66.7% (6)	510.5 ± 9.9 (6)
Non-pathogenic mutation	5	35.4 ± 19.9 (5)	60.0% (5)	60.0% (5)	40.0% (5)	40.0% (5)	473.0 ± 26.8 (5)
VUS mutation	7	35.0 ± 21.8 (7)	42.9% (5)	57.1% (7)	0.0% (6)	42.9%	453.2 ± 37.4 (6)[c]
Pathogenic mutation	41	28.2 ± 15.9 (40)	73.2% (41)	57.9% (38)	16.7% (36)	57.1% (35)	515.9 ± 64.1 (36)

[a]Non-carriers *vs* carriers: $P<0.01$.
[b]Carriers with *SCN5A* mutation *vs* carriers with *KCNH2* mutation: $P<0.05$.
[c]VUS carriers *vs* pathogenic mutations carriers: $P<0.05$.
Values are presented as mean ± SD. Complete clinical information was not available for each patient; thus the number of patients used to obtain the percentage or mean values are specified in parentheses.

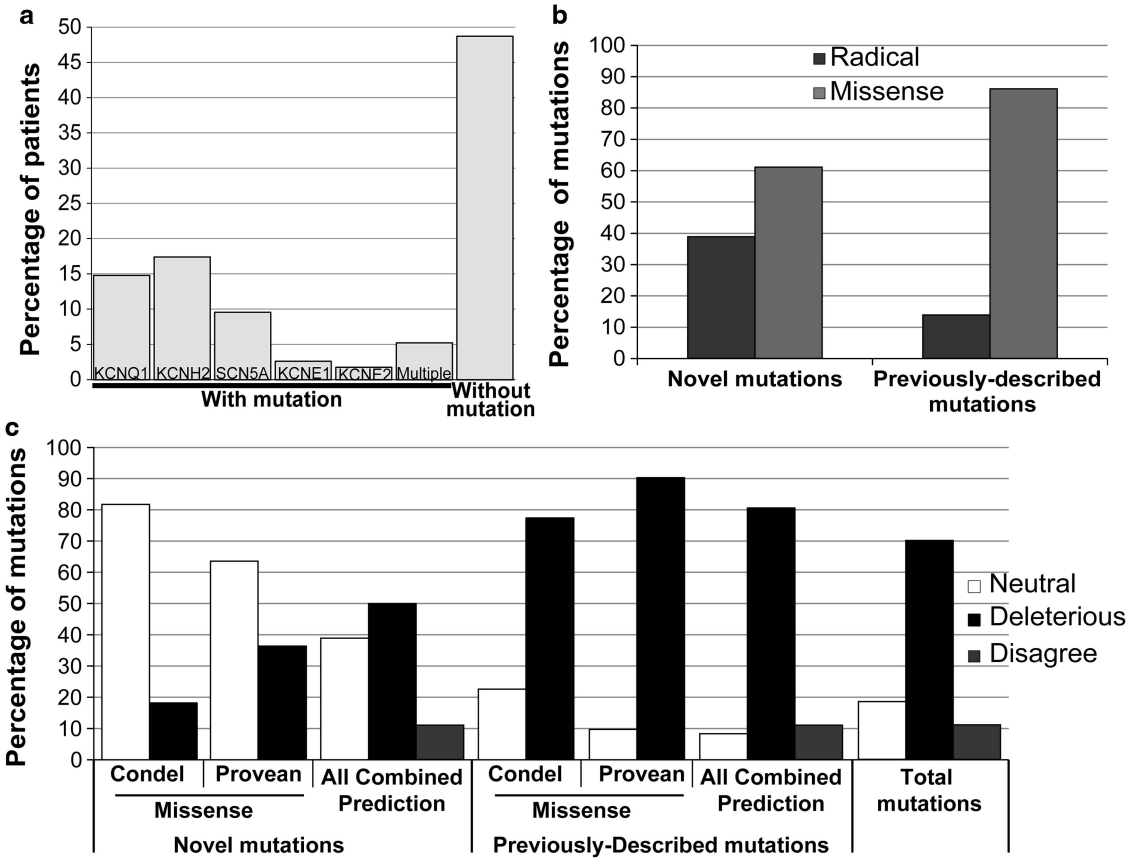

Figure 1 Mutation distribution in non-related LQTS patients. (**a**) Distribution of all mutations in our non-related LQTS cohort ($n=115$). Bars represent the percentages of patients with mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* or with multiple mutations (≥ 2 mutations in ≥ 1 genes) and without mutations in the five analyzed genes. (**b**) Distribution of radical mutations (in-frame deletions, frameshift deletions, and nonsense mutations; dark gray bars) and missense mutations (light gray bars) of the novel mutations ($n=18$) and previously described mutations ($n=36$) for the 54 mutations identified from 59 patients. (**c**) Distribution of *in silico* predictions for novel missense mutations ($n=11$) and previously described missense mutations ($n=31$). Mutations were classified as neutral (white bars) or deleterious (black bars). In combined prediction, bars represent radical (considered deleterious) and missense mutations predicted from both novel ($n=18$) and previously described ($n=36$) mutations. Disagreement bars represent the mutations with inconclusive data due to a different prediction between the two used tools (gray bars). Total mutations are the combined prediction for both novel and previously described radical and missense mutations.

and one mutation did not segregate with the clinical data. However, four index cases with novel mutations with IP also harbored previously described mutations in the same or other genes (see 'Pathogenicity of multiple mutations' paragraph and Figure 2).

