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Phenotypic Correlation of Genetic Mutations with Ventricular Arrhythmias

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1. Introduction

Much progress has been made in identifying genetic loci linked to hereditary arrhythmia syndromes over the past decade and a half. Linkage analyses for Mendelian diseases have been powerful in the discovery phases. Considerable challenges remain however, for the clinician faced with individual patients and families when the clinical symptoms are atypical or intermediate and when novel mutations or polymorphisms are reported in the course of genetic testing. To unambiguously define the deleterious nature of any given mutation, additional functional analyses are required. Such studies should not only detect the functional consequence of mutations but also the degree of severity and mechanisms that bring about the deleterious behavior. These principles apply not only to cardiac arrhythmia syndromes but also to any hereditary genetic disease. In practice, this is not always feasible or possible with current technology. This is particularly problematic when standard genetically manipulable animals (mouse) differ considerably from human, as they do in cardiac electrophysiology. An additional obstacle occurs when the target organ is not amenable to biopsy without considerable risk (e.g. heart, brain, etc.). For evaluation of genetic mutations in cardiac arrhythmia syndromes, heterologous expression of affected genes has helped tremendously.

Hereditary arrhythmia syndromes include: the long QT syndrome, the Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, the short QT syndrome, and arrhythmogenic right ventricular dysplasia. We will restrict our discussion to the long QT syndrome; however, the basic principles of verifying functional consequences of mutations also applies to the other syndromes. In this chapter we will review the progress in characterizing arrhythmia-linked genetic mutations. Several areas of recent technical advancement have been achieved which we will discuss in detail. We will also highlight how biophysical, biochemical and cell-biological studies may be used to help inform clinicians in managing the more subtle and varied aspects of patients with specific mutations. Lastly, we will discuss how such studies may eventually point to therapeutic modalities that will lead to gene-specific, or personalized medicine.

2. Overview of the long QT syndrome

Congenital long QT syndrome (LQTS) was first described by Jervell and Lange-Nielsen in 1957, who presented a family in which four of six children were born deaf, had episodes of syncope, prolonged QT interval and early sudden death (Jervell & Lange-Nielsen, 1957). In 1963 and 1964, independent reports of a similar constellation of findings in patients, but without hearing loss, were made (Romano et al., 1963; Ward, 1964). Subsequently, these were classified as autosomal-recessive (Jervell-Lange-Nielsen syndrome, with hearing loss) and autosomal dominant (Romano-Ward syndrome) forms of LQTS. The incidence of hereditary LQTS has been estimated to be as high as 1 in 2500 (Crotti et al., 2008). Notably, disease severity varies widely—from patients who are mostly asymptomatic, to ones who suffer multiple episodes of syncope and/or sudden cardiac death at a young age.

The common pathophysiological feature of LQTS is delayed repolarization, manifest on electrocardiogram (ECG) as a prolonged QT interval corresponding to a prolonged action potential duration (APD). Delayed repolarization occurs either due to an excess of sodium (Na^+) or calcium (Ca^{2+}) influx, or to deficient potassium (K^+) efflux. This disruption in the normal ionic currents across the cell membrane undermines the highly regulated electrical activity in the heart required for normal, rhythmic beating, and leaves patients at risk for potentially lethal arrhythmias. Abnormal currents can result from congenital mutations in the ion channels, or from pharmacological agents and acquired disease that can alter cardiac ion channel function.

When a ventricular myocyte action potential is prolonged, abnormal depolarizations may develop, known as early afterdepolarizations (EAD) that occur during the plateau or repolarization phases of the action potential (i.e. a type of depolarization that occurs before an action potential has completed repolarization). An EAD can then trigger an action potential that is self-perpetuating, leading to a particularly deadly type of arrhythmia known as polymorphic ventricular tachycardia or "*torsade de pointes*" which may degenerate into ventricular fibrillation. Furthermore, intracardiac imbalances of ion currents may lead to disperson of refractoriness that may play a role in susceptibility to micro-reentry. Symptoms include syncope (fainting), palpitations and sudden cardiac death.

