

LETTER TO JMG

Mutation in *KCNQ1* that has both recessive and dominant characteristics

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Inherited forms of long QT syndrome (LQTS) are characterised by an extended QT interval and clinical manifestations that include syncope and sudden death. The known genes in which mutations give rise to LQTS all produce components of cardiac ion channels.¹⁻⁵ The two genes mutated in the majority of cases are *KCNQ1* or *HERG*.⁵ The proteins produced from these genes are subunits which form tetrameric transmembrane voltage gated potassium channels. *KCNQ1* interacts with IsK (also called minK), the product of the *KCNE1* gene, to produce ion channels that are responsible for the cardiac I_{Ks} current, which is one of the major delayed rectifying potassium currents responsible for phase 3 repolarisation of the heart. LQTS has been subdivided clinically into the dominantly inherited Romano-Ward syndrome (RWS)^{7,8} and recessively inherited Jervell-Lange-Nielsen syndrome (JLNS).⁹ Although the cardiac abnormalities are similar in the two conditions, JLNS also presents with bilateral deafness. Mutations in *KCNQ1* produce JLNS as well as RWS,¹⁰⁻¹² although in the latter case there appears to be a strongly dominant negative effect exerted by the mutant protein, while this is very weak or absent in mutant proteins which produce JLNS.¹³

Three reports have shown that RWS can be inherited in a recessive manner.¹⁴⁻¹⁶ One of these families was a compound heterozygote, where there were extended QT intervals seen for both heterozygotes,¹⁴ but the other two families had no heterozygotes with QTc above 450 ms.^{15,16} These were therefore recessive both for the effect on QT interval and for clinical manifestations. None of these families showed deafness. The family described in the present report was first reported by Reardon *et al*¹⁷ in 1993, the proband having had a cardiac arrest at 4 years, and she and her brother were then found to have a QTc of 490 ms. The parents of the proband were first cousins and there were hearing abnormalities reported in several family members. It was therefore difficult to determine whether the diagnosis should be RWS or JLNS. We looked for mutations in *KCNQ1* to see if we could explain these diagnostic problems.

METHODS**Patient ascertainment**

The family was originally reported as a result of a cardiac arrest in the proband that led to irreversible brain damage.¹⁷ Both the proband and her treated, asymptomatic brother, who is now aged 16 years, were shown to have a prolonged QT interval (0.49 seconds). The proband, now deceased, was not known to have a hearing problem and audiometric testing of the brother shows no evidence of hearing loss.

Mutation analysis

Amplifications of the exons of *KCNQ1* by PCR and subsequent SSCP and sequence analysis was exactly as reported previously.¹⁸

Production of mutant cDNAs

A mutation in KvLQT1 isoform 1 was created in PCI-CMV-iso1, a PCI plasmid containing isoform 1 of *KCNQ1* using the

Transformer™ Site-directed Mutagenesis Kit from Clontech. A 23 nucleotide primer sequence with the mutated nucleotide in the centre was used to mutate the normal *KCNQ1* gene in the plasmid. A 26 nucleotide primer was used to co-mutate the unique restriction site in the plasmid. The primer was 5' CAGATCACTAGACGCTTATGCGG 3' which changed the normal *Hind*III site by substituting a C for the normal A. Minipreps were checked using PCR primers which amplified through the exon 5 region, followed by cutting of the amplicons with restriction enzyme to determine the presence of the mutation. After isoform 1 of *KCNQ1* was changed to incorporate the mutation found in the proband and checked using restriction enzymes and sequencing, isoform 2 was obtained. The mutated isoform 1 was cut with the restriction enzymes *Eco*RI and *Afl*III and electrophoresed on a 1% agarose gel. The iso-1 specific band of 717 bp was excised and discarded while the upper plasmid band was purified from the gel. Cutting the normal PCI-*KCNQ1* isoform 2 with the same restriction enzymes excises the iso-2 specific band of 280 bp. Extraction then ligation of this 280 bp fragment obtained from isoform 2, with the upper plasmid band obtained in the iso-1 restriction digest, gives a plasmid containing iso-2 *KCNQ1* with the mutation. The plasmids were sequenced through their cloning sites to check that there had been no endonuclease activity and the two *KCNQ1* DNA clones were complete.

