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Multiple different missense mutations in the pore region of *HERG* in patients with long QT syndrome

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Abstract Long QT syndrome (LQTS), is an inherited cardiac disorder in which ventricular tachyarrhythmias predispose affected individuals to syncope, seizures, and sudden death. Characteristic electrocardiographic findings include a prolonged QT interval, T wave alternans, and notched T waves. We have screened LQTS patients from 89 families for mutations in the pore region of *HERG* , the K^+ channel gene previously associated with chromosome 7-linked LQT2. In six unrelated LQTS kindreds, single-strand conformation polymorphism analyses identified aberrant conformers in all affected family members. These conformers were not seen in over 100 unaffected, unrelated control individuals, suggesting that they represent pathogenic LQTS mutations. DNA sequence analyses of the aberrant conformers demonstrated that they reflect five different missense mutations: V612L, A614V, N629D, N629S, and N633S. The missense mutation A614V was found in two unrelated families. Further functional studies will be required to determine what effect each of these changes may have on HERG channel function.

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Introduction

Autosomal dominant long QT syndrome (LQTS), or Romano-Ward syndrome, is an inherited cardiac disorder that predisposes affected individuals to sudden death from ventricular tachyarrhythmias (reviewed in Roden et al. 1996). LQTS is characterized clinically by a spectrum of symptoms including recurrent syncope, seizures, and sudden death, often triggered by emotional or physical stress (Romano et al. 1963; Ward 1964; Jackman et al. 1988). Abnormalities of electrocardiographic repolarization, including a prolongation of the QT interval, asymmetric or notched T or U waves, and T wave alternans are characteristic of this disease and sometimes may be the only sign of affected status.

Ventricular repolarization involves several distinct currents controlled by a number of different ion channels. Not surprisingly, defects in any of several of these channel genes can result in altered repolarization leading to LQTS. Presently four different genetic loci are associated with LQTS: LQT1 on chromosome 11p15.5, LQT2 on chromosome 7q35–36, LQT3 on chromosome 3p21–24, and LQT4 on chromosome 4q25–27 (Keating et al. 1991a, b; Benhorin et al. 1993; Curran et al. 1993; Jiang et al. 1994; Towbin et al. 1994; Schott et al. 1995). Two of these loci, LQT1 and LQT2, encode the cardiac potassium channel genes *KVLQT1* and *HERG* and are thought to be the first and second most common disease genes, respectively (Curran et al. 1995; Wang et al. 1996). The third locus, LQT3, has been shown to be the cardiac sodium channel gene, *SCN5A*, mutations of which are relatively rare (Wang et al. 1995). The chromosome 4 locus, LQT4, is known only from linkage analysis in a single family and the gene responsible remains to be identified (Schott et al. 1995).

HERG, the human *ether-a-go-go* (*eag*)-related gene, is a member of the *eag* family of potassium channels (Warmke et al. 1993). Family members all contain six putative transmembrane segments, an ion-conducting pore region and a putative cyclic nucleotide-binding domain.