2.1 Linkage studies

The hereditary long QT syndrome (LQTS) is now recognized as a genetically heterogeneous disorder with at least 13 different proposed loci (Table 1). Most of the loci contain genes of cardiac ion channels, accessory subunits, or channel-associated scaffolding proteins. The approaches taken by researchers in the 1990s to initially characterize hereditary LQTS relied on classical genetics with pedigree analysis of large families using microsatellite markers and logarithm of odds (LOD) score calculation. LOD scores indicate the likelihood of linkage of two loci by comparing the calculated recombination frequency against chance. A positive LOD score signifies linkage, whereas a negative score signifies the absence of linkage. The major goal of the early studies was to connect symptomatic LQTS patients with a common genetic feature.

Originally, LQTS was thought to be a single-gene disorder linked to chromosome 11 (Keating et al., 1991a; Keating et al., 1991b). Subsequent refinement revealed that heterogeneity and multiple loci were involved (Worley et al., 1992; Benhorin et al., 1993). In 1994, analysis of multiple LQTS families using LOD scores showed that some had linkage to chromosome 7, others linked to chromosome 3, and both excluded chromosome 11 linkage (Jiang et al., 1994). Other of the families in the study did not show linkage to any of the three known loci, suggesting the existence of additional loci. Once the first three LQTS loci were identified, several groups worked to identify the genes responsible for the phenotypes. In

1995, Wang et al. used linkage analysis to show that locus LQT3 contained SCN5A, a Na$^+$ channel that was previously cloned and characterized in 1992 (Gellens et al., 1992; Wang et al., 1995a; Wang et al., 1995b).

Locus	Gene	Protein Function	Chromosome	Other Diseases
LQT1	KCNQ1	KvLQT1 K$^+$ channel α subunit	11p15.5	Short QT Syndrome (SQTS1) Familial Atrial Fibrillation (FAF)
LQT2	KCNH2	HERG K$^+$ channel α subunit	7q35-q36	SQT1
LQT3	SCN5A	Na$^+$ Channel α subunit	3p21	Brugada Syndrome (BrS1) Conduction & Sinus node disease
LQT4	ANK2	Ankyrin B adaptor protein	4q25-q27	LQTS
LQT5	KCNE1	minK β subunit	21q22.1-2	Atrial Fibrillation, Deafness
LQT6	KCNE2	MiRP1 β subunit	21q22.1-22.2	Hypothyroidism, Periodic paralysis
LQT7	KCNJ2	Kir2.1 K$^+$ Channel	17q23.1-q24.2	Andersen's Syndrome, myotonia
LQT8	CACNA1c	Ca^{2+} channel α subunit	12p13.3	Timothy Syndrome, BrS3
LQT9	CAV3	Caveolin 3 membrane scaffold	3p25	Cardiomyopathy
LQT10	SCN4B	Na$^+$ Channel β subunit	11q23	Conduction Disease
LQT11	AKAP9	Yotiao PKA scaffold	7q21-q22	LQTS
LQT12	SNTA1	Syntrophin α 1 scaffold protein	20q11.2	LQTS
LQT13	KCNJ5	Kir3.4 K$^+$ Channel	11q24	Neonatal hyper-insulinemia

Table 1. The Hereditary Long QT Syndrome Loci

In 1995 Curran et al. analyzed LQTS families using markers linked to locus LQT2 on chromosome 7q35-36 (Curran et al., 1995). Physical mapping using yeast artificial chromosomes (YACs) and fluorescent in situ hybridization (FISH) indicated that a candidate gene with homology to potassium ion channels (K$^+$ channels) resided in that position. This gene had been previously identified as the human *ether-à-go-go* related gene (HERG or KCNH2) (Warmke & Ganetzky, 1994). Patient sample analysis for mutations in HERG with single-strand conformation polymorphisms (SSCP) detected the presence of genetic variants and functional expression of the cDNA in *Xenopus* oocytes showed that HERG encoded a channel that carried the rapidly activating delayed rectifier K$^+$ current (I_{Kr}) and confirmed the deleterious nature of the mutations. Further positional cloning showed that LQT1 on chromosome 11 encoded KvLQT1/KCNQ1, another K$^+$ channel (Wang et al., 1996). Concurrently, Schott et al. used similar linkage techniques to map the LQT4 locus to chromosome 4q25-27 (Schott et al., 1995). The gene responsible for LQT4 was identified in 2003 by the Mohler group as ankyrin-B, a scaffolding protein which when mutated causes aberrant targeting of essential cardiac channel proteins (Mohler et al., 2004).

