Published in final edited form as:

Heart Rhythm. 2007 March ; 4(3): 323–329.

KCNJ2 mutations in arrhythmia patients referred for LQT testing: a mutation T305A with novel effect on rectification properties

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Abstract

BACKGROUND—Loss-of-function mutations in the *KCNJ2* cause ~50% of Andersen-Tawil Syndrome (ATS) characterized by a classic triad of periodic paralysis, ventricular arrhythmia, and dysmorphic features. Do *KCNJ2* mutations occur in patients lacking this triad and lacking a family history of ATS?

OBJECTIVES—The purpose of this study was to identify and characterize mutations in the *KCNJ2*-encoded inward rectifier potassium channel Kir2.1 from patients referred for genetic arrhythmia testing.

METHODS—Mutational analysis of *KCNJ2* was performed for 541 unrelated patients. The mutations were made in wild type (WT) and expressed in COS-1 cells and voltage clamped for ion currents.

RESULTS—Three novel missense mutations (R67Q, R85W, and T305A) and one known mutation (T75M) were identified in 4/249 (1.6%) patients genotype-negative for other known arrhythmia genes with overall incidence 4/541 (0.74%). They had prominent U-waves, marked ventricular ectopy, and polymorphic ventricular tachycardia but no facial/skeletal abnormalities. Periodic paralysis was present in only one case. Outward current was decreased to less than 5% of WT for all mutants expressed alone. Co-expression with WT (simulating heterozygosity) caused a marked dominant negative effect for T75M and R82W, no dominant negative effect for R67Q, and a novel selective enhancement of inward rectification for T305A.

CONCLUSIONS—*KCNJ2* loss of function mutations were found in ~1% of patients referred for genetic arrhythmia testing that lacked criteria for ATS. Characterization of three new mutations identified a novel dominant negative effect selectively reducing outward current for T305A. These results extend the range of clinical phenotype and molecular phenotype associated with *KCNJ2* mutations.

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Keywords

K-channel; Long QT syndrome; Ion channels; Ventricular Arrhythmia; Gene expression; Gene testing; inward rectification; KIR2.1

Mutations in the *KCNJ2*-encoded inwardly rectifying potassium channel, Kir2.1, that cause loss of function have been linked to the uncommon clinical phenotype called Andersen Tawil syndrome¹. In 1971, Andersen described an 8-year old with short stature, hypertelorism, broad nasal root, and defect of soft and hard palate². The term Andersen Syndrome was first used by Tawil in 1994 in reference to a rare, autosomal dominant disorder consisting of three major features: dysmorphic features (similar to those described by Andersen in 1971), periodic paralysis, and abnormal repolarization³. The repolarization/arrhythmia phenotype included frequent ventricular ectopy, polymorphic ventricular tachycardia (VT), bidirectional VT, and recurrent torsades and also the presence of asymptomatic QT prolongation, perhaps more specifically QU prolongation⁴.

KCNJ2 mutations were first reported in patients selected for the classic ATS clinical phenotype^{1,5,6,7,8}. Most studies required 2 out of the 3 features of the classic triad. Early on, however, it was noted that the mutation presented in a very heterogeneous way, with related family members carrying the *KCNJ2* mutation displaying only one or none of the classic triad^{5,7}. Do such patients present with a cardiac phenotype alone? To answer this question, a cohort of 541 unrelated patients referred for genetic arrhythmia testing was genotyped for *KCNJ2*. A second aim of this study was to characterize the molecular function of the mutations found by expressing them in a heterologous cell culture system. Of the four distinct mutations found, three were novel, and one of these (T305A) showed a novel selective dominant negative effect on outward currents, in effect, an enhancement of the inward rectification only when co-expressed with wild type (WT) channels.