We identified 7 radical mutations (in-frame deletion, frameshift deletion, and nonsense novel mutations) and 11 missense mutations (Table 2 and Figure 1b). Nonsense and frameshift mutations were considered deleterious, as in the literature,[28] because no *in silico* pathogenicity predictors exist for them. In-frame deletions were predicted as deleterious by Provean tool. *In silico* pathogenicity prediction from the 11 novel missense mutations revealed that 2 mutations had a deleterious effect; 3 mutations localized in the *KCNH2* C-terminus, 2 were predicted as neutral but predictions for the third were discordant; and 4 of the 5 mutations in the *KCNH2* N-terminus or *SCN5A* IDL were predicted as neutral, the fifth had discordant predictions (Figure 1c). Nevertheless, none of these variants were found in the general population, and alignment analysis revealed all but one (*KCNH2* p.(Gly238Arg)) as conserved amino acids.

Pathogenicity of previously described mutations. Of the known mutations confirmed in our population, 5 were radical mutations and 31 were missense mutations (Figure 1b). Three mutations occurred in the general population with an MAF $<1\%$ (Supplementary Table S2). Alignment analysis showed conservation of all but three of the affected amino acids. Family members were available for 19 index cases. Segregation analysis revealed that 10 mutations segregated with the disease, 6 segregated with IP, 1 did not segregate, and 2 were *de novo* (Supplementary Tables S1 and S2).

Based on these analyses and other reports, we considered 25 previously described mutations as probably pathogenic mutations (radical mutations or missense mutations without frequency in the general population, amino-acid conservation, and positive segregation from the families available) (Supplementary Table S1). Our *in silico* analysis predicted 23 out of the 25 probably pathogenic mutations as deleterious, and 2 mutations were predicted differently between the tools used (Figure 1c and Supplementary Table S1).

In addition, we considered four previously described mutations as having uncertain significance (Supplementary Table S2): amino acids *KCNQ1* p.Pro73 and *SCN5A* p.Pro1008 were not conserved between species. Further, *KCNQ1* p.(Pro73Thr) was previously described as a VUS,[28] and *SCN5A* p.(Pro1008Ser) had been associated with other arrhythmogenic diseases, similar to *SCN5A* p.(Arg620Cys).[29,30] Accordingly, *in silico* programs predicted these three mutations as neutral (Supplementary Table S2). In contrast, although *SCN5A*

Table 2 Eighteen novel mutations were found in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, and *KCNE2* from our LQTS patient cohort

