Even pore-localizing missense variants at highly conserved sites in *KCNQ1*-encoded $K_v7.1$ channels may have wild-type function and not cause type 1 long QT syndrome: Do not rely solely on the genetic test company's interpretation

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Introduction

Long QT syndrome (LQTS) is an inheritable cardiac channelopathy characterized by delayed ventricular cardiomyocyte repolarization and cardiac action potential prolongation that often presents as a prolonged QT interval on a 12-lead surface electrocardiogram (ECG).[1,2] With a prevalence as high as 1:2000,[3] LQTS may manifest with episodes of syncope, seizures, or sudden cardiac arrest/sudden cardiac death typically triggered by exertion, extreme emotion, or auditory stimuli, although events during rest can also occur. However, LQTS is characterized by marked clinical heterogeneity ranging from a lifelong asymptomatic course to sudden death during infancy.[3,4] The potential for sudden cardiac arrest/sudden cardiac death without prior symptoms underscores the need for prompt and accurate diagnosis and prophylactic treatment.

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LQTS is typically inherited in an autosomal dominant manner. About 75%–80% of patients with LQTS host mutations in 1 of 3 genes (*KCNQ1*, *KCNH2*, and *SCN5A*) that encode for ion channel α subunits responsible for maintaining proper cardiac action potential and normal heart rhythm. The *KCNQ1* gene encodes for the $K_v7.1$ pore-forming voltage-gated potassium channel α subunits responsible for the slow delayed rectifier potassium current (I_{Ks}) and is responsible for the most common LQTS subtype (LQTS type 1 [LQT1]) that accounts for 35%–40% of cases with the disorder.[1,4]

Clinical genetic testing for LQTS has been available commercially since 2004. In order to assist physicians in the interpretation of genetic findings, case-control studies demonstrated that the probability of pathogenicity of rare variants identified within the major genes can be predicted on the basis of the topological location of the variant within known structural domains.[5,6] For example, *KCNQ1* missense mutations localizing to the transmembrane region confer a relatively high (>90%) probability of pathogenicity when originating from a case of clinically probable LQTS.[5] Since the estimated pathogenicity of mutations is highly correlated with protein topology, knowledge of the mutational location within the $K_v7.1$ potassium channel can provide significant diagnostic probability for patients with mutations that have not been characterized functionally. However, despite high probabilities, extreme caution must still be exercised when diagnosing patients, especially those with a borderline or weak LQTS phenotype since a topology-derived estimate does not guarantee pathogenicity.[7]

Here, we present a case of a patient diagnosed elsewhere with LQTS but with a clinically equivocal, nondiagnostic evaluation who had a rare *KCNQ1*-A300S missense variant localizing to the pore domain. This variant was classified as

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KEY TEACHING POINTS

- Since genetic testing for long QT syndrome exerts a substantial diagnostic, prognostic, and therapeutic impact for the index cases and their affected family members, it is of critical importance to identify the exact disease-causing mutation and to properly rule out benign variants.
- The article contains a fundamentally important message and dispels a commonly held perception, namely, that identification of a rare variant does not automatically equate to a disease diagnosis.
- Even *KCNQ1* mutations within areas of high probability of pathogenicity (ie, transmembrane spanning or pore-forming regions) or indicated by a genetic test company as "deleterious" should be interpreted with caution, especially if the variant-positive individuals have insufficient clinical evidence for a diagnosis of long QT syndrome in the first place.
- It is important that physicians analyze the veracity and concordance of the evidence underpinning both the alleged genotype and the alleged phenotype. If discordance exists, one must be critical of any genetic test company's interpretation and tread carefully with the use of the genotypic data in their clinical decision making.
- Genetic testing continues to be perceived as the ultimate diagnostic arbiter, yet the perils of testing in a poorly phenotyped family can be significant as illustrated in this case, with major implications for the family in terms of medical care, potential restrictions, insurance, and the psychological burden such a diagnosis brings.

a "predicted deleterious mutation" by the genetic testing company, which seemingly solidified the diagnosis of LQT1 with the primary heart rhythm specialist. However, after further phenotypic characterization of the patient and her family (their second opinion evaluation) as well as a functional validation assay using the patch clamp technique for this variant in question, the diagnosis of LQTS in general and LQT1 in particular was reversed and *KCNQ1*-A300S was demoted to a benign variant despite its topological location.