Intranuclear injection of plasmids

COS-7 cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's medium supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, all from GIBCO, Paisley, Scotland) at 37°C in a humidified incubator. Cells were microinjected into the nucleus with plasmids at day 1 after plating. The protocol to microinject cultured cells using the Eppendorf ECET microinjector 5246 system has previously been reported.¹⁹ Briefly, plasmids were diluted in a buffer made of (mmol/l): NaCl 40, HEPES 50, NaOH 50, pH 7.4, supplemented with 0.5% fluorescein isothiocyanate-dextran (150 kDa). Human cardiac KvLQT1 isoform 1 and KvLQT1 isoform 2 were subcloned into the mammalian expression vector pCI (Promega, Madison, WI) under the control of a cytomegalovirus enhancer/promoter. Human IsK cDNA was subcloned into the mammalian expression vector pRC under the control of a cytomegalovirus enhancer/promoter. A green fluorescence protein pCI plasmid (a gift from Dr Rainer Waldmann, Sophia-Antipolis, France) was used as an inert plasmid to ensure that cells were always injected with a constant 15 µg/ml plasmid concentration. It has been previously calculated that in our conditions, 5 µg/ml injection corresponds to about 500 plasmid copies per cell.²⁰

Abbreviations: LQTS, long QT syndrome; RWS, Romano-Ward syndrome; JLNS, Jervell-Lange-Nielsen syndrome; WT, wild type

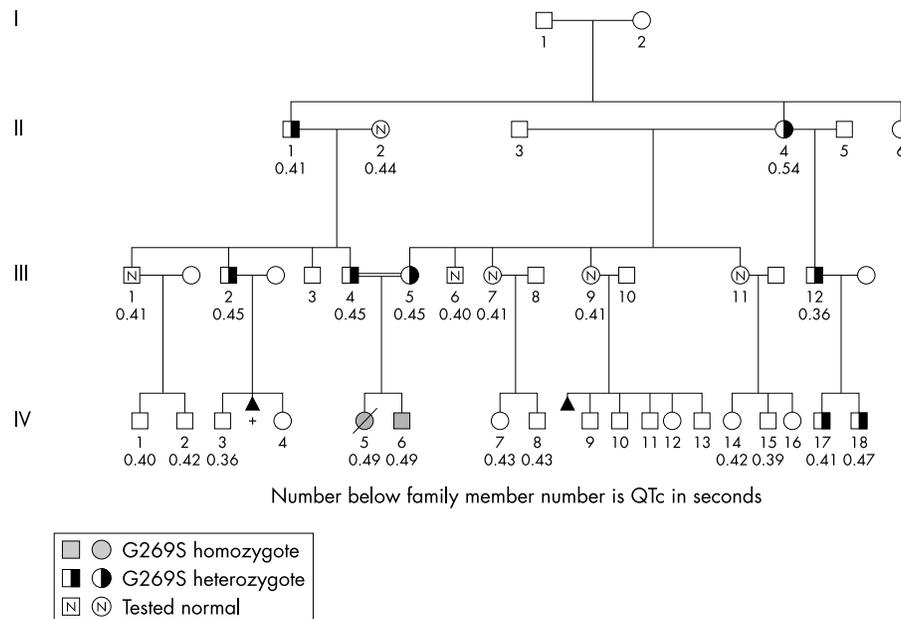


Figure 1 Family pedigree. Shaded symbols are homozygotes for the G269S mutation, those half filled are heterozygotes, those with an 'N' in them have been analysed and have no copies of the mutation.

Electrophysiological analysis

Whole cell currents were recorded as previously described.¹⁹ Cells were placed on the stage of an inverted microscope and continuously superfused with the standard extracellular solution. Patch pipettes with a tip resistance of 2.5–5 M Ω were electrically connected to a patch clamp amplifier (Axopatch 200A; Axon instruments, Foster City, CA). Stimulation data recording and analysis were performed through an analogue to digital converter (Tecmar TM100 Labmaster, Scientific Solutions, Solon, OH) and Acquis1 software (Bio-Logic, Claix, France). A microperfusion system allowed local application of experimental solution at 35°C. Current measurements were normalised using the cell capacitance. Patch clamp measurements were presented as the mean (\pm SEM). Statistical significance of the observed effects was assessed by means of the Student *t* test, Mann-Whitney rank sum test, or two way ANOVA when needed. A value of $p < 0.05$ was considered significant.