							In silico prediction				
Patient no.	*Gene*	*Ex*	*Protein region*	*Aa change (p.)*	*Base-pair change (c.)*	*Mutation type*	*Zygosity*	*Condel*	*Provean*	Align	*Family segregation*	*Prediction*[a]
10	*KCNQ1*	7	Pore-forming (H5)	Tyr315His	943T>C	Missense	het	DEL	DEL	YES	YES	Pathogenic
73	*KCNQ1*	7	Transmembrane S6	Ile328_Ser330del	982_990del 5′-ATCGCCTCC-3′	Deletion in frame	het	—	DEL	YES	IP	Pathogenic
33	*KCNQ1*	10	Cytoplasmic C-term	Asp454Thrfs*7	1360_1375 del 5′-GACCACTTCTCTGTCG-3′	Frameshift/deletion	het	—	—	YES	IP (Figure 2a)	Pathogenic[b]
115	*KCNQ1*	12	Cytoplasmic N-term	Tyr522*	1566C>G	Nonsense	het	—	—	YES	N/A	Pathogenic
15	*KCNH2*	4	Cytoplasmic N-term	Ala172Val	515C>T	Missense	het	NTR	NTR	YES	N/A	VUS
81	*KCNH2*	4	Cytoplasmic N-term	Gly238Arg	712G>C	Missense	het	NTR	NTR	NO	NO	Non-pathogenic
31	*KCNH2*	5	Cytoplasmic N-term	Leu343Serfs*17	1027delC	Frameshift/deletion	het	—	—	YES	N/A	Pathogenic
5	*KCNH2*	5	Cytoplasmic N-term	Arg356His	1067G>A*	Missense	het	NTR	NTR	YES	IP (Figure 2b)	VUS[b]
40	*KCNH2*	8	Cytoplasmic C-term	Trp705Cysfs*9	2115delG	Frameshift/deletion	het	—	—	YES	N/A	Pathogenic
94	*KCNH2*	10	Cytoplasmic C-term	Glu807*	2419G>T	Nonsense	het	—	—	YES	YES	Pathogenic
21	*KCNH2*	10	Cytoplasmic C-term	Ser855Arg	2565C>G	Missense	het	NTR	NTR	YES	N/A	VUS
50	*KCNH2*	11	Cytoplasmic C-term	Gly880Val	2639G>T	Missense	het	NTR	DEL	YES	IP (Figure 2c)	VUS[b]
97	*KCNH2*	11	Cytoplasmic C-term	Ser890Cys	2669C>G	Missense	hom	NTR	NTR	YES	N/A	VUS[b]
3	*SCN5A*	19	Cyt. loop DII-DIII	Ser1135Ile	3404G>T	Missense	het	NTR	DEL	YES	N/A	VUS
92	*SCN5A*	19	Cyt. loop DII-DIII	Asp1163Glu	3489C>A	Missense	het	NTR	NTR	YES	N/A	VUS
61	*SCN5A*	23	Extr.loop DIII S5-S6	Gly1329Ser	3985G>A	Missense	het	DEL	DEL	YES	IP (Figure 2d)	Pathogenic[b]
1	*SCN5A*	26	Cyt. loop DIII-DIV	Lys1505_Gln1507del	4513_4521del 5′-AAGCCCCAG-3′	Deletion in frame	het	—	DEL	YES	N/A	Pathogenic
8	*KCNE1*	3	Cytoplasmic C-term	Val80Ile	238G>A	Missense	het	NTR	NTR	YES	N/A	VUS

Abbreviations: Aa, amino-acid; Cyt., cytoplasmatic; Ex, exon; Extr., extracellular; het, heterozygous genotype; hom, homozygous genotype.
[a]Our prediction after the analysis of the results obtained from segregation analysis, amino acid sequence alignment, and *in silico* tools. The prediction was in terms of probability.
[b]The novel mutation was found together with other mutations in the family.
Pathogenic predictions were obtained from Condel and Provean (see Materials and Methods). Frameshift/deletions and nonsense mutations could not be predicted by either of the predictor programs; we assumed that these types of mutations are deleterious. In-frame deletions could only be predicted by Provean program. We described the pathogenicity with the same scores as given by the programs: NTR, neutral; DEL, deleterious; N/A, not available; IP, incomplete penetrance.

p.(Ser216Leu) was found in the general population (MAF = 0.1%) and was described as a VUS,[24] our *in silico* analysis predicted it as deleterious (Supplementary Table S2).