Methods

Study Cohort

Informed written consent was obtained in accordance with study protocols approved by the Mayo Foundation Institutional Review Board. Between August 1997 and July 2004, 541 consecutive, unrelated patients with a suspected clinical diagnosis of congenital long QT syndrome (LQTS) were referred to Mayo Clinic's Sudden Death Genomics Laboratory for molecular genetic testing⁹. See Table 1 for characteristics of the total cohort. Comprehensive mutational analysis of all 60 translated exons of the 5 cardiac channel LQTS-associated genes (LQT1-3, 5-6) and targeted analysis of *ANKB*-associated LQT4 and *RyR2*-associated catecholaminergic polymorphic ventricular tachycardia (CPVT) was performed previously^{9,10,11}. The investigation conforms with the principles obtained in the Declaration of Helsinki¹².

KCNJ2 Mutational Analysis

Comprehensive mutational analysis of *KCNJ2* was performed using polymerase chain reaction (PCR), denaturing high performance liquid chromatography (DHPLC), and DNA sequencing. DNA amplification of the entire single exon coding region of the *KCNJ2* gene was conducted using five overlapping fragments with PCR primers designed using Oligo Primer Analysis Software version 6.63 (Molecular Biology Insights, Inc., Cascade, Colorado) (primers, PCR and DHPLC conditions are available upon request). Control genomic DNA, comprised of 100 healthy white and 100 healthy black subjects, was obtained from the Human Genetic Cell

Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, New Jersey).

The investigators constructing *KCNJ2* mutations, performing and analyzing cell trafficking and electrophysiological experiments, were blinded to the clinical data until after the experiments were completed and analyzed.

KCNJ2 construction and mutagenesis

Wild-type (WT) human Kir2.1 was isolated from human cardiac cDNA using PCR, forward primer *atgggcagtggtgcaaccaac* and reverse primer *tcatatctccgactctgcgctaagg*. Sequence integrity was verified with sequence analysis. *KCNJ2* mutations were constructed using the Stratagene ExSite site directed mutagenesis kit using the following primers: for R67Q forward *agtacctgcgacacatcttca* and reverse *gttgccccctctcacccac*, T75M forward *ccatgtgtgtggacattcgct* and reverse *tgaagatgtctgcgaggtacc*, R82W forward *gtgggtgatgctggttatcttct* and reverse *cagcgaatgtccacacacgt*, T305A forward *gctgccatgacgacacagt* and reverse *ggcttccaccatttgccctc*. WT and mutant DNA was sub-cloned into mammalian expression vector pcDNA3.1 (Invitrogen). The WT and mutant DNA were also sub-cloned into pcDNA3.1-NT-GFP-TOPO vector for sub-cellular localization. All constructs were verified by sequence analysis.

Transfection and cell culture

COS-1 cells were cultured in DMEM (Invitrogen) with 10% FBS. For electrophysiological experiments, cells were transiently transfected using SuperFect method (Qiagen) using 2.5µg of WT and/or mutant *KCNJ2* both with green fluorescent protein cDNA in a pRK5 vector (GFP-pRK5, Clontech, Palo Alto, CA). For fluorescence studies, WT or mutant *KCNJ2* DNA in pcDNA3.1-NT-GFP-TOPO were transfected using the SuperFect into COS-1 cells using 2.5µg DNA. After 48 hours, the cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min.

Electrophysiological experiments

Patch clamp experiments were carried out 24 hours after transfection using the ruptured patch whole cell technique at room temperature and recorded with an Axopatch 200B amplifier. Borosilica glass pipettes were pulled to resistances of 2–4 MΩ. Cells were identified by GFP fluorescence under fluorescent microscopy (Olympus Optical Corp). Bath solution contained (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, HEPES 5, NaH₂PO₄ 0.33, D-glucose 5.5 and pH adjusted to 7.4. Pipette solution contained (mM): KCl 30, K Aspartate 85, MgCl₂ 5, KH₂PO₄ 10, K₂EGTA 2, K₂ATP 2, and HEPES 5 and pH adjusted to 7.2. Control experiments in WT were done contemporaneously with the mutations. From a holding potential of −70mV, voltages were applied from −140 to 40mV in 20mV increments for 100ms. Data were filtered at 10kHz and digitized using a Digidata 1200 (Axon Instruments). Analysis of data was done using pClamp 8 and Origin 6.1.