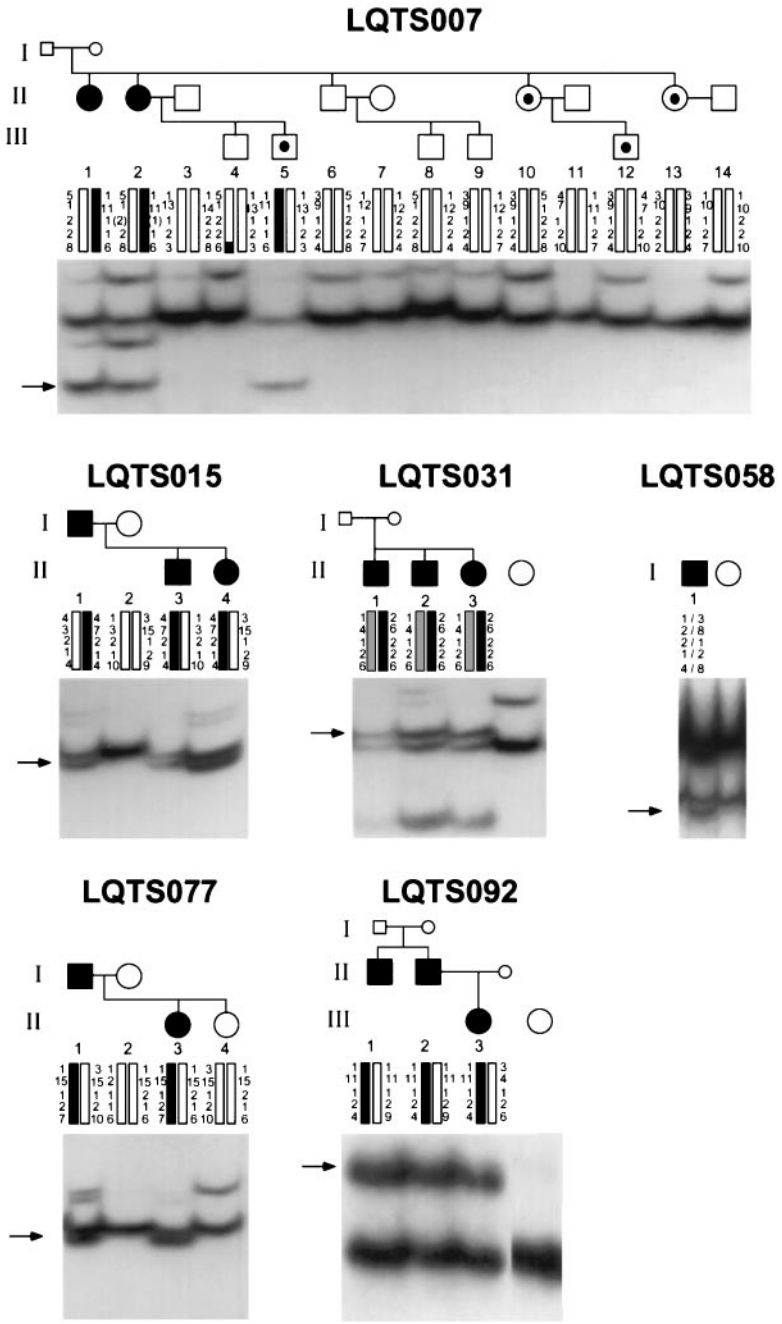

Fig. 1 Pedigree structures and genotypic data on six LQTS kindreds with *HERG* mutations. *Clear circles* unaffected females, *clear squares* unaffected males. *Filled symbols* represent affected individuals and *bulleted symbols* those of ambiguous phenotype. *Smaller symbols* indicate individuals not studied. *Unattached circles* indicate unrelated control individuals for several families. Polymorphic markers (D7S505, D7S636, HERG 3–9, HERG 5–11, D7S483) are listed in centromere-to-telomere order. HERG 3–9 and HERG 5–11 are intragenic polymorphisms while D7S636 and D7S483 flank the gene and are 2 cM from each other (Curran et al. 1995). Genotypes are grouped in the most likely paternal and maternal haplotypes. Alleles from the original nonrecombinant chromosome haplotype that cosegregated with the disease gene are marked by a *blackened bar*. *Grey bars* under pedigree LQTS031 indicate uncertainty as to which haplotype is disease-associated. The identification number of each individual is shown below his or her pedigree symbol. The results of single-strand conformation polymorphism (SSCP) analyses using 7.5% nondenaturing polyacrylamide gels to analyze polymerase chain reaction products of primer pair HERG 1894–12 are shown below each pedigree. Aberrant SSCP conformers (*arrow*) cosegregate with the disease in each kindred

The pore region is analogous to those found in the *shaker* family of voltage-gated potassium channels, and is likely important for determining ion selectivity, conductivity, and channel inactivation (Choi et al. 1991; Hoshi et al. 1991; Heginbotham et al. 1992, 1994; DeBiasi et al. 1993; Yellen et al. 1994; Lu and Miller 1995; Smith et al. 1996).