Identification of other LQTS loci was done through a variety of techniques ranging from classical genetics to modern genomic methods (Chevillard et al., 1993; Duggal et al., 1998; Abbott et al., 1999; Fodstad et al., 2004; Vatta et al., 2006; Ueda et al., 2008). The loci include other channel proteins such as the Kir2.1 channel encoded by KCNJ2 and the voltage-gated Cav1.2 calcium channel encoded by CACNA1c, K^+ channel accessory subunits (KCNE1 and KCNE2), as well as scaffolding proteins such as AKAP9 and syntrophin. While the genes are numerous and diverse, the overall themes of cardiac ion channel function/dysfunction and alterations in regulation unify the genetic causes of LQTS.

3. Heterologous expression of arrhythmia-linked genes

The ideal system for studying behavior of cardiac ion channels would be isolated cardiac myocytes that survive in culture for a long time period. Such primary cells however, entail significant risk to patients and are extremely difficult to maintain long term in culture. The next option is to express the channel proteins in a cell type that can be maintained and manipulated as necessary.

3.1 *Xenopus* oocytes

Xenopus laevis oocytes are an established system for studying ion channels using electrophysiological techniques. The procedure consists of creating cRNAs of the gene of interest followed by injection into oocytes, which contain all the necessary cellular machinery for protein expression (Gurdon et al., 1971; Barnard et al., 1982). Two-electrode voltage clamp is a relatively easy method to use with oocytes given their large size and provides a rapid way to functionally characterize many of the genes involved in LQTS, and many of the first studies utilized this method. *Xenopus* oocytes however, contain an endogenous K^+ channel similar to KCNQ1, thus confounding some of the early studies on KCNE1 and KCNQ1. Moreover, oocytes are maintained at 16-19ºC, a temperature that may permit mutant proteins to properly fold and traffic to the cell surface thereby masking a misfolding phenotype that would normally occur at human physiological temperatures. Such an occurrence was noted in the initial analysis of the cystic fibrosis transmembrane conductance regulator protein (CFTR)(Cheng et al., 1990; Denning et al., 1992).

3.2 Mammalian cultured cell systems

Another approach is to use immortalized mammalian cell lines such as human embryonic kidney (HEK 293), Chinese hamster ovary (CHO), or COS-7 cells. Unlike primary cell lines, immortalized cell lines can be propagated many times and maintain baseline characteristics. The cells are incubated at 37°C and contain all the necessary components for protein transcription, translation, trafficking and degradation. They are more amenable than oocytes for immunoblotting, immuno-precipitation, high-resolution immuno-fluorescence, trafficking assays, cell-surface expression assays, and patch clamp electrophysiology. The cells may also have endogenous K^+ current; however, the magnitude is small and does not usually interfere with measurements of over-expressed channels currents. Mammalian cell lines more closely mimic native systems than oocytes and are useful for analyzing biological consequences of LQTS mutations. A caveat to this system is that the LQT-linked channels may exist in macromolecular complexes in vivo. Such complexes may comprise accessory subunits and regulatory proteins, which may not be recapitulated by heterologous expression system.

3.3 Purified proteins for biochemistry and structural analysis

Functional expression in oocytes and cells allows the study of many aspects of mutations but the fundamental mechanism of mutational effects ultimately relies on structural analysis. The primary challenge is finding conditions under which a large quantity of protein can be expressed and purified. This process can be relatively straightforward for soluble, cytosolic proteins, but is more difficult for membrane proteins such as ion channels. Because of the large amount of protein needed for purification, transfection of mammalian cells, or even the use of stably transfected mammalian cells lines, may not be feasible. Alternative systems of expression have been developed for bacteria, yeast, and insect cells, but determining the best host for producing a particular protein is usually an empirical process.