Imaging

Cells obtained 24 hours after transfection were fixed to coverslips with 4% paraformaldehyde and washed with PBS. The fixed cells were washed again with PBS and the coverslips containing the cells were mounted on slides using a 50% glycerol/50% PBS solution. A Bio-Rad MRC 1024 laser scanning system with 15 mW mixed gas (krypton/argon) laser was utilized to view GFP labeled cells. The Bio-Rad MRC 1024 system was mounted on a Nikon Diaphot 200 inverted microscope. Images of the fluorescent-labeled cells were scanned under a x40 objective with normal speed, x2 zoom. The confocal system was set to 3.6 for iris, laser

power at 100%, and camera sensitivity gain to 900. A Kalman collection filter with three frames per image was applied to record the image.

Results

Demographics and clinical presentation

Table 1 summarizes the demographics of the 541 unrelated patients referred for LQTS genetic testing and the frequency of a positive mutational analysis of genes for LQTS1–6, ryanodine receptor gene *RYR2* for catecholaminergic polymorphic VT, and *KCNJ2*. No examples of compound heterozygosity involving *KCNJ2* were identified as none of the 292 patients with a previously established LQTS1–6 or *RYR2*-associated mutation hosted a *KCNJ2* mutation. Instead, comprehensive open reading frame/splice mutational analysis of *KCNJ2* revealed 4 distinct missense mutations (R67Q, T75M, R82W, and T305A) in 4/249 (1.6%) genotype negative patients (Figure 1) for an overall incidence of 0.7% (4/541). Except for T75M, the mutations were novel. Each mutation involved a highly conserved amino acid residue and was absent in 400 reference alleles.

Table 2 details the genotype-phenotype correlations of the *KCNJ2* mutation positive cases including age at diagnosis, presentation, cardiac electrical abnormalities and a history of periodic paralysis or presence of a physical malformation. Briefly, all *KCNJ2* harboring individuals were young white females with a normal QTc and a negative family history, two had prominent U waves (Figure 2), two were symptomatic (syncope or palpitations), four displayed ECG abnormalities such as ventricular ectopy, premature ventricular beats, or polymorphic VT (Figure 2), and one had a history of periodic paralysis. Facial/skeletal dysmorphism was unremarkable in all.

KCNJ2 mutations caused non-functioning channels in COS-1 cells

The R67Q, T75M, R82W, and T305A *KCNJ2* mutations were compared with wild type (WT) in COS-1 cells and analyzed by whole cell patch clamp technique. Representative current traces from WT and two of the mutations (T75M and R82W) expressed singly, or from the mutations each co-expressed with WT are shown in Figure 3. Summary data for the current-voltage relationships are shown in Figure 4A (R67Q, T75M, R82W) and Figure 5 (T305A). WT current expression was robust and exhibited a typical inward rectifier N-shaped curve on the current-voltage plot. In contrast, all of the mutant channels: R67Q, T75M, R82W, and T305A, were non-functional channels, showing no measurable current.

Dominant negative effects of mutations when co-expressed with WT

Kir2.1 channels assemble in a tetramer comprised of four separate subunits that could be transcribed from either gene. Individuals with *KCNJ2* mutations are heterozygous for the mutation. Therefore, we also analyzed the effect on Kir2.1 current when the WT gene was co-expressed with each of the *KCNJ2* mutant alleles. Equal concentrations of mutant and WT DNA (2.5 µg) were transfected in COS-1 cells to investigate for dominant negative effects. T75M and R82W both caused a significant ($p < .05$) dominant-negative type effect on WT current to 37% and 18% respectively of maximal WT inward current at -140 mV, and abolished outward current at -40 mV (Figure 4B). T305A caused a smaller dominant negative suppression to 74% of maximal WT inward current at -140 mV that did not reach statistical significance (Figure 5), but abolished outward current at -40 mV (Figure 5 inset). Despite the dramatic molecular phenotype when expressed alone, R67Q exerted no apparent dominant negative effect when co-expressed with WT.