By analogy with *shaker* potassium channels, which have similar structure, active HERG channels are thought to be formed by homotetramers (MacKinnon 1991). Expression of wild-type HERG proteins in *Xenopus* oocytes has demonstrated that *HERG* encodes the rapidly activating, inwardly rectifying K^+ channel responsible for the cardiac myocyte current, I_{Kr}. This current is an important component of ventricular repolarization, irregularities of which were hypothesized to produce the repolarization abnormalities found in LQTS.

Based on the colocalization of LQT2 and *HERG* on chromosome 7q35–36, Curran et al. (1995) searched for and found mutations in the *HERG* genes of six families with LQTS. As the *HERG* gene is strongly expressed in cardiac muscle, Curran et al. concluded that the *HERG* gene is LQT2. Expression of LQTS-associated mutant HERG channels in *Xenopus* oocytes revealed that they either cause loss of function or dominant-negative suppression of HERG function. Resulting decreases in I_{Kr} explain the prolonged QT interval observed in LQTS patients. Additional studies by Smith et al. (1996) demonstrated that specific gating properties of wild-type HERG channels

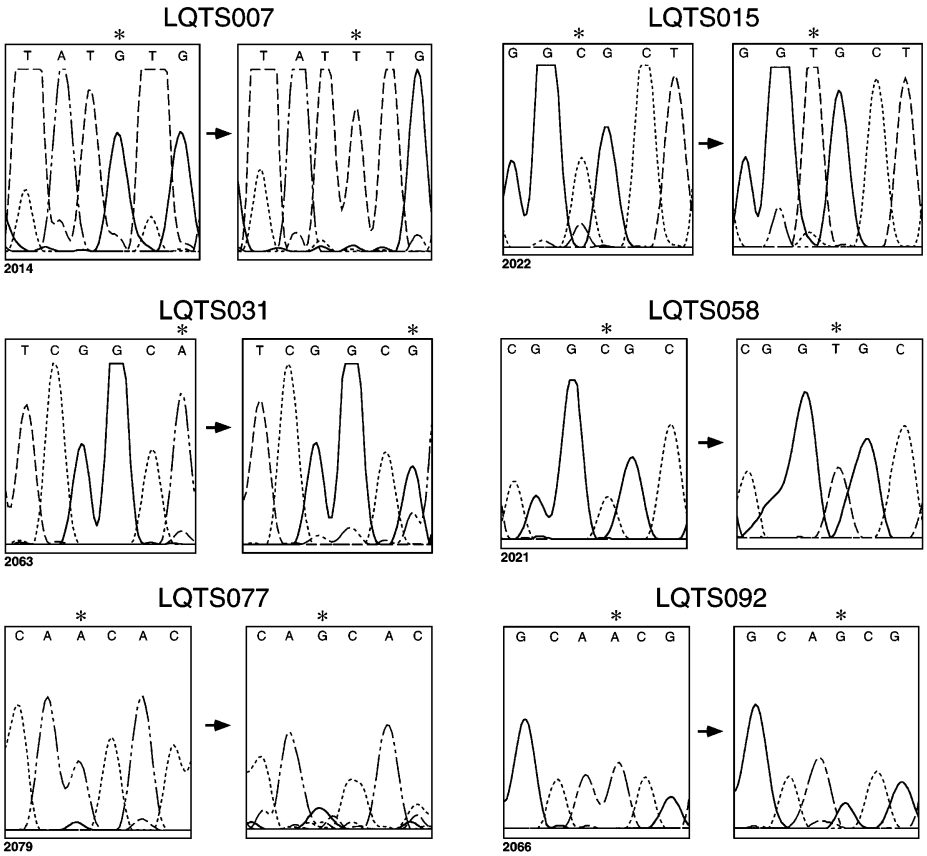

Fig. 2 Comparisons of the mutant and wild-type *HERG* sequences: DNA sequence analyses of the normal and aberrant SSCP conformers shown in Fig. 1 for each family. Positions from the region corresponding to residues 612–633 of HERG for LQTS007, LQTS015, LQTS031, LQTS058, LQTS077 and LQTS092 are shown. (The first base is indicated below each normal sequence.) The wild-type and corresponding mutant nucleotides are marked by an *asterisk* in each chromatogram. In LQTS007, a GT substitution results in a valine (GTG) to leucine (TTG) transition at codon 612, (V612L). In LQTS015 and LQTS058, a CT transition results in an alanine (GCG) to valine (GTG) transition at codon 614, (A614V). In LQTS031, an AG substitution results in an asparagine (AAC) to aspartic acid (GAC) transition at codon 629, (N629D). In LQTS077, an AG substitution results in an asparagine (AAC) to serine (AGC) transition at codon 633, (N633S). In LQTS092, an AG substitution results in an asparagine (AAC) to serine (AGC) transition at codon 629, (N629S). Each missense mutation shown is found in the pore region of *HERG*