One of the most commonly used expression systems is the bacteria *Escherichia coli*. There are several technical and economic advantages: the ease of introducing DNA via transformation with a plasmid expression vector, rapid growth, and simple growth media. Problems do exist, though, in expressing mammalian membrane proteins in bacteria. These include alternative translation, posttranslational modification and trafficking mechanisms. Certain limitations can be overcome by changing growth conditions, co-expressing necessary chaperones, or creating fusions with prokaryotic partners such as maltose-binding protein (MBP) or glutathione *S*-transferase (GST) to improve their solubility and stability. Even with these modifications, it can still be difficult to express the full length of a protein (for example, the full length of KCNQ1 is nearly 700 amino acids, and Nav1.5 is around 2000 amino acids). An alternative expression host is the yeast *Pichia pastoris*, which has a eukaryotic protein synthesis pathway and is capable of post-translational modifications, though it is not entirely equivalent to a mammalian system. The first (and thus far, only) mammalian K^+ channel to be crystallized was expressed in *P. pastoris*, whereas several previously crystallized bacterial channels were expressed in *E. coli* (Doyle et al., 1998; Jiang et al., 2002; Jiang et al., 2003; Long et al., 2005). A higher eukaryotic system that may be used is insect cells, with baculovirus as the vector for protein expression. Insect cells are even better equipped with the machinery needed for proper protein folding and for post-translational modifications. While they provide high expression levels and can be grown to high density, a disadvantage is that the growth media is more expensive than for bacteria or yeast.

If a system for high-yield expression and purification of a protein can be achieved, the protein can then be used in a multitude of biochemical and structural experiments. The highest resolution is crystal structure; however, this is a difficult and time consuming task. The difficulty of this task is evident in the small fraction of membrane proteins that have been crystallized, compared to soluble proteins. An alternative that has been used successfully is solution nuclear magnetic resonance (NMR) structure. Besides the obvious advantage of not needing crystals, NMR may yield structure that is closer to native form, since formation of crystals may impose non-native constraints on the protein.

4. Animal models of inherited human arrhythmias

Ideally, it is desirable to create an animal model of a disease—acquired or hereditary—in order to study pathophysiological mechanisms, and to design and test therapeutic options. To accomplish this it is important that the model recapitulate the human condition as closely as possible. For hereditary diseases it is necessary that the animal be genetically manipulable and that homologues of the genes of interest exist and be expressed in the same tissues as humans. Here we will discuss animal models that have been proposed and used for LQTS.

4.1 Rodent

Mice and rats are valuable systems for modeling a variety of human diseases, especially in terms of organ system pathophysiology and immune diseases. Since they can be easily genetically manipulated, they are good surrogates and provide clues to the hierarchy of genetic pathways and regulation that occur in the healthy and disease states.

Once the genetic loci were identified, investigators created knock-out (null) and knock-in mice to model the LQTS phenotype. The knock-out is done by creating an exogenous construct based on the sequence of the mouse gene, but where the relevant allele has been inactivated or nullified by inserting a stop codon or deletion/insertion to inhibit expression of the native protein. This construct is then injected into mouse embryos where homologous recombination occurs and the endogenous mouse gene is replaced by the null construct, which contains a marker so that recombinant mice may be distinguished. The recombinant mouse must then be bred to create heterozygous and homozygous null mice in subsequent generations. A knock-in mouse is created by a similar method, where the construct is a human gene (or mouse ortholog) that contains a known functional mutation. The engineered heterozygous mice will express one copy of endogenous mouse gene and one of the transgenic mutated gene.

There are at least 40 mouse models of LQTS genes. Two mouse models were created that disrupted exons 1 and 2 in KCNQ1 (Lee et al., 2000; Casimiro et al., 2001). Interestingly, the mouse with mutation in exon 1 did not show any ECG abnormalities. However, this mouse did have auditory-vestibular aspects of the Jervell and Lange-Nielsen syndrome. The mouse with KCNQ1 exon 2 disruption showed abnormal T-wave morphology on in vivo ECGs and inner ear abnormalities. In a third study, mice were created that expressed a dominant negative isoform of KCNQ1; these mice had QT prolongation on ECG as well as *torsade de pointes* arrhythmias (Demolombe et al., 2001). For KCNE1 null mice, the models exhibit deafness, but no baseline QT prolongation (Schulze-Bahr et al., 1997). One KCNE1 null mouse showed abnormal rate adaptation, which is similar to the phenotype seen in humans with KCNQ1/KCNE1 mutations upon exercise challenge (Charpentier et al., 1998; Warth & Barhanin, 2002). Mice express the HERG ortholog Merg1 in the heart. The Merg1 homozygous knock-out mouse is embryonic lethal as it dies early in development (London, 1998). A mouse model that expresses the dominant-negative HERG-G628S mutation showed a normal ECG phenotype (Babij et al., 1998).