Finally, we considered two mutations as potentially non-causative mutations: we found *KCNE1* p.(Asp85Asn) in three non-related index cases; in one case, this mutation occurred with other mutations (Figure 2d and Supplementary Table S2). Although this mutation is found in <1% of the general population, it has been described as an LQTS modulator[31,32] and associated with a QT prolongation.[33] Similarly, *KCNE2* p.(Thr8Ala) was found in 0.5% of the general population but was also described as a VUS.[24] We found this variant in two unrelated index cases; segregation analysis from one of the index cases revealed that the mutation did not segregate with the disease, excluding it as the causal mutation (Supplementary Table S2). The *in silico* tools did not agree in the pathogenicity of those mutations.

Pathogenicity of multiple mutations. We found six index cases with multiple mutations; segregation was assessed for five of these cases (no family information was available for patient no. 97) (Figure 2). Three families had two mutations, which occurred in the same gene in two families (Figure 2b and c) and on a different gene in one (Figure 2e); two families had three mutations, and in both families one of the mutations was a VUS or a modulator variant (Figures 2a and d).

The index cases of families a, c, and d, carrying multiple mutations, showed a more severe phenotype within their own family: In family a the index case (II.2, Figure 2a) had LQTS, which was diagnosed at the age of 8 years; in family c the index case (III.1, Figure 2c) was the only family member with LQTS; and in family d the index case (IV.3, Figure 2d) was the only family member with LQTS, diagnosed after an aborted SCD. In contrast, the index case in family b (II.1, Figure 2b), who was the only member harboring the two mutations, had no symptoms until she was 74 years old (Figure 2b). Finally, the three sisters from family e carried the two mutations and presented prolonged QT interval and previous syncope (Figure 2e).

Clinical data analysis

Differences in the clinical phenotype between carrier or non-carrier patients. Of all the genetic carriers, 66.1% were females, and significant differences were observed in mean QTc value and age of diagnosis between patients with or without an identified mutation (470.8 ± 48.1 ms and 38.9 ± 19.1 years; and 504 ± 58.8 ms and 29.6 ± 17.9 years, respectively). In contrast, there were no significant differences in the remaining severity predictors between genotypically positive and negative patients. Multiple mutations were present in 5.2% of the index cases. No differences were detected in the clinical data between patients with single or multiple mutations (Table 1).

Differences in the clinical phenotype depending on mutation pathogenicity. We compared clinical data from patients with VUS mutations and from those with probably pathogenic mutations. Indeed, QTc was significantly longer in patients with pathogenic mutations than in