may allow this channel to play a protective role against arrhythmias. Dysfunctional channels, therefore, may not only be responsible for the observed prolonged ventricular repolarization, but may also leave affected individuals at higher risk for life-threatening arrhythmias.

To date, only 14 different HERG mutations in LQTS patients have been reported (Curran et al. 1995; Akimoto et al. 1996; Benson et al. 1996; Dausse et al. 1996; Satler et al. 1996; Tanaka et al. 1997). Two of these cause translational reading frame shifts leading to truncated proteins while a third is an intragenic deletion of nine codons within the third membrane-spanning domain (Curran et al. 1995) The remaining 11 mutations are all missense changes of which 6 are in the pore region. Only one of these pore region mutations, G628S, has been tested in a functional assay (Sanguinetti et al. 1996), but it is noteworthy that the mutant channels were nonfunctional and that coinjection of G628S and wild-type *HERG* cRNA demonstrated that this mutation acted in a dominant fashion. Thus apparently subtle alterations to the pore region can have profound effects on HERG function and I_{Kr} and such mutations may act in a dominant fashion.

To determine the frequency of mutations of the *HERG* pore region in LQTS, we have screened this area in 110 individuals from 89 different families. Here we report five distinct missense mutations of *HERG* associated with LQTS in six previously unreported families of various descent. Clinical and genetic analyses of each family are consistent with chromosome 7-linked LQT2. Further functional studies of these mutations will be important for understanding the structure-function relationships of amino acids in this domain.

Materials and methods

Identification and clinical evaluation of LQTS patients

LQTS families were identified from medical clinics throughout the United States. Informed consent was obtained from all participants in accordance with the Children's Hospital Committee on Clinical Investigation. All relatives were evaluated by family history, physical examination, and 12-lead electrocardiography. Measurement of the corrected QT interval, QTc, was performed using established methods (Bazett 1920). Diagnosis of LQTS was made according to a di-