While these studies yielded valuable information about pathogenesis of LQTS, they also highlighted how mice have limited value in studying inherited cardiac arrhythmias resulting from mutations in delayed rectifier K+ current channels. Mice have a baseline heart rate of ~600 beats per minute. As such, they have a short action potential and repolarization phase that is largely dependent on the transient outward K+ current (I_{to}) and have little to no I_{Ks} or I_{Kr} (Nerbonne, 2004; Milan & MacRae, 2005). So while they are genetically tractable, they may not be electrophysiologically similar enough to humans to provide a good model system. In contrast to the limitations of modeling human repolarization in the mouse, more success has been achieved for the depolarizing currents, which are more akin to those in the human. A LQTS mouse model generated by knock-in of an LQT3 mutation (KPQ deletion in SCN5A) (Nuyens et al., 2001). The transgenic mice had prolonged APD and polymorphic ventricular tachycardia.

Early studies of guinea pig ventricular myocytes revealed that two components making up the repolarization current I_{Kr} and I_{Ks} (Sanguinetti, 1990). This work was the original characterization of two repolarizing K+ currents and forms the basis for many of the

subsequent studies. Considering that isolated guinea pig ventricular myocytes was the in vitro system that launched a whole field of study, some groups have used an interesting approach by injecting adenoviral vectors containing wild-type or mutant KCNE1 or HERG into guinea pig myocardium (Hoppe et al., 2001). This group found that myocytes expressing the HERG G628S mutant, I_{Kr} was reduced, but action potential duration was not shortened however, beat-to-beat variability increased as did EADs. They also expressed the KCNE1-D76N mutant which suppressed I_{Ks}, significantly slowing repolarization, leading to frequent EADs and QT prolongation on ECG.

4.2 Rabbit

Given the limitations of rodent models, larger animals with cardiac electrophysiology more similar to humans might be considered. These included study of dogs, ferrets and rabbits. The Koren group has developed transgenic rabbits expressing human LQT mutations (Brunner et al., 2008). To create the transgenic rabbits the investigators injected embryos with a cDNA construct that contained either mutant HERG or KCNQ1 under a cardiac specific promoter, so that the transgene will only be active in the heart. These animals have enabled the investigators to gain significant insights by ECG analysis in awake freely moving animals, optical mapping of repolarization waves using voltage-sensitive dyes, and at the cellular level by recording from isolated rabbit myocytes. To date, this may be the most accurate model system that exists for hereditary LQTS.

4.3 Zebrafish

The newest model system to be explored is the zebrafish, *Danio rerio*. These are genetically tractable animals that expresses an endogenous ortholog of HERG (zERG) (Langheinrich et al., 2003). zERG is expressed specifically in both heart chambers of zebrafish embryos, is similarly composed of six transmembrane domains, and displays a particularly high degree of amino acid conservation in the S6 helix and pore domain. One specific mutant that was characterized named *breakdance* displayed prolonged ventricular APD, spontaneous EADs, and 2:1 atrio-ventricular block in the embryonic stages of development. The group of Scholz et al. expressed cloned zERG in *Xenopus* oocytes and showed current characteristics similar to the human channel however the details of its kinetics and gating were distinctly different (Scholz et al., 2009). Arnaout et al. recently performed a forward genetic screen and identified two zebrafish HERG mutants s213 and s290. They showed that homozygous animals had virtually no ventricular contraction and impaired calcium handling in the ventricles. Heterozygous animals showed increased APD and prolonged QT-interval on ECG (Arnaout et al., 2007). These studies show that given the conserved channel function, zebrafish does represent a valuable genetic model system to investigate HERG channel mutations.

4.4 Primary isolated myocytes

To find a more native system to study ion channels, researchers have sought methods to isolate and maintain primary cardiac myocytes. Primary isolated myocytes are best suited for short-term culture (approximately four days) and electrophysiological or immunofluorescence experiments that require only a low yield of viable cells (10s compared to 10,000s needed for biochemistry experiments) (Nuss & Marban, 1994). Some of the technical challenges involved include obtaining fresh healthy heart samples,