Solutions

The standard extracellular medium contained (mmol/l): NaCl 145, KCl 4, MgCl₂ 1, CaCl₂ 1, HEPES 5, glucose 5, pH adjusted to 7.4 with NaOH. The intracellular medium contained (mmol/l): K-gluconate 145, HEPES 5, EGTA 2, hemi Mg-gluconate 2 (free-Mg²⁺: 0.1), K₂ATP 2, pH 7.2 with KOH, whereas the extracellular medium used to record K⁺ currents contained (mmol/l): Na-gluconate 145, K-gluconate 4, hemi Ca-gluconate 7 (free-Ca²⁺: 1), hemi Mg-gluconate 4 (free-Mg²⁺: 1), HEPES 5, glucose 5, pH 7.4 with NaOH. Free activities were calculated using a software designed by G L Smith (University of Glasgow, Scotland).

RESULTS

Patient phenotypes

The proband, the product of a consanguineous first cousin marriage, presented with repeated syncopal attacks at the age of 4 years, and was found to have a QTc of 0.49 seconds. Of the other family members, six have a QTc >0.44 seconds (fig 1), and three have a QTc >0.46 seconds (fig 1). No-one other than the proband has a history of syncope. Nine subjects have had detailed audiometric testing (fig 1, II.1, II.4, III.2, III.4, III.5, III.12, IV.6, IV.17, IV.18). II.4 and IV.6 have normal hearing,

II.1, III.4, and III.12 have high frequency loss attributable to industrial noise, while IV.17 and IV.18 have mild sensorineural loss related to recurrent childhood infection. There is otosclerosis in III.2 and mild sensorineural loss in III.5, but these were also seen in other family members without long QT. There appear to be different factors, such as industrial exposure and the possibility of another gene, influencing the development of hearing loss in this family, as a number of other relatives with normal QTc have documented audiometric abnormalities. There is no association with the occurrence of LQTS.

Only one of the heterozygotes for the mutation described below swims regularly (fig 1, III.5), and she has never experienced any clinical problems.

Molecular analysis

SSCP analysis of *KCNQ1* produced a band shift in exon 5 for both the proband and her brother, which suggested a homozygous base change compared to the normal pattern. Band shifts which suggested a heterozygous change were identified in II.1, II.4, III.2, III.4, III.5, III.12, IV.17, and IV.18 (fig 1). Sequencing of exon 5 from the proband showed that there was a homozygous change, G805A, which altered the codon from GGC to AGC, producing an amino acid substitution, G269S. This creates an additional *AluI* site. After digestion with *AluI*, one of the fragments produced from the restriction digest of the normal PCR product, 110 bp, is cut into 23 bp and 87 bp bands. The fragments from the restriction digests with *AluI* of the amplified exon 5 from family members were electrophoresed in a 10% polyacrylamide gel, which was silver stained. This confirmed that the proband and her brother were homozygous for the mutation, while the other family members with an SSCP band shift all carried one copy of the mutation.

Analysis of *KCNE1* showed SSCP shifts in several subjects, including the proband, but not her brother. Sequencing showed this to be a previously reported polymorphism G \rightarrow A, which produces an amino acid substitution of glycine to serine at amino acid 38. A gene frequency of 0.29 has been reported for this polymorphism.²¹

At the time of the initial studies on this family, G269S was not reported as a mutation in *KCNQ1*, although it was known