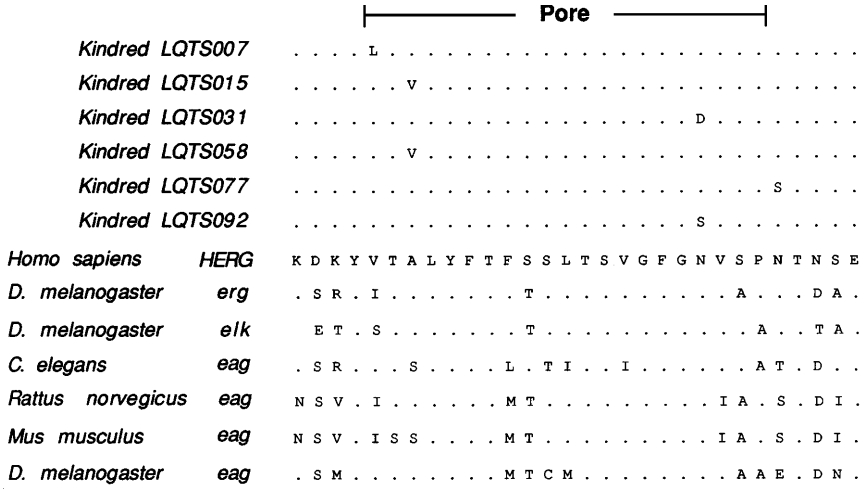

Fig. 3 Amino acid sequence alignment of the pore region of *HERG* and other K^+ channels of the *eag* family from various species. The complete *HERG* sequence for amino acids 608 to 637 is shown and variant residues in other *eag* channels are indicated below. There is 49% identity of the hydrophobic domains (S1–S6, and the pore region) between the *HERG* sequence and the other noted *eag* channels (Warmke and Ganetzky 1993). Mutations found in each family are indicated above. All sequences were obtained from the Genome Data Bank. Accession numbers are: *Homo sapiens HERG*, U04270; *Drosophila melanogaster erg*, U36925; *D. melanogaster elk*, U04246; *Caenorhabditis elegans eag*, Z35596; *Rattus norvegicus eag*, Z34264; *Mus musculus eag*, U04294; *D. melanogaster eag*, Q02280

agnostic criteria system as described (Schwartz et al. 1993). The relatives evaluated had no history of neurologic abnormalities or documented use of drugs or medications known to prolong the QT interval.

Single-strand conformation polymorphism (SSCP) and sequence analyses of genomic DNA

Genomic DNA was isolated from peripheral lymphocytes using the Puregene DNA Isolation kit (Gentra Systems, Research Triangle Park, N.C.). Samples were stored at –20°C. For SSCP screening, DNA samples from one or two affected family members were arrayed into 96-well microtiter plates. Genomic DNA samples were amplified by polymerase chain reaction (PCR) and used in SSCP analyses as described (Satler et al. 1996). Nucleotide and amino acid sequence numbers for *HERG* are based on U04270 (Warmke and Ganetzky 1993). The primer HERG-1894 was designed based on published sequence and paired with the previously described primer HERG-12, which is located in an intron one base away from the exon that ends at nucleotide 2128 (Warmke and Ganetzky 1993; Curran et al. 1995). The oligonucleotide sequence for HERG-1894 is 5′-ATCGGCAACATGGAGCAGCCACACAT-3′, which is complementary to *HERG* cDNA sequence spanning positions 1894 to 1919. Thus, our analysis screened bases encoding residues 580–648 as well as the first G at the 5′ splice site in the intron lying between nucleotides 2128 and 2129. All PCR products were screened for mutations under three different SSCP conditions. Three microliters of each sample was electrophoresed on two types of 0.5 × MDE gels (FMC Bioproducts, Rockland Me.) with and without 5% glycerol using 0.6 × TBE running buffer at room temperature at 8 W for 14 h. Products were also analyzed on 7.5% nondenaturing polyacrylamide gels (49:1 polyacrylamide:bisacrylamide) at 4°C and 40 W for 2–5 h. Gels were transferred to filter paper, dried, and exposed to X-ray film for 4–12 h. Normal and aberrant SSCP conformers were cut directly from dried gels and eluted in 100 μl water at 37°C for 1 h. Eluted products (5 μl) were reamplified using the original primer pair and sequenced on an Applied Biosystems model 370A DNA sequencer (Applied Biosystems, Foster City, Calif.) as described (Satler et al. 1996).