Methods

Pedigree

The index case is a young woman of Indian ethnicity who was noted to have a "borderline QT interval" on a routine sports physical ECG at the age of 14. Repeat ECG over the next 4 years as well as exercise and stress tests repeatedly showed "borderline" results as interpreted elsewhere. The family history was unremarkable; both parents had normal ECGs. However, a maternal first-degree male cousin died suddenly at the age of 7 while ill with a fever; an autopsy was performed, but it was inconclusive. A clinical LQTS genetic test was ordered, and a rare *KCNQ1*-A300S variant interpreted as a "predicted deleterious mutation" was identified in the patient, her 2 sisters, and their father. The study was approved by the Mayo Clinic Institutional Review Board, and all participants were consented appropriately.

In silico pathogenicity prediction of *KCNQ1*-A300S

Eight in silico tools (paralog conservation, ortholog conservation, Grantham values, SIFT, PolyPhen2, KvSNP, APPRAISE, and ConDel) were used to assess the predicted pathogenicity of A300S as previously described.[8]

Molecular modeling and molecular dynamics

The initial configuration of our 3-dimensional structural model leveraged the previous computational work of Smith et al.[9] An all-atom explicit environment composed of 173,794 atoms was generated using VMD[10] (Supplemental Figure S1). This environment included a square phosphatidylcholine (POPC) membrane patch (1.5 nm sides) with *KCNQ1* embedded in the center, transferable intermolecular potential with 3 points (TIP3) water, and 150 nm KCl. In silico mutagenesis was performed using the Mutator (version 1.3) VMD plugin. Molecular dynamics simulation (MDS) were carried out using NAMD[11] and the CHARMM27 with CMAP[12] force field. Wild-type (WT) and A300S simulations were independently energy minimized for 5000 steps, followed by heating to 300 K over 300 ps at a constant pressure via a Langevin thermostat and equilibration for 5 ns. We used a simulation time step of 1 fs and conformations were recorded every 2 ps. At a constant volume, a further 10 ns of simulation trajectory was generated. All trajectories were first aligned to the initial WT conformation using C^{α} atoms. Analysis was carried out using custom scripts, leveraging VMD and Bio3D (an R package).[13] Visualizations were performed using PyMOL[14] and VMD.

KCNQ1 and *KCNE1* mammalian expression vectors and mutagenesis

WT *KCNQ1* complementary DNA (cDNA) was subcloned into pIRES2-EGFP (Clontech, Mountain View, CA) to produce pIRES2-*KCNQ1*-WT-EGFP, and WT *KCNE1* cDNA was subcloned into pIRES2-dsRed2 (Clontech) to produce pIRES2-*KCNE1*-WT-dsRed2. The A300S variant was engineered into pIRES2-*KCNQ1*-WT-EGFP using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). DNA sequencing was used to confirm the integrity of all vectors.

TSA201 cell culture and transfection

TSA201 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine

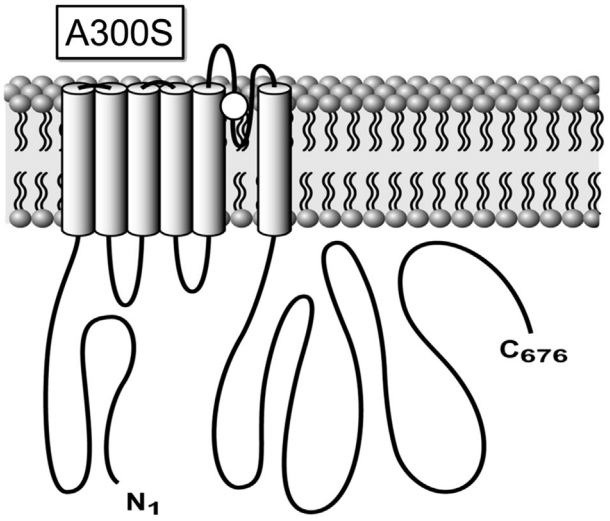

Figure 1 *KCNQ1* topology with A300S variant location. Depicted is a schematic representation of the *KCNQ1*-encoded potassium channel α subunit (K_v7.1) with A300S localized to the channel's pore region.

serum, 1.0% L-glutamine, and 1.2% penicillin/streptomycin solution in a 5% CO_2 incubator at 37°C. Heterologous expression of the I_{Ks} channel was accomplished by cotransfecting 1 μg of *KCNQ1-pIRES2*-GFP WT or mutant (*KCNQ1*-A300S-pIRES2-GFP) cDNA with 1 μg of *KCNE1*-pIRES2-dsRed2 with the use of 5 μL of Lipofectamine 2000. Transfected TSA201 cells were cultured in Opti-MEM and incubated for 48 hours before electrophysiological experiments.