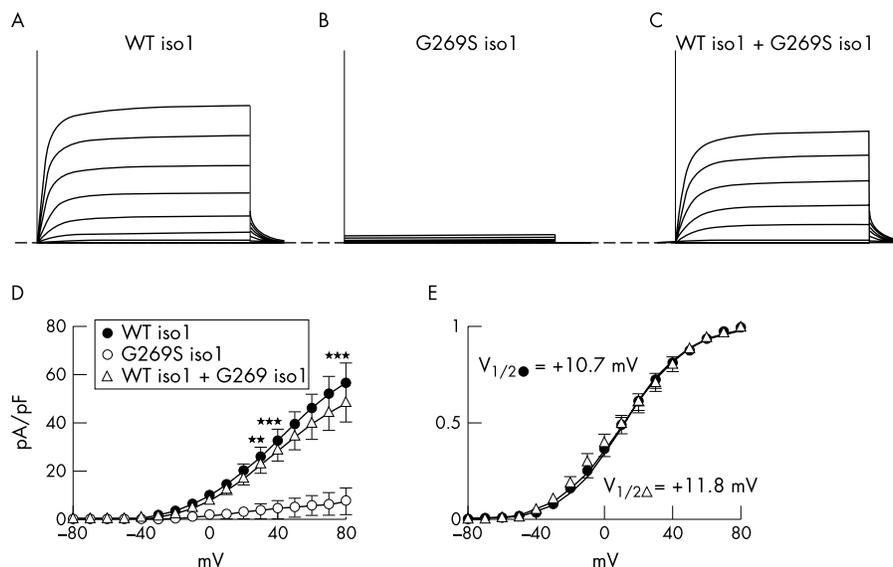


Figure 2 Expression of G269S mutated KvLQT1 isoform 1. (A, B) Superimposed whole cell currents in COS-7 cells injected with wild type (WT) KvLQT1 isoform 1 cDNA (5 $\mu\text{g/ml}$) and G269S mutated KvLQT1 isoform 1 cDNA (5 $\mu\text{g/ml}$) respectively in the presence of IsK cDNA (5 $\mu\text{g/ml}$). (C) Superimposed currents in cells injected with WT KvLQT1 isoform 1 cDNA (5 $\mu\text{g/ml}$) plus IsK cDNA (5 $\mu\text{g/ml}$) in the presence of G269S mutated KvLQT1 isoform 1 cDNA (5 $\mu\text{g/ml}$). Voltage steps applied in sequence from -80 mV to various voltages in the range of -100 mV to $+60$ mV, and then to -40 mV where tail currents were recorded. Current scale: 20 pA/pF; time scale: 500 ms. (D) Current-voltage (I-V) relation for cells expressing WT KvLQT1 isoform 1 plus IsK (black circles, $n = 19$), G269S mutated KvLQT1 isoform 1 plus IsK (white circles, $n = 6$) and WT KvLQT1 isoform 1 plus G269S mutated KvLQT1 isoform 1 plus IsK (triangles, $n = 20$). WT v G269S: ** denotes $p < 0.01$ and *** $p < 0.001$. (E) Averaged KvLQT1/IsK activation curves in the absence (black circles, $n = 9$) and presence (triangles, $n = 20$) of G269S mutated KvLQT1 isoform 1. $V_{1/2}$ denotes the potential for half maximal activation. In all experiments, injected plasmid total concentration was maintained constant at 15 $\mu\text{g/ml}$ with pCI-GFP.

that G269D produced a severe form of dominantly inherited RWS, with five sudden deaths out of eight members of the family with this mutation.²² A mutation of glycine to serine might be expected to produce a much less deleterious change to the protein than glycine to aspartic acid, but it is very difficult to extrapolate changes in protein function from point mutations. BLAST searches showed this residue to be moderately conserved, being present in mouse, rat, rabbit, bovine, and *C. elegans* orthologues, as well as mammalian KCNQ2, KCNQ3, and KCNQ4, but absent from the *Drosophila shaker* channel. To see if the mutation did affect the ion channel, we produced mutant copies of the two isoforms of KCNQ1, and used these to study the currents produced.

Functional expression of mutated isoform 1 KvLQT1 channel

Human mutated KvLQT1 isoform 1 cDNA plasmids (G269S, 5 $\mu\text{g/ml}$) were coinjected together with a human IsK cDNA plasmid (5 $\mu\text{g/ml}$) into the nucleus of COS-7 cells. Twenty-four hours after injection, the cells were analysed for K^+ channel expression. G269S-KvLQT1 isoform 1 produced a K^+ current with a reduced amplitude (tail current at -40 mV elicited by a test pulse to $+40$ mV for 1 sec: 1.06 ± 0.9 pA/pF v 11.2 ± 1.3 pA/pF with wild type isoform 1; 5 $\mu\text{g/ml}$; $n = 8$ and 22 cells, respectively; $p < 0.001$, Mann-Whitney rank sum test).