Genotypic analyses

Polymorphic microsatellite markers that span the HERG locus were amplified with the PCR as described (Satler et al. 1996). The markers analyzed include D7S505, D7S636, and D7S483 (Jiang et al. 1994). The primer pairs HERG 3–9 and HERG 5–11, which recognize silent polymorphisms at I489 and L564, respectively, were previously described (Curran et al. 1995). Radiolabeled PCR products were electrophoresed on 6% polyacrylamide gels and visualized following autoradiography. Alleles were scored by two investigators, independently and without knowledge of each individual's phenotypic status. Genotypes are grouped in most likely paternal and maternal haplotypes.

Results

Mutations in the pore region of *HERG*

Eighty-nine families and sporadic cases in whom linkage analyses for the four known loci would not be definitive were screened for mutations in the pore region of *HERG*, using SSCP analyses. Two additional large families in this series were previously shown to have *HERG* mutations, one of which was in the pore region (Benson et al. 1996; Satler et al. 1996). The primer pair HERG-1894 and HERG-12(Curran et al. 1995) amplifies a 246-bp fragment encoding amino acids 580–648, corresponding to the S5-S6 linker, the pore domain, and a portion of S6. Aberrant conformers were identified in affected individuals from 7 of the 89 families studied. In each case, the aberrant conformer cosegregated with LQTS in all available family members (Fig. 1). For each family, the normal and aberrant conformers were sequenced (Fig. 2). In six of these families, five distinct missense mutations were identified, each resulting in a different single-residue substitution in the pore region of the HERG K^+ channel α-subunit (Fig. 3, Table 1). None of these anomalies were found in over 100 unrelated, unaffected individuals (data not shown).

Table 1 *HERG* gene mutations in seven families with LQTS

Kindred	Ethnic background	DNA change	Predicted amino acid change
LQTS007	Irish, Italian, Dutch	G2017T	V612L
LQTS015	English, Irish, French, Filipino	C2024T	A614V
LQTS021	Mexican	C1983T	Unkown
LQTS031	German, Irish, Native American	A2068G	N629D
LQTS058	Irish, Czech, English	C2024T	A614V
LQTS077	English	A2081G	N633S
LQTS092	Unknown	A2069G	N629S

A seventh aberrant conformer was identified in two affected individuals from family LQTS021 (data not shown). Additional members of this family were not available to determine whether this anomaly cosegregates with LQTS, although the variant was not seen in the control population. Sequence analysis demonstrated a C to T substitution at position 1983, resulting in an AGC to AGT shift at codon 600. This transition does not lead to a residue substitution at codon 600 (S600S). However, it does create a GT dinucleotide that could potentially serve as an alternative 5′ splice site, which, if used, would result in an in-frame deletion of 50 amino acids and the creation of an additional threonine residue at the deletion junction (*Δ*S600–S649+T).

Genotypic analyses

To ascertain whether there might be any common haplotypes associated with *HERG* gene mutations, genotypes were determined for each individual with microsatellite markers within and around the *HERG* gene (Curran et al. 1995). Haplotypes were constructed where possible. For each family with informative meioses, segregation of the affected status with the LQT2 locus was evident (Fig. 1). Segregation analysis identified one recombination event between HERG 5–11 and D7S483 in individual III-4 of kindred LQTS007. No other recombination events were identified in the six families. Although it is not possible to establish phase in the single affected individual of LQTS058, it may be that the haplotype 2/1/4 for HERG 3–9/HERG 5–11/D7S483 is associated with A614V in both the LQTS015 and LQTS058 kindreds. There were no other apparent associations of any particular haplotype(s) with the other *HERG* mutations but further interpretation of these data will require population studies to determine allele and haplotype frequencies.