Electrophysiological measurements

The standard whole-cell patch clamp technique was used to measure I_{Ks} *KCNQ1* WT and mutant currents at room temperature (22°C–24°C) with the use of Axopatch 200B amplifier, Digidata 1440A system, and pCLAMP version 10.2 software (Axon Instruments, Sunnyvale, CA). The extracellular (bath) solution contained (mmol/L) the following: 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 1 mM Na-pyruvate, and 15 HEPES (pH adjusted to 7.4 with NaOH). The intracellular (pipette) solution contained (mmol/L) the following: 20 KCl, 125 K-aspartate, 1 $MgCl_2$, 10 EGTA, 5 MgATP, 5 HEPES, 2 Na_2-phosphocreatine, and 2 Na_2-GTP (pH adjusted to 7.2 with KOH).[15] Microelectrodes were pulled on a P-97 puller (Sutter Instruments, Novato, CA) and fire polished to a final resistance of 2–3 MΩ. The series resistance was compensated by 80%–85%. Currents were filtered at 1 kHz and digitized at 5 kHz with an 8-pole Bessel filter. The voltage dependence of activation was determined using voltage-clamp protocols described in the figure legend. Data were analyzed using Clampfit (Axon Instruments) and Excel (Microsoft, Redmond, WA) and fitted with Origin 9.1 (OriginLab Corporation, Northampton, MA) software.

Statistical analysis

All data points are shown as the mean value, and bars represent the standard error of the mean. A Student *t* test was performed to determine statistical significance between the 2 groups. A *P* value of $<.05$ was considered to be significant.

Results

Initial patient history and clinical presentation

The patient presented to her primary care physician at the age of 14 for a routine sports physical examination. She had a history of medically controlled hypothyroidism and postural orthostatic tachycardia. Her symptoms of lightheadedness improved dramatically with an increased fluid intake. At this evaluation, ECG showed a "borderline QT interval" that prompted follow-up. Holter monitoring and exercise stress test were performed at that time and were considered normal. Four repeat studies over 4 years showed resting corrected QT (QTc) values ranging between 449 and 478 ms.

She never had a clear LQTS-attributable event. She had 1 event in the setting of exercise, in which she stood up quickly and fainted after lightheadedness and spontaneously recovered. This was assessed clinically and was not considered an arrhythmia-mediated episode. At the age of 17, she presented with symptoms of palpitation, lightheadedness, light flashes, and fatigue. A repeat ECG again showed QTc interval in the upper limits of normal/borderline. The local cardiologist then ordered LQTS genetic testing. Genetic testing revealed a "predicted deleterious mutation" in *KCNQ1* (c.898G>T, p.A300S, Figure 1). At this time, she was put on a LQT1 treatment program and initiated on a daily dose of 10 mg of nadolol.

Family history

The patient's family history is largely unremarkable (Figure 2A). Both parents have normal ECGs. A maternal first-degree male cousin died suspiciously at the age of 7 while ill with a fever; an autopsy was performed but was inconclusive. However, it was determined via genetic testing that the *KCNQ1*-A300S variant was inherited paternally. The patient's 2 asymptomatic sisters also underwent variant-specific genetic testing and were found to be *KCNQ1*-A300S positive. Both siblings were asymptomatic from an LQTS standpoint.

Second opinion patient history and clinical presentation

The patient was referred to Mayo Clinic for a second opinion clinical evaluation. Serial ECGs were performed while at rest and during an exercise stress test. Resting QTc values were 451, 438, 416, and 440 ms over 2 consecutive days. The T waves appeared normal and did not show the late-developing delay in upslope that is often observed in patients with LQT1. A representative ECG tracing is shown in Figure 2B. At the start of the treadmill stress test, QTc values were 419 and 425 ms while sitting and lying. Her QTc values were 434 ms at peak exercise and 353, 445, 429, and 461 ms at 1, 2, 3, and 5 minutes of recovery. Although the patient was taking nadolol during this test, the 10 mg dose is too low to interfere with this observation.