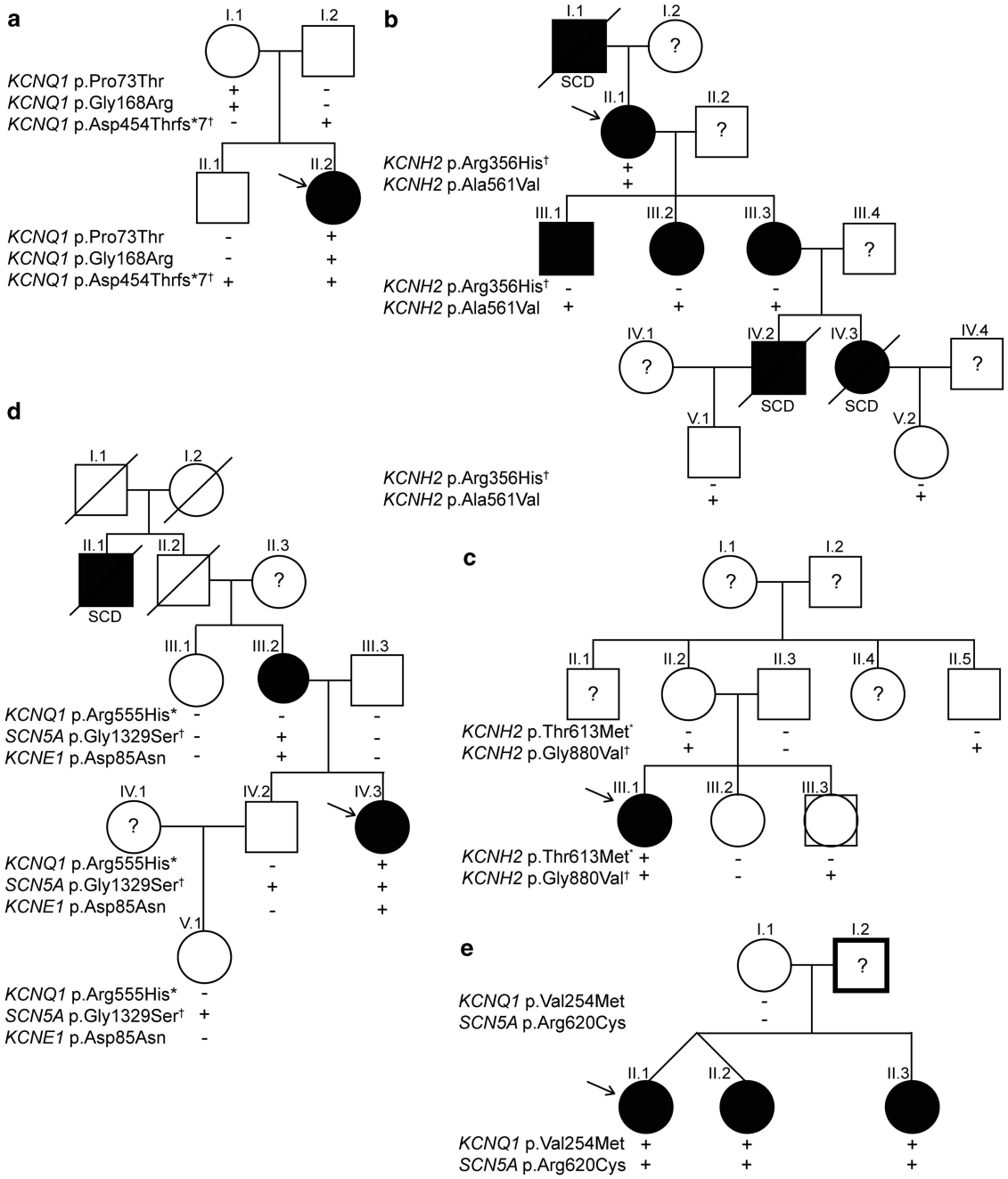

Figure 2 Familial segregation analysis from patients with multiple mutations. Families (**a**–**e**): Arrows indicate the index cases. Circles represent female, and squares represent male; circle-within-squares represent unknown sex. Clinically affected individuals are shown in black. Symbols with a thick black line represent symptomatic carriers without LQTS at the time of the study but susceptible of exhibiting the disorder. The + symbol represents mutation carriers and − symbol the non-carriers from the different mutations found in the index case. Interrogation marks (?) represent family members for whom genetic information was not available. Asterisk (*) signifies *de novo* mutations; and † signifies novel mutations.

patients with VUS (515.9 ± 64.1 and 453.2 ± 37.4 ms, respectively; Table 1).

To better understand this finding, we assessed the prevalence of mutation identification according to Schwartz Score (SS) (Figure 3). The percentage of patients with a genetic diagnosis increased to 63.6% in SS ≥ 3.5. In addition, the percentage rose to 75.7% when we limited to QTc ≥ 500 ms. In this patient population (SS ≥ 3.5 and QTc ≥ 500 ms), the presence of probably pathogenic mutations was higher and the number of VUS mutations was lower compared with patients with SS < 3.5 and QTc < 500 ms (Figure 3).