appropriate and not over-digestion of the tissue by enzymes, purification of myocytes from fibroblasts and matrix, calcium tolerance of the freshly isolated myocytes, and finding the correct conditions for culture. Most adult myocytes have been isolated from mouse, rat, guinea pig, and rabbit since the animals are readily available and economical. Fresh human heart samples for cardiomyocyte isolation are difficult to obtain routinely for ethical reasons. Rat neonatal cardiomyocytes have provided a fairly easy-to-obtain and widely applicable system in recent years (Chlopcikova et al., 2001). Since the rat neonatal cardiomyocytes may only transiently express the relevant channel, another approach is to use adenoviral or lentiviral vectors containing the cDNA of interest to infect the cells and allow adequate expression for study in a more native system. Comparing the behavior of wild type HERG and KCNQ1 channels with previously characterized deleterious mutants in rat neonatal myocytes has confirmed initial phenotypic characterization (Li et al., 2001; Lin et al., 2010). These groups found that the wild-type and mutant channel behaved generally the same as in cultured cells with some slight differences. Additionally some groups used the neonatal cardiomyocyte system to understand localization and interaction of the HERG, KCNQ1 and β accessory subunits (Rasmussen et al., 2004; Wu et al., 2006).

5. Human phenotypic studies

5.1 Locus-specific triggers

While QT interval prolongation puts patients at risk for abnormal heart rhythms, most patients are asymptomatic on a daily basis, with arrhythmias triggered by certain conditions or stimuli. In a 2001 study of 670 patients with known symptomatic LQT1, LQT2, or LQT3, a correlation between genotype and one of three specific triggers: exercise, emotion, or sleep was found. LQT1 patients had most events (syncope, cardiac arrest, or sudden death) triggered by exercise (62% of cases), while LQT2 patients had most events triggered by emotion (43% of cases), and LQT3 patients had most events during sleep (39% of cases) (Schwartz et al., 2001). In another study exercise induced significant further prolongation of QTc in LQT1 patients compared to LQT2 (Takenaka et al., 2003). In mice with an LQT3 knock-in mutation, bradycardia induced by cholinergic stimulation provoked *torsade de pointes*, while physical stress, mental stress, isoproterenol, and atropine did not (Fabritz et al., 2010). In female LQT2 patients, the post-partum period is a time of increased risk for arrhythmia (Khositseth et al., 2004). These efforts to categorize locus-specific triggers help clinicians in initial diagnostic phases and to better advise patients diagnosed with a specific LQTS genotype. There are some overlaps in triggers; for example, a certain percentage of LQT2 patients have cardiac events triggered by exercise. One study found a correlation of mutation location within HERG and the type of trigger causing symptoms: pore-loop mutations correlated with arousal-triggered events, non-pore mutations more often associated with exercise-triggered events (Kim et al., 2010).

5.2 Therapeutic approaches

Currently, there are five main avenues for treatment for adult patients with LQTS: (1) β-blockers, (2) gene-specific therapy, (3) pacemakers, (4) left cervico-thoracic sympathetic ganglionectomy, and (5) implanted cardio-verter defibrillators (ICDs). The primary goal of these therapies is to prevent life-threatening ventricular tachyarrhythmias and sudden cardiac death.

Given the correlation of LQTS locus and specific arrhythmia triggers, an important part of LQTS management is avoidance of triggers. LQT1 patients are advised to avoid competitive and endurance athletics, especially swimming. LQT2 patients are advised to reduce exposure to startle-stimuli, such as loud alarm clocks. LQT3 patients may have a pacemaker implanted to prevent bradycardia during sleep. For all patients with a LQTS diagnosis, the first line treatments demand avoidance of all potentially QT-prolonging drugs and the correction of electrolyte imbalances or other precipitating metabolic conditions.

Pharmacological treatment may used in combination with trigger avoidance. β-blocker therapy is widely used for treatment of LQT1 and LQT2, having been associated with significant risk-reduction in adult and pediatric cases and is considered a treatment with very low risk of adverse effects (Goldenberg et al., 2010). Mortality of patients on β-blockers is around 0.5% (Schwartz, Priori et al., 2001; Priori et al., 2004). Channel blockers or openers may also be used, though they can be pro-arrhythmic if not properly monitored. A study examining the effects of the K^+ channel opener nicorandil on canine models of LQT1, 2 and 3 showed that the drug may be effective in shortening the QT interval and preventing *torsade de pointes* in LQT1 and LQT2, but not LQT3 (Shimizu & Antzelevitch, 2000). For LQT3 patients where cardiac events are more likely to happen at low heart rates, β-blocker therapy is generally less helpful. LQT3 who have mutations in SCN5A where the defect is a persistent late current, channel blockers such as mexilitene, or flecainide are potentially helpful (Rosero et al., 1997).