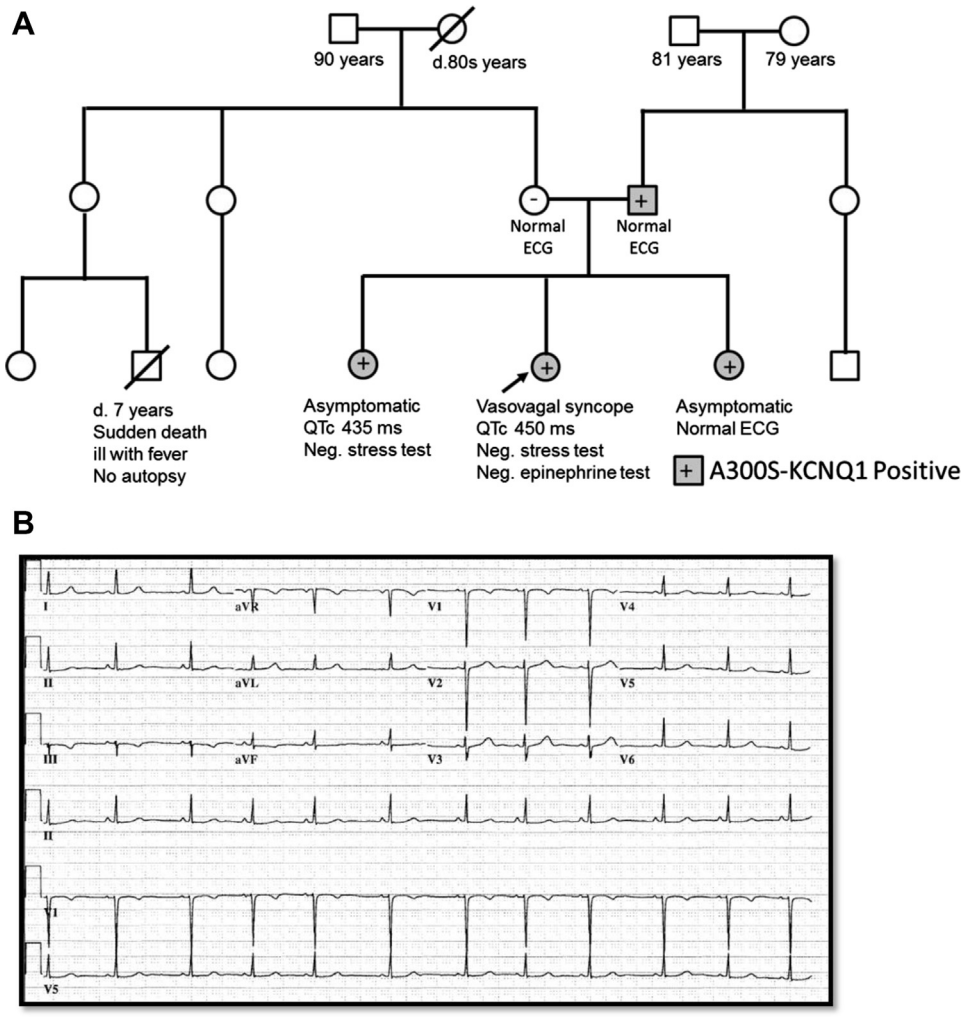

Figure 2 Patient and pedigree summary. Illustrated are the pedigree for our index case (arrow) (**A**) and a representative ECG tracing (**B**). The plus sign represents those family members (index case, her father, and her 2 sisters) who were positive for A300S. The negative sign represents the family member (mother) who was negative for A300S. ECG = electrocardiogram; QTc = corrected QT interval.

It was noted that for therapeutic intervention, the patient would need to be taking 40–60 mg of nadolol daily. A transthoracic echocardiogram was recorded, which confirmed a structurally normal heart.

An ECG recorded in the older sister, after the index patient's visit, for her own second opinion evaluation showed normal T-wave morphology and serial QTc values of 435 and 438 ms, which is well within the normal range. Her stress test showed normal QTc parameters before, during, and after exercise (data not shown).