N-terminus *KCNJ2* mutations demonstrate normal cellular trafficking

COS-1 cells transfected with WT and mutant DNA were analyzed by confocal microscopy. Light and fluorescent microscopy (Figure 6) demonstrated normal cellular trafficking for WT *KCNJ2* with a clearly delineated membrane distribution of green fluorescence. Identical patterns were found for R67Q-, T75M-, R82W-, and T305A-*KCNJ2* in at least 5 experiments (T305A data not shown).

Discussion

The prevalence of loss of function *KCNJ2* mutations in suspected LQT inherited arrhythmia syndromes

We found 4 *KCNJ2* mutations (R67Q, T75M, R82W, and T305A), three of them novel, in nearly 2% of LQTS genotype negative subjects who had been referred for genetic arrhythmia testing. None of the subjects had been diagnosed with ATS. No *KCNJ2* mutations were detected in patients hosting an established LQT1-6 or RyR2 receptor mutation. Previous studies of *KCNJ2* selected patients based on probands exhibiting strong features of Andersen-Tawil syndrome (ATS)^{1,5,6,7,8}. A study of 22 individuals from 11 families derived from a cohort of more than 140 subjects with a diagnosis of periodic paralysis¹³ found eleven mutations in *KCNJ2*, five of them novel, including T75M that we found in our cohort as well. All patients from that cohort of periodic paralysis also had dysmorphic features, but only 10/22 had cardiac signs or symptoms and only 4/22 had a long QTc. In a study with a design similar to ours, 188 unrelated patients with clinically suspected LQTS were screened for *KCNJ2* mutations and 2 novel loss of function mutations (T75A, 163–164delFQ) were found¹⁴. Neither family had dysmorphic features, one family relative had periodic paralysis, and the mean QTc intervals were < 460 ms. Our results from a larger cohort with a similar referral pattern also showed an overall ~2% incidence of *KCNJ2* mutations.

Comparison with previous *KCNJ2* mutations

To date, 35 unique loss of function mutations (Figure 1) have been described in *KCNJ2*, and 24 of these have been expressed for voltage clamp data. With this study, these totals come to 38 and 27 respectively. We confirm the previous report¹³ that T75M shows a dominant negative effect on current, and additionally we show that it traffics normally. This T75M mutation and two of the new mutations (R67Q, R82W) are located at the same amino acid positions as previous mutations in a clustering of previously published N-terminal mutations: R67W, D71V, D71N, T74A, T75R, T75M, D78G, R82Q^{7,5,8,15,13}. Of note, we could not demonstrate a dominant negative effect for the new mutation R67Q. This is in contrast to a mutation at the same position R67W which showed a dominant negative effect in oocytes⁵. This lack of suppression or even haploid insufficiency for R67Q makes it difficult to explain how a patient heterozygous for R67Q would have loss of function, but a common caveat is that these mutations are studied in cell culture or oocytes, not the native cardiac muscle environment, where effects may be different. In addition, perhaps environmental influences such as acidosis¹⁶ are necessary to bring out the abnormality. Perhaps also this is simply a rare variant and not related to the patient's arrhythmia. All the mutations reported here traffic normally in the COS cells (Figure 6), as do most *KCNJ2* mutations that have been studied. Only four mutations are trafficking defective, two in the M1 region (delta95–9817 and C101R¹⁸) and two in the C-terminus (delta 314–315 and V302M)¹⁷. Residues in the N- and C- termini are important for PIP₂ binding⁸ which is important for the function of inward rectifier channels¹⁹ and this has been proposed as the mechanism for loss of function⁸.