Phenotype related to mutated gene. We analyzed clinical data to identify phenotypic differences associated with variants of the five genes (Table 1). The mean age of diagnosis and QTc value were not influenced by the analyzed genes. However, we found that patients with a mutation in *SCN5A* were significantly less likely to have previous syncope compared with patients carrying mutations in *KCNH2* (27.3 and 70%, respectively).

Phenotype related to mutation type, protein region, and zygosity. Missense mutations, heterozygous genotype, and mutations in the C-terminal

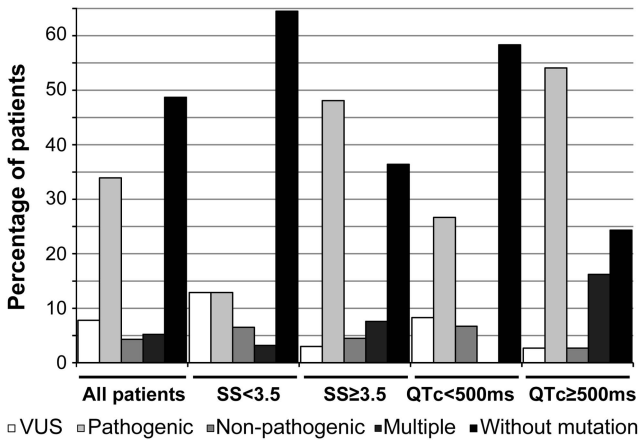

Figure 3 Mutation pathogenicity yield correlates with LQTS prolongation. Bars represent the percentages determined for all patients ($n=115$), patients with Schwartz Score (SS)<3.5 ($n=31$), SS≥ 3.5 ($n=66$), QTc<500 ms ($n=60$), or QTc≥ 500 ms ($n=37$) for different mutation groups according to their pathogenicity: VUS, probably pathogenic, probably non-pathogenic, multiple variations, and non-carriers.

domain were the most common identified (Supplementary Table S3). No significant differences were seen when we evaluated whether the mutation characteristics influenced the risk factors or the severity of clinical characteristics (Supplementary Table S3).

DISCUSSION

In our cohort of patients with LQTS, the mean age of diagnosis and the mean QTc values were similar to previous studies.[12–14,16] In addition, also similar to a recent report,[34] we found a ratio near to 2:1 between women and men. Thus our population appears to be a representative sampling of Caucasian LQTS patients.

We identified mutations in the five most prevalent disease-related genes in 51.3% of the LQTS-affected patients. Previous studies indicated that 70–75% of patients carry a mutation in one of these five genes,[11–13] but recent studies have reported a similar genetic finding in close to 50% of LQTS patients.[14,16] The percentage increased to 75% when patients had a SS≥ 4.[13] According to the modified risk scores,[17] we analyzed patients with SS≥ 3.5. Our percentage of patients with an identified mutation rose to 63.6%, increasing to 75.7% when we analyzed only patients with QTc≥ 500 ms. Importantly, the likelihood of finding a probably pathogenic mutation increased in both cases.

The number of patients with identified mutations increases in younger patients.[12,13,16] Our population age ranged from birth to 77 years old. However, if we analyzed patients within an age range of diagnosis from birth to ≤ 40 years old, the percentage of patients with an identified mutation increased to 62.3%.

Therefore, in agreement with previous studies,[13,14] our data indicate the genetic diagnosis of LQTS syndrome is more effective in more severe cases, in younger patients, and in those with higher QTc values.

Although we found significant differences in the occurrence of syncope between patients carrying mutations in *SCN5A* and *KCNH2*, in contrast to other studies we did not see differences in life-threatening cardiac events between patients carrying mutations in *KCNQ1*, *KCNH2*, and *SCN5A*.[22,35] The size of the cohort may have precluded a conclusive genotype-phenotype analysis.