In silico assessment of *KCNQ1*-A300S' pathogenicity

The *KCNQ1*-A300S pore region (amino acids 300–320) localizing variant (Figure 1) involves a residue that is highly conserved across species and the K_v7.x family of proteins. The variant was predicted to be pathogenic by 7 of 8 in silico tools. Furthermore, A300S is absent in 141,353 individuals in the Genome Aggregation Database, 15,378 of whom are from South Asia.[16] However, *KCNQ1*-A300T was seen in 12 of 125,745 ([minor allele frequency] MAF = 4.772×10^{-5}) exomes overall and specifically in 9 of 17,850 (MAF = 0.00025) Latino, 2 of 55,866 (MAF = 1.790×10^{-5}) European Caucasian, and 1 of 4922 (MAF = 0.0001) Ashkenazi Jewish exomes. In addition, 6 other nonsynonymous variants (A302V, W305L, W305S, W305X, T311I, and V319L) localizing to the channel pore region were observed in the Genome Aggregation Database. The 4 missense variants were each seen in only 1 of 141,353 exomes, and the nonsense variant W305X was seen in only 5 exomes. In addition, 5 of the 6 variants (all except V319L) have been previously identified in a patient with LQTS and functionally characterized with a loss-of-function in vitro phenotype.[17–21]

Taken together, the in silico evidence for A300S and its topological location highly supported the rendered conclusion that this variant was a "predicted deleterious mutation." However, given the clinical absence of a discernible clinical phenotype for either LQTS in general or LQT1 in particular for both the index case and another A300S-positive sibling, the pathogenicity of the *KCNQ1*-A300S was called into