A novel molecular phenotype for T305A

The newly described C-terminal mutation, T305A, is interesting in that the dominant negative suppression of outward current was more marked than that of inward current, suggesting a selective effect on the rectification mechanism. This mechanism is distinct from other mutations in the region that have been studied (G300V, V302M, E303K, T309I)^{7,8,20} which show dominant negative suppression equally of inward and outward currents. Residues in the C-terminus have been implicated in the rectification mechanism^{21,22}. For T305A the effect on rectification involves an interaction of WT and mutant subunits as the mutant alone showed no currents (Figure 5). A full biophysical explanation for this effect requires further study. Clinically, the patient with T305A presented with ventricular ectopy and no other features, whereas patients with other mutations in this region show dysmorphic features²⁰.

Implications for classification

The clinical heterogeneity of *KCNJ2* mutation poses some difficulties for classification as well as diagnosis. The common abnormality is a loss of function mutation in the *KCNJ2* gene, but up to 40% of patients with clinical features of ATS are *KCNJ2* genotype negative²³. Therefore, there is anticipated genetic heterogeneity for ATS, and designations as ATS1, ATS2, etc might be reasonable. More recently, mutations in *KCNJ2* have been implicated in the pathogenesis of familial atrial fibrillation²⁴ and short QT syndrome²⁵. These, however, are gain-of-function mutations and should cause no confusion with ATS. Recently, we described a loss-of-function *KCNJ2* mutation in a cohort referred for evaluation of CPVT²⁶. Should this be considered a new CPVT mutation (CPVT3) or as a manifestation of ATS1? None of the *KCNJ2* positive individuals had a long QT but had rather had prominent U-waves, a feature previously recognized⁴. This brings to question whether or not ATS or loss of function *KCNJ2* mutations should be classified as LQT7⁷. As more studies characterize the range of genotypes, clinical phenotypes, and the mechanistic molecular and cellular phenotypes, a naming consensus may emerge.

Study caveats

The patients studied were from a referral cohort that was based upon the referring physician's suspicion of inherited arrhythmia of the long QT type, and with a presumed clinical diagnosis of autosomal dominant, Romano-Ward LQTS. While this represents a real world patient selection process, entry into this cohort was not prospectively defined by strict criteria. Also, as mentioned above, the molecular phenotype was determined in heterologous expression system, not in cardiac cells. Since Kir2.1 is a hetero-tetramer and can also combine with Kir2.2 and 2.3²⁷, there may be compensation or other effects for these mutant channels. Ultimately, variation in other genes, as well as broader environmental and developmental considerations may be required to fully define the phenotype at both the molecular and the clinical level.

Conclusions

Three novel *KCNJ2* arrhythmia mutations are reported and the ionic currents characterized. A novel biophysical mechanism, enhanced inward rectification, was found for T305A. Mutations in *KCNJ2* may underlie arrhythmia in a small number of patients lacking criteria for ATS yet suspected of an inherited arrhythmia.

Acknowledgements

LLE and ALF were funded by T32 HL07936 (JCM, PI) "Translational Research Training in Cardiovascular Sciences". Other support from HL-57414 (JCM) and HD42569 (MJA).

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Abbreviations used

ATS	Andersen Tawil Syndrome
WT	wild-type
LQTS	long QT syndrome
VT	ventricular tachycardia

Figure 1.

Abnormal *KCNJ2* genotypes of 4 patients referred for LQTS testing and a channel diagram showing location of new and previously reported mutations. Examples of the sequencing reaction are shown in the insets along with the predicted amino acid substitution. A diagram of the secondary structure of the *KCNJ2*/KIR2.1 channel in the membrane shows locations of the 4 characterized mutations R67Q, T75M, R82W and T305A represented with large circles. Previously reported *KCNJ2* mutations are also noted: D71V, R218W, 95–98del, S136F, G144S, R218Q, G300V, E303K, 314–315del¹, R67W⁵, T192A⁶, P186L, N216H, V302M⁷, D71N, T75R, G146D, R189I, G300D, R312C⁸, G215D¹⁴, T75A, 163–164delFQ¹⁵, T74A, C101R, G144A⁴, T75M, D78G, R82Q, V123G, L217P¹³, C154F, T309I²⁰, and G144A¹⁸.