In most cases, RW syndrome shows an autosomal dominant pattern of inheritance, and patients are heterozygous carriers having both WT and mutated alleles. Thus, coexpression experiments were performed to assess the eventual dominant negative effect of G269S mutated KvLQT1 isoform 1 on WT KvLQT1 isoform 1. COS-7 cells were coinjected with a plasmid combination made of WT and mutated isoform 1 (each at 5 $\mu\text{g/ml}$) cDNAs in the presence of IsK cDNA (5 $\mu\text{g/ml}$). As illustrated in fig 2, cells coinjected with WT and G269S mutated KvLQT1 isoform 1 produced a K^+ current that was indistinguishable from the current recorded in cells expressing WT KvLQT1 isoform 1 alone (5 $\mu\text{g/ml}$; tail current: 9.37 ± 1.5 pA/pF; $n = 27$ and 11.2 ± 1.3 pA/pF; $n = 22$ in the presence or

absence of mutated KvLQT1 isoform 1, respectively). Furthermore half maximal activation potential of wild type KvLQT1 isoform 1 was not modified by the presence of the G269S mutated KvLQT1 isoform 1 (11.8 ± 3.1 mV; $n = 20$; in presence and 10.6 ± 3.3 ; $n = 19$; in absence of the G269S mutated KvLQT1 isoform 1).

Functional expression of mutated isoform 2

It has been shown that KvLQT1 isoform 2 (an N-terminus truncated KvLQT1 isoform) is endogenously expressed in the human heart and exerts strong dominant negative effects on the KvLQT1 isoform 1 protein.²³ We explored whether the G269S mutation, which is situated in an exon common to isoform 1 and 2, would affect the dominant negative activity of isoform 2. We thus investigated the dominant negative properties of G269S mutated isoform 2 construct on the current produced by wild type isoform 1 expression in the presence of IsK. As shown in fig 3, coinjection of wild type isoform 2 (5 $\mu\text{g/ml}$) markedly reduced the K^+ current related to WT isoform 1 expression (5 $\mu\text{g/ml}$). By contrast, the G269S mutation abolished the dominant negative properties of isoform 2 (tail current at -40 mV were 3.4 ± 0.6 pA/pF with wild type KvLQT1 isoform 2 and 11.7 ± 2.1 pA/pF with G269S mutated KvLQT1 isoform 2; $n = 9$ and 14 cells, respectively, $p < 0.01$). The effects of WT isoform 2 were not restricted to a strong reduction in the amplitude of the K^+ current. Wild type isoform 2 also shifted to a more depolarised potential of the activation curve of the KvLQT1 current ($V_{1/2} = 10.6 \pm 3.2$ mV in the absence and 24.6 ± 3.1 mV in the presence of wild type isoform 2; $n = 19$ and six cells, respectively, $p < 0.05$). By contrast, expression of G269S mutated KvLQT1 isoform 2 did not shift the half maximum activation potential ($V_{1/2} = 13.6 \pm 2.3$ mV, $n = 9$).

DISCUSSION

Owing to a complex clinical phenotype, this family posed a diagnostic problem when first ascertained eight years ago. Discovery of the underlying mutation has provided a means of