Clinical findings in mutation carriers

Pedigrees of the six families described are shown in Fig. 1 and clinical findings are summarized in Table 2. Kindred LQTS007 is of European descent. Two members of this kindred carried clinical diagnoses of LQTS, four were unaffected, and four were of indeterminate phenotype (e.g., clinically asymptomatic with a QTc between 0.41 and 0.44 s). SSCP analysis demonstrated that one of these four

Table 2 Clinical findings in individuals with mutant *HERG* genes

Kindred	Subject no.[a]	Age (years)	Sex	QT_c[b] $ms^{1/2}$	Palpitations	Syncope	Seizure	Aborted sudden death
LQTS007	II–1	33	F	620	+	+	–	–
	II–2	35	F	520	–	–	–	–
	II–3	3	M	445	–	–	–	–
LQTS015	I–1	40	M	575	+	–	+	–
	II–3	10	M	515	–	–	–	–
	II–4	14	F	510	–	+	–	+
LQTS031	II–1	19	M	485	+	+	–	+
	II–2	13	M	600	–	–	–	+
	II–3	16	F	485	+	–	–	–
LQTS058	I–1	8	M	480	–	+	+	–
LQTS077	I–1	36	M	565	–	+	–	–
	II–3	6	F	500	–	–	–	–
LQTS092	II–1	37	M	550	–	–	–	–
	II–2	35	M	530	–	–	–	–
	II–3	12	F	540	+	+	+	+

[a] Numbering according to pedigrees in Fig. 1
[b] QT_c, corrected QT interval (Bazett 1920)

indeterminate individuals carried the *HERG* V612L mutation. Kindred LQTS015 is of European and Pacific Islander descent. Three of four relatives had clinical findings indicative of LQTS and all three carried the A614V mutation. The single affected individual of kindred LQTS058 is of European descent and also has the *HERG* A614V mutation, but there is no evidence that this individual is related to kindred LQTS015. Kindred LQTS031 is of American-Indian and European descent and all three relatives were clinically affected and were heterozygous for *HERG* N629D. Kindred LQTS077 is of Northern European and Ukrainian descent, with two of four relatives clinically affected and carriers for *HERG* N633S. The three available members of kindred LQTS092, of unknown ancestry, all have markedly prolonged QT intervals and are all heterozygous for *HERG* N629S.

The measured QTc values of the 15 *HERG* mutation carriers ranged from 445 to 620 $ms^{1/2}$, with an average of 529±64 $ms^{1/2}$ (Table 2). Six of these 15 individuals (4 males, 2 females) were asymptomatic, including 3 adults over the age of 35 years. Each family had at least one member with overt clinical symptoms who was responsible for bringing the family to attention. A history of palpitations was documented in five individuals, syncopal episodes were reported by six individuals, three individuals experienced seizures and four experienced aborted sudden cardiac death. Although the numbers are small, the intra- and interfamilial variabilities in clinical findings are similar, suggesting that none of these mutations are associated with particularly severe or mild prognoses.

Discussion

We conclude that the five missense mutations described above likely cause the chromosome 7-linked form of LQTS. Each mutation segregates with LQTS disease status for each of the six respective families and none of the mutations were found in over 100 unrelated, control individuals. To date, 14 distinct *HERG* LQTS mutations have been previously reported (Akimoto et al. 1996; Benson et al. 1996; Curran et al. 1995; Dausse et al. 1996; Satler et al. 1996; Tanaka et al. 1997). A614V has been found in one Japanese family as well as in two of the families described here, making a total of 18 different known *HERG* mutations. Fifteen of these are missense mutations of which ten are in or near the pore region. However, the apparently high incidence of pore region mutations is due to a bias of ascertainment as all studies to date have screened only a limited portion of the *HERG* gene. We are currently determining the genomic structure for the entire *HERG* gene to allow a complete mutation analysis of the entire coding region.

Mutation of *FGFR2* exonic sequences to create a new splice site has been reported to alter splicing and cause Crouzon syndrome (Del Gatto and Breathnach 1995). However, the pathogenic status of the C to T transition at base 1983 (S600S) in family LQTS021 is unclear. The exon/intron boundary at the 3′ end of this exon (at base 2128) is CT/GTGAGT, which has a consensus score of 75.7 using the scoring system of Shapiro and Senapathy (1987). The altered sequence creates a potential 5′ splice site with the sequence CA/GTGGCC; however, the consensus score for this sequence is only 45.3, suggesting that it is unlikely to function as an alternative 5′ splice site for this exon. Reverse transcription (RT)-PCR studies of mRNA splicing would be necessary to test this hypothesis directly but, unfortunately, appropriate tissue specimens are unavailable.