Figure 2. Representative 12-lead electrocardiograms from the four cases. Note prominent U waves, and polymorphic ventricular triplet in Case 2 (T75M).

Figure 3.

Representative current traces. From a holding potential of -70 mV, step pulses from -140 to 40 mV were applied to the cells in 20 mV increments for 100 ms (see protocol diagram). Zero current level is indicated by the dotted line in each tracing. The representative current traces for WT, T75M and R82W are shown, as well as co-expression of the mutations with WT to show dominant negative suppression of current.

Figure 4. Summary data represented as current voltage plots for WT and mutant *KCNJ2* (T75M, R82Q, R67Q) expressed alone (A) or co-expressed with WT (B) to assess for dominant negative interactions. Mutant channels showed no current (A), whereas dominant negative suppression was shown for T75M and R82Q but not R67Q (B). Peak currents for each voltage are shown as the mean with standard error of the mean at each voltage ($n=5-8$ cells).

Figure 5.

Summary data represented as current voltage plots for WT and mutant *KCNJ2* T305A. The mutant expressed alone showed no current. When co-expressed with WT the inward currents were largely preserved suggesting minimal dominant negative suppression, but the inset shows that outward currents in the physiological range of voltages were dramatically reduced. Peak currents for each voltage are shown as the mean with standard error of the mean at each voltage (n=15–17 cells).

Figure 6.

KCNJ2 mutations exhibit normal trafficking. Representative WT and mutant *KCNJ2* DNA transfected into COS-1 cells and analyzed by confocal microscopy. Light microscopy (bottom panels) and fluorescent microscopy (top panels) are shown; the fluorescent images show a rim of fluorescence at the cell membrane typical of normal trafficking to the surface membrane.

Table 1

Phenotypic comparison of LQTS gene-positive, LQTS gene-negative, RyR2-positive, and KCNJ2-positive cohorts

	Total Cohort	LQT1-6 gene positive	LQTS gene negative	RyR2 positive	KCNJ2 positive
Number of Unrelated Patients	541	275	245	17	4
Age at diagnosis (range in years)	24 ± 16 (0–78)	23 ± 16 (0–75)	24 ± 17 (0–78)	23 ± 15 (0–58)	11.5 ± 4 (11–17)
Sex (male/female)	183/358	94/181	82/163	7/10	0/4
Ethnicity (% white)	93	90	96	94	100
Average QTc (ms) (range)	482 ± 57 (365–759)	494 ± 51 (402–700)	471 ± 61 (365–759)	422 ± 24 (380–460)	418 ± 2 (417–420)
% with QTc ≥ 480 ms	46	57	37	0	0
% with syncope	42	46	36	80	20
% with cardiac arrest	12	13	10	47	0
% with positive family history	42	46	37	80	0
% with positive family history of SCD	48	29	24	67	0
% with Schwartz and Moss score ≥ 4	29	41	17	0	0

Table 2

Genotype-phenotype summary of ATS1-associated *KCNJ2* mutations

Case	Age at Dx (yrs)/ Sex	Nucleotide Change	Amino Acid Change	QTc (ms)	QTU (ms)	Presentation / ECG abnormalities	Facial or Skeletal features	Periodic Paralysis	FH
1	15/F	200 G>A	R67Q*	417	NA	Asymptomatic / polymorphic PVCs non-specific inferior and lateral T-wave changes	No	No	No
2	14/F	224 C>T	T75M	420	650	Syncope / PVCs, bigeminy, couplets	No	Yes	No
3	6/F	244 C>T	R82W*	418	Prominent U-wave	Irregular heart rhythm and palpitations / Frequent premature ventricular beats, couplets, and short runs of VT. Ventricular Ectopy with exercise	No	No	No
4	11/F	913 A>G	T305A*	NA	NA	Asymptomatic / Ventricular ectopy Polymorphic VT Flattening of T-waves	No	No	No

Dx – diagnosis; F – female; M – male; ms – milliseconds; NA – not available; PVCs – premature ventricular contractions; VT – ventricular tachycardia; FH – family history;

* – novel