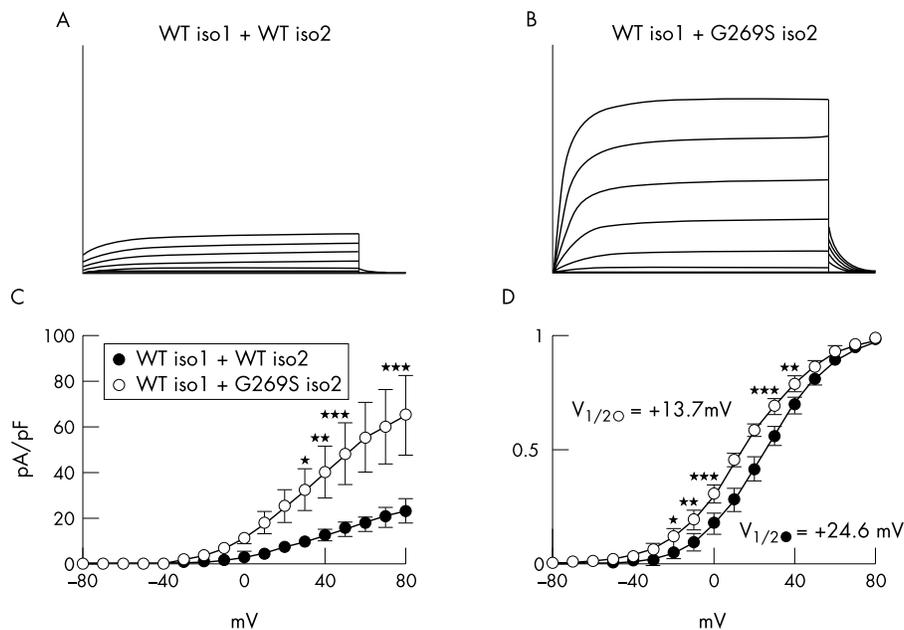


Figure 3 Expression of G269S mutated KvLQT1 isoform 2. (A, B) Superimposed whole cell currents in cells injected with WT isoform 1 cDNA (5 $\mu\text{g}/\text{ml}$) plus WT isoform 2 cDNA (5 $\mu\text{g}/\text{ml}$) or G269S mutated KvLQT1 isoform 2 cDNA (5 $\mu\text{g}/\text{ml}$) respectively in the presence of IsK cDNA (5 $\mu\text{g}/\text{ml}$). Current scale: 20 pA/pF; time scale: 500 ms. (C) Current-voltage (I-V) relation for cells expressing WT KvLQT1 isoform 1 plus WT KvLQT1 isoform 2 plus IsK (black circles, n=6) and WT KvLQT1 isoform 1 plus G269S mutated KvLQT1 isoform 2 plus IsK (white circles, n=9). (D) Averaged KvLQT1/IsK activation curves in the presence of WT KvLQT1 isoform 2 (black circles, n=6) or G269S mutated KvLQT1 isoform 2 (white circles, n=9). $V_{1/2}$ denotes the potential for half maximal activation. *denotes $p < 0.05$, **denotes $p < 0.01$, and ***denotes $p < 0.001$. In all experiments, injected plasmid total concentration was maintained constant at 15 $\mu\text{g}/\text{ml}$ with pCI-GFP.

analysing its presence or absence in family members, but has also posed additional questions. Clinically, the family shows recessively inherited RWS. This in itself would have been impossible to diagnose in 1993, as there was not thought to be such a disease entity, and this is only the third family with a homozygous mutation in *KCNQ1* that fits into this category. The other two families were also recessive in terms of the QT interval exhibited for heterozygotes, in that none of the gene carriers had a QTc greater than 450 ms.^{15, 16} In the family presented here, there are two in excess of 460 ms, the top cut off for any studies on LQTS. Thus, for ECG analysis this is a dominantly inherited trait with reduced penetrance, but clinically it shows recessive inheritance. The deafness in this family was almost certainly the result of a combination of noise exposure at work, and a different gene in the two cousins, as the surviving homozygote for G269S shows no hearing loss.

Comparing the electrophysiological data from this family with the two other recessive RWS families described above shows that, in terms of isoform 1 expression alone, the G269S mutation cannot be distinguished from R533W¹⁵ and A300T.²⁴ Like A300T mutated isoform 1,²⁴ no dominant negative effect that resulted in a decrease of the current density was observed with the G269S mutated isoform 1 subunit. This is unlike the R533W mutated isoform 1, which induced a dominant negative effect on wild type isoform 1.¹⁵ Mohammad-Panah *et al*²⁵ have shown that mutations on KvLQT1 isoform 2 can explain the different phenotypes found in RW and JLN heterozygous carriers. The G269S mutated KvLQT1 isoform 2 had lost its dominant negative effect on the current produced by wild type isoform 1 expression in the presence of IsK. Our patch clamping data strongly suggest that G269S would have a phenotypic effect only when present on both alleles.

The confusion over patterns of inheritance in this family in some ways simply shows the arbitrary nature of dividing inheritance into either dominant or recessive categories, but it clearly is very important for the patient to understand what the clinical effects of this mutation would be. From our data we would say that a single copy of this mutation would not be

deleterious. However, the only other recorded occurrence of the same mutation is in a single person in a paper describing mutations in *KCNQ1*, which provoke drowning or near drowning events during swimming.²⁶ By implication these are mutations with a particularly poor prognosis. The subject in the report had a QTc of 470 ms, and a cousin who had experienced a near death event while swimming. The proband himself was asymptomatic, however, and the cousin did not appear to have been tested. It could be that the cousin carried another mutation, or, as in this family, was homozygous for the same mutation, but this information is unfortunately not known. The electrophysiological results clearly put this mutation as most likely to produce a recessive pattern of inheritance clinically, although there is evidently sufficient interference with the potassium channel function to extend the QT above the top limit of the normal range.

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