By analogy with *shaker* channels, the HERG pore region is responsible for several important K^+ channel functions. Three channel characteristics regulated by the pore region are selectivity, conduction, and inactivation. In *shaker* channels, residues that strongly affect ion selectivity have only been found in the pore region (DeBiasi et al. 1993; Heginbotham et al. 1992, 1994). Conductivity is also determined by specific residues within the pore region; however, the definitive mechanism involved has yet to be identified (Lu and Miller 1995). C-type inactivation of K^+ channels, like HERG, is postulated to be the result of conformational changes in the pore and its surrounding regions (Choi et al. 1991; Hoshi et al. 1991; Yellen et al. 1994; Smith et al. 1996). It is possible that each mutation described here may alter HERG channels by affecting one or more of these critical pore region functions. Since HERG presumably associates as a tetramer, four-fifths of mature HERG channels in heterozygous individuals will contain at least one mutant monomer, explaining the dominant-negative disruption of I_{Kr} (Sanguinetti et al. 1995, 1996). Slight variations in the relative levels at which two alleles are expressed should result in larger differences in the proportion of functional and nonfunctional channels. This phenomenon has been demonstrated to be at least partially responsible for the clinical variability exhibited by Quarter Horses with hyperkalemic periodic paralysis due to mutations in a sodium channel gene (Zhou et al. 1994) and it may partly explain the intrafamilial clinical variability seen in our LQTS patients. Knowing the specific mutations will make it possible to test this hypothesis by allele-specific quantitative RT-PCR analysis of myocardial specimens from heterozygous carriers.

It is interesting to note the same C to T transition causing A614V has previously been reported in an unrelated Japanese LQTS family (Tanaka et al. 1997). Although the two families with the A614V mutation reported here are of European descent, kindred LQTS015 resides in Ohio and kindred LQTS058 resides in Florida and there is no known relationship between the two families. It may be that this site represents a mutational hotspot due to deamination of 5-methylcytosine at this CpG dinucleotide (Cooper and Krawczak 1990), but further studies will be necessary to exclude the possibility of ancestral links between these three kindreds.

Remarkably, seven of the ten different mutations in the pore region are conservative changes that do not alter charge or polarity of the amino acids (Curran et al. 1995; Akimoto et al. 1996; Benson et al. 1996; Tanaka et al. 1997) (Fig. 3). All five of the changes reported here are at

positions that are conserved between human *HERG* and *Drosophila erg* but three of these, V612L, A614V and N633S are sites that vary between *erg*, *elk* and *eag* in different species. In fact, serine at position 633 is a conserved residue in *Drosophila elk* and murine and rat *eag* channels, suggesting that asparagine at this position may be important for HERG function and may differentiate *HERG* from other *eag* family members. The highly conserved asparagine at position 629 has been changed to aspartic acid in kindred LQTS031 and to serine in kindred LQTS092. Aspartic acid is an invariant residue at this position in *shaker* voltage-dependent channels and some calcium-activated channels (Heginbotham et al. 1994). It would be interesting to see whether the *HERG* N629D mutation confers *shaker*-like electrophysiological properties to mutant HERG channels. The subtle nature of all these changes supports the notion that pore function is highly structure dependent and easily altered (Heginbotham et al. 1994).

In conclusion, we have identified five different LQTS-associated missense mutations in the pore region of HERG. Electrophysiological studies of the corresponding mutant channels in oocytes will be important to determine whether they have pathologic significance and to improve our understanding of the structure-function relationship of wild-type and mutant HERG.

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