Novel approaches include potassium supplementation for LQT2 patients. In vitro experiments have showed that proper intracellular K^+ concentration is a requirement for normal HERG channel trafficking to the membrane, and that extracellular potassium modulates HERG current (Guo et al., 2009; Wang et al., 2009). These findings correlate with earlier studies that focused on HERG current density, showing that I_{Kr} current paradoxically increased when extracellular K^+ concentration was increased (Sanguinetti & Jurkiewicz, 1992). One group administered spironolactone to eight LQT2 patients for four weeks, and observed a decrease in QT interval, (Etheridge et al., 2003) while another treated seven subjects with potassium supplementation and had similar findings (Compton et al., 1996). Such approaches may be considered in LQT2 patients.

Invasive therapies include left cardiac sympathetic denervation (LCSD), stellate ganglionectomy, and implantable cardioverter defibrillators (ICDs). LCSD involves the removal or ablation of the first four thoracic ganglia (which includes the stellate ganglion). In a 2004 study that included 174 high-risk, symptomatic LQTS patients who underwent LCSD, post-surgical QT intervals were shortened, and there was a 91% reduction in cardiac events over eight years of follow-up (Schwartz et al., 2004). These types of surgical interventions decrease sympathetic stimulation to the heart and may be recommended for patients who have not experienced cardiac arrest, but still experience syncope while on β-blocker therapy. ICD placement in such patients may be problematic because they may receive an intolerably high number of shocks. ICDs are most appropriate for patients who have already had an episode of cardiac arrest and are at higher risk for recurrence.

5.3 Male / female differences

To date, all LQTS loci are autosomal and not sex-linked. There are however, interesting differences between male and female LQTS patients. The QTc for women during the reproductive years (age 16-45) is longer than that for men of the same age (Bazett, 1920).

Women also have a higher resting heart rate than men (Ashman, 1942; Jose & Collison, 1970). The QTc intervals for males and females under age 16 are comparable as are those of post-menopausal women and men of the same age (Locati et al., 1998). There is also an increased risk for women of reproductive age with LQT1 and LQT2 mutations to have arrhythmic events (Zareba et al., 1995); (Lehmann et al., 1997). These findings implicate differential affects of the sex hormone pathways on cardiac electrophysiology.

Interestingly, there is an increased risk of having a cardiac event for female LQT1 and LQT2 patients in the immediate post-partum period (Seth et al., 2007). Another recent report described a patient with KCNE1 mutation who experienced aborted sudden cardiac death in the post-partum period (Nakajima et al., 2010). The current recommendation is to continue β-blocker therapy throughout the pregnancy and post-partum period to avoid cardiac events. While LQT1 and LQT2 mutations seem to adversely affect women more, the LQT3 (and Brugada syndrome mutations) event rate is greater in men (Priori et al., 2003). Among LQT3 genotyped individuals, men have a longer QTc than women. Another important condition where there are significant male/female differences is in acquired LQTS that may occur with drugs that block K^+ channels, mainly HERG. Multiple studies found that women are more likely to have adverse events when taking a QT-prolonging medication (Woosley & Sale, 1993; Drici et al., 1996; Reinoehl et al., 1996). This should be a key consideration when prescribing medications to patients with LQTS and in the general population.

6. New model systems: Induced patient-specific stem cells (iPSCs)

A novel model that has been under recent investigation to better understand the pathophysiology of LQTS is induced patient-specific stem cells. This process consists of obtaining skin cells (dermal fibroblasts) from patients with known LQTS mutations as well as unaffected control subjects, culturing them, de-differentiating them into pluripotent stem cells, and re-differentiating them into cardiomyocytes in vitro. The dermal fibroblasts are infected with retroviruses or lentiviruses containing specific transcription factors that convert and reprogram the dermal cell to a pluripotent stem cell without affecting the other genomic DNA containing the LQTS mutation. The pluripotent stem cells are then given specific growth factors in a precise order and grown on feeder cells until they form embryoid bodies: aggregates of cells that can differentiate into cardiomyocytes of three distinct types: "nodal", "atrial" and "ventricular" (Zhang et al., 2009). The cells were also shown