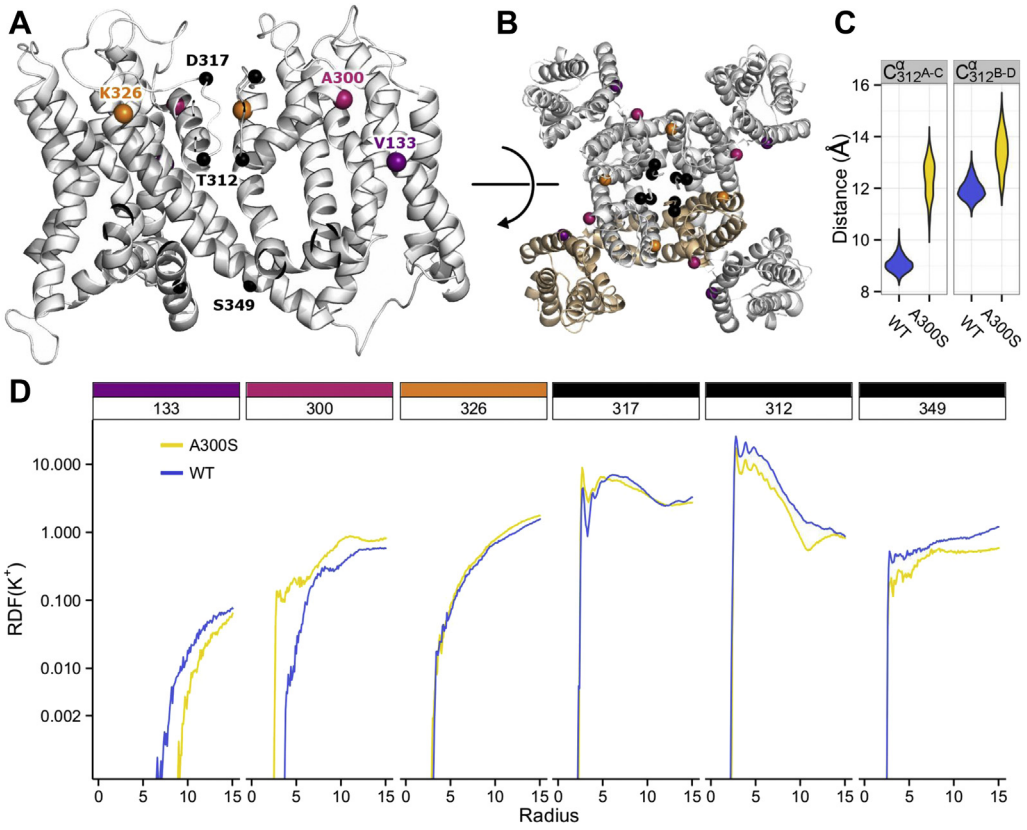

Figure 3 Simulations based on the *KCNQ1* molecular structure indicate similarities and modest differences for A300S. **A:** The position within the 3-dimensional tetrameric structure of A300 and 5 additional reference amino acids are indicated—2 along the interior and 2 at sites flanking A300. The view is along the membrane plane, and the "front" half of the tetramer has been hidden for clarity. **B:** From the extracellular side, the position of reference sites is indicated and 1 monomer of the tetramer colored tan. **C:** T312 lies at the base of the selectivity filter. The distance between T312 from one monomer to another is used to quantify how open the selectivity filter is. In our simulations, A300S lead to a greater propensity for a more open conformation of the selectivity filter. **D**: We quantified the distribution of K^+ around each reference amino acid; the reference residue number is shown above each subplot and colored as in panel A. RDF = radial distribution function; WT = wild type.

question. A 2-pronged approach, with molecular modeling and a heterologous expression functional validation assay using the patch clamp technique, was then pursued in an attempt to mount additional independent evidence in an effort to either confirm the genetic test company's rendered interpretation (despite the absence of a clinical phenotype) or demote the variant's classification from deleterious to benign (concordant with the clinical evidence).

Molecular modeling and in silico dynamic assessment

In order to increase the resolution with which the effects of A300S could be investigated, we used molecular modeling followed by MDS. Molecular modeling produced an atomic structure that could be used to interpret the potential mechanistic role of each amino acid. The model showed that A300 is positioned on the lipid-facing side of the pore region near the extracellular interface and lipid head groups and across from the voltage sensor (Figure 3). Thus, substitution of polar amino acids may not interfere with the protein's structure, nor be highly disfavored by the membrane environment. However, its proximity to the voltage sensor and selectivity filter raised the possibility for amino acid substitutions to lead to impact dynamics.

Analysis of our MDS revealed a slight difference in the overall conformation of the *KCNQ1* tetramer, including differences in the orientation of the voltage sensor with respect to the pore, and in the diameter of the pore, with the WT exhibiting a narrower annulus (Figure 3). Principal component analysis was used to summarize differences in the motions observed across WT and A300S. The first principal component indicated motions throughout the structure, including tilting of the voltage sensor domains relative to the pore-forming domains and alternating opening and closing of the selectivity filter (Supplemental Figure S2). We quantified the distribution of K^+ ions throughout the system, hypothesizing that if A300S were pathogenic, it would affect the presence of K^+ within the pore. Although moderate decreases in K^+ ions in the pore were observed for A300S, the overall profile was similar to that of WT (Figure 3).

Functional characterization of *KCNQ1*-A300S

Given that *KCNQ1* variant adjudication has not relied on MDS-derived predictions by themselves thus far, we proceeded with a conventional heterologous expression