Pathogenicity prediction with *in silico* tools

With the use of genetics as a diagnostic tool, often performed solely in the index case without segregation analysis, the challenge to define true pathogenic association is tremendous.[23,25,28] However, new *in silico* tools are helping define the pathogenicity of variants. Here, we used two such programs: Condel (including Polyphen2, SIFT, and Mutation assessor) and Provean (including prediction of in-frame deletions and insertions). Based on literature, MAF, segregation, and conservation data, we considered 25 previously described mutations as probably pathogenic. Condel and Provean predicted as deleterious 92 and 100% of them, respectively. This accuracy is even higher than previously claimed with those predictors, believed to be near 80%.[26,27,36]

Of all the mutations, three were considered non-causative from segregation analysis, conservation, and previous studies. Condel predicted all of them as neutral. In contrast, Provean predicted two of them as deleterious. These results are in accordance with the nearly 20% false-positive rates expected from these programs.[36]

In silico pathogenicity prediction of the 11 novel missense mutations revealed that 2 novel mutations had a deleterious effect, according to the high probability of pathogenicity for the region (pore-forming region and the loop DIII S5-S6),[23] amino-acid conservation, and family segregation. However, the *in silico* predictions obtained for novel mutations in protein areas with lower pathogenicity probability (50–80% probability in C-terminal mutations *vs* $<50\%$ in N-terminal or interdomain loop mutations (IDL) in *SCN5A*)[23] were more ambiguous. Moreover, from our analysis, Condel and Provean differed in 6 out of the 54 mutations evaluated (11%). Some studies have shown discordance between Polyphen2 and SIFT *in silico* prediction programs to be more frequent than the accordance on their predictions.[28] Although in our analysis the percentage of discordance was low, segregation analysis and/or functional studies are necessary to verify the pathogenicity or neutrality of these mutations.[28,37] Taking together, without validation, some of these mutations predicted as neutral or discordance will remain of uncertain significance, confounding genetic diagnosis.

Segregation analysis: key to validating the predictions

In several different situations, segregation analysis is critical to understanding the contribution of a mutation to LQTS. First, we identified mutations associated with Brugada syndrome (*SCN5A* p.(Arg620Cys))[29] and cardiac conduction disease (*SCN5A* p.(Pro1008Ser)),[30] which result from sodium channel defects. The discovery of mutations that are associated with different arrhythmogenic diseases could be consistent with the overlapping syndromes seen in these sodium channel diseases.[38,39] However, the contribution of these mutations remain unclear; thus their presence makes diagnosis, and especially medical therapy, difficult. Therefore, additional familial evaluation is critical for further medical decisions.

Additionally, five of the identified mutations were segregating with other mutations. Segregation analysis suggested that some mutations with uncertain effects could modulate the phenotype when occurring with other mutations (*KCNQ1* p.(Pro73Thr) in Family a, *KCNH2* p.(Arg356His) in Family b, and *KCNH2* p.(Gly880Val) in Family c).

Finally, nine novel missense mutations had neutral or discordant predictions, but only one, *KCNH2* p.(Gly238Arg), exhibited a lack of segregation that excluded it as the main cause of the disease in the family. The rest of them were considered VUS. Therefore, in these situations, prediction only is not sufficient for understanding the contribution of variants to disease.

CONCLUSIONS

Our data support that *in silico* predictors, in general, are useful for determining pathogenicity. However, prediction may be uncertain or inaccurate in ~22% of cases. In our cohort of LQTS, with a rate of identified mutations near 50%, such uncertainty is not to be taken lightly, especially considering that the genes of interest that have been widely studied for their clear association with pathology. Thus, *in silico* predictors should be combined with other tools and family segregation analyses to verify pathogenicity.[25]

In this era of rapid genetic sequencing, the lesson from these studies is twofold. First, we are not yet ready to use genetic testing as a diagnostic tool in the least-studied genes. The lack of robust genetic associations with these genes will still hamper clinical decision-making. Second, segregation studies and family investigation continue to be critical in any pathogenicity study. If the genotype does not fit the family phenotype, no matter what, genetic causality cannot be proven. This has important implications in genetic diagnosis, in which the analysis is often performed solely in the index patient, without family investigation.

CONFLICT OF INTEREST

RB is a consultant for Ferrer Incode. The other authors declare no conflict of interest.

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