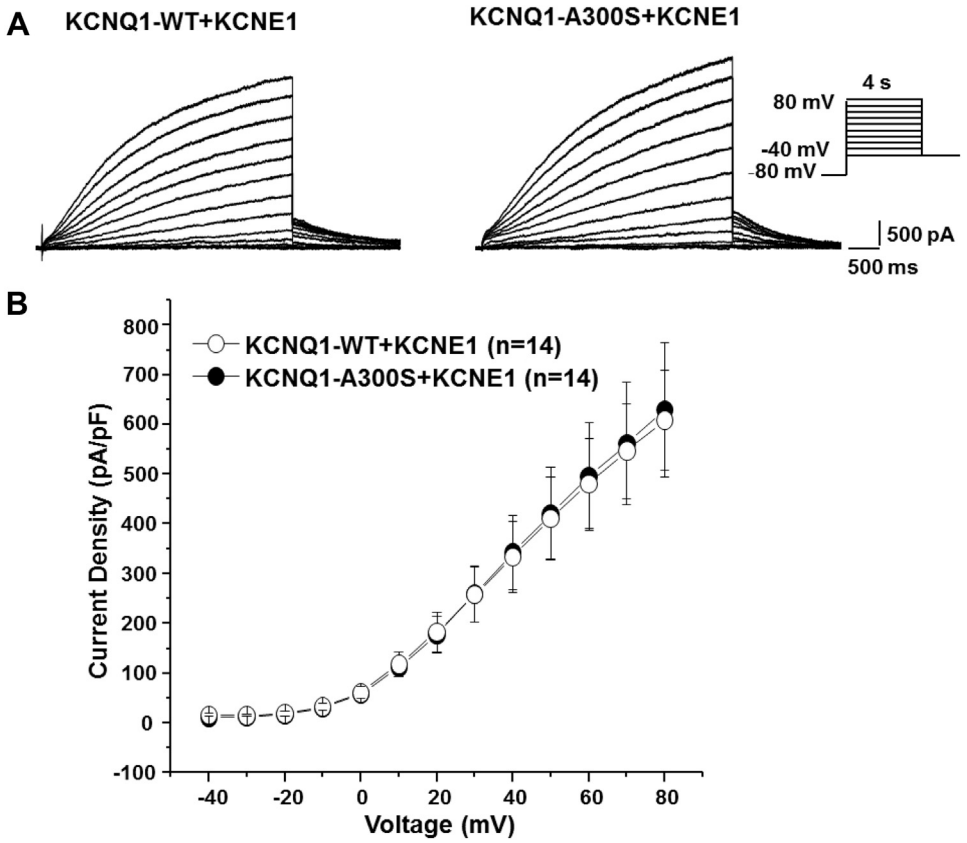

Figure 4 *KCNQ1*-A300S missense variant did not alter I_{Ks} current density in heterologous TSA201 cells. **A:** Whole-cell I_{Ks} representative tracings from TSA201 cells expressing *KCNQ1*-WT or *KCNQ1*-A300S determined from a holding potential of −80 mV and testing potentials from −40 to +80 mV in 10 mV increments with a 4-second duration. **B:** Current-voltage relationship for I_{Ks} *KCNQ1*-WT (n = 14) and -A300S missense variant (n = 14). All values represent mean ± standard error of the mean. I_{Ks} = slow delayed rectifier potassium current; WT = wild type.

functional validation assay using the patch clamp technique and heterologous expression studies, to determine whether this A300S variant produced a discernible biogenic or biophysical loss of function. Typical I_{Ks} *KCNQ1* tracings of voltage-dependent activation from *KCNQ1*-WT and -A300S variant are shown in Figure 4A with a holding potential at −80 mV to various depolarization potentials (see Figure 4A, inset and figure legend). Analysis of the current-voltage relationship revealed no significant differences in current density across the voltage from −40 to +80 mV between *KCNQ1*-WT and *KCNQ1*-A300S. At +80 mV, the current density of *KCNQ1*-A300S was 629.4 ± 135.5 pA/pF (n = 14) and that of the *KCNQ1*-WT channel was 608.4 ± 100.3 pA/pF (n = 14) ($P = 0.902$) (Figure 4B).

Discussion

Because of rapid scientific advancements, genetic testing for LQTS has gone from gene discovery to readily available commercial testing within the past 20 years.[22] Since genetic testing for LQTS exerts a substantial diagnostic, prognostic, and therapeutic impact for the index cases and their affected family members, it is of critical importance to identify the exact disease-causing mutation and to properly rule out benign variants.

Several case-control studies have been undertaken in order to distinguish rare pathogenic variants from similar rare yet benign variants in cardiac channelopathy genes.[5,6] For example, in *KCNQ1*-encoded $K_v7.1$, radical variants (ie, nonsense, frame-shift, splice-error) occurring in any location of the protein had a >99% estimated predictive value (EPV) for pathogenicity while rare missense variants localizing to the N terminus had only a 71% EPV.[5] Rare missense variants within the transmembrane and channel pore (including A300S) have a >90% EPV,[5] suggesting that rare variants localizing to these domains should be considered pathogenic. In addition, studies have shown that the use of multiple in silico mutation prediction tools can assist in variant interpretation.[8] These studies have greatly aided in the risk classification of rare variants identified within *KCNQ1*, thus providing a scientific basis for variant interpretation by genetic testing companies and the specific rendering of the A300S mutation as a deleterious mutation with a >90% probability of being pathogenic. However, both the topology-derived predictions and the usefulness of the in silico prediction tools were established by comparing the features of variants found in controls with those properties of variants found in cases where the clinical evidence for the rendered clinical diagnosis of LQTS was robust.

While the A300S variant is extremely rare (absent in >60,000 individuals from the Exome Aggregation Consortium), an amino acid substitution involving the same exact residue, A300T, has been reported previously in 1 of the 172 ostensibly healthy Asian individuals[5] and may have a heterozygous prevalence as high as 1 in every 2000 Latino individuals,[16] suggesting that A300T is simply too common to be a pathogenic substrate for autosomal dominant LQT1. However, A300T seems to exhibit a mildly dysfunctional in vitro phenotype[23,24] and is associated with recessively inherited LQTS within a single pedigree involving a consanguineous marriage.[23] In contrast, heterozygous A300T-positive individuals were asymptomatic and have normal QT intervals.[23,24] These findings suggest that although the canonical amino acid resides within a critical $K_v7.1$ potassium channel domain, it may not be vital for function.

Despite a high probability of pathogenicity, conservation across species, extreme rarity, and multiple in silico tools predicting a damaging effect, our in vitro electrophysiological data suggest that the $K_v7.1$ pore-localizing A300S variant may in fact be benign. Moreover, the clinical phenotype observed within this A300S positive family was underwhelming for LQTS in general and LQT1 in particular. In fact, based on the repeat clinical evaluations of the index case and her sister, the index of suspicion for LQTS was so low that LQTS genetic testing would not have been indicated clinically.

Genetic variants such as *KCNQ1*-A300S represent a real conundrum in genetic test interpretation. While all current analytical tools and published LQTS mutation calling algorithms strongly suggest that A300S should be pathogenic, the genotype did not match either the observed clinical phenotype or the in vitro electrophysiological phenotype. Unfortunately, in vitro cellular electrophysiological studies using the patch clamp technique as performed herein are labor intensive, are performed in only select research laboratories, and are not conducive to a rapid output of information that can assist in variant interpretation in a timely manner. It is our hope that technological advances toward the goal of creating new tools for variant interpretation will continue rapidly. Perhaps these tools will come in the form of computational biophysics and biochemistry with the development of sophisticated molecular protein modeling and dynamic simulation techniques capable of assessing the atomic level biophysical changes that may affect a protein's 3-dimensional conformational structure and biochemical function.[25]

In this work, we have performed a proof-of-principle comparison between WT and A300S using computational biophysics and revealing the possibility for high-resolution structure-based modeling to provide significantly greater interpretive power compared to sequence-based methods. We observed A300S to alter the average pore diameter; however, the K^+ ion distribution was similar to WT. Greater interpretive power is likely with additional technical replicates, increased simulation time, and variants with known effects as comparators, so that we can better differentiate between differences in the motions of the tetramer that do and do not alter the protein's function. Furthermore, molecular modeling may reveal alterations that are not pathogenic for LQTS, but may still impact the protein's function and potentially confer some level of susceptibility for a different phenotype. Application of molecular modeling and MDS to a large collection of variants well established to be associated with either WT, gain-of-function, or loss-of-function properties may provide the resolution required for clinical interpretation of novel variants and additionally reveal the detailed atomic mechanisms of pathogenicity.

Along with the great promise of genetic testing and precision medicine comes potential perils.[26] It is important that physicians analyze the veracity and concordance of the evidence underpinning both the alleged genotype and the alleged phenotype. If discordance exists, one must be critical of any genetic test company's interpretation and tread carefully with the use of genotypic data in their clinical decision making.[7,26] Currently, none of the genetic test companies have incorporated the clinician's phenotypic evidence or even a qualitative gestalt of his or her clinical index of suspicion into their interpretive algorithms. This huge divide between the clinical evidence and the genotypic evidence must be addressed urgently to further improve the adjudication of a variant's pathogenicity.

Study limitations

While heterologous cell systems like the use of TSA201 cells have been successfully used to identify ion channel functionality, these cells do not recapitulate the native cardiomyocyte environment where other cardiac specific ion channel interacting proteins are expressed and may have a modifying effect on the overall activity of the ion channel being studied. Therefore while important, these results should be interpreted with some caution until they can be confirmed in other clinical cases.

Conclusion

The combination of comprehensive clinical phenotyping of 2 A300S-positive siblings, molecular modeling and MDS, and an in vitro functional validation assay facilitated the reclassification/demotion of *KCNQ1*-A300S from a rare "predicted deleterious mutation" to a nonpathogenic variant despite its rarity, topological location, and predictions of pathogenicity from a variety of in silico tools. This suggests that even *KCNQ1* mutations within areas of high probability of pathogenicity (ie, transmembrane spanning or pore-forming regions) or indicated by a genetic test company as "deleterious" should be interpreted with caution, especially if the variant-positive individuals have insufficient clinical evidence for a diagnosis of LQTS in the first place.

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Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrcr.2017.04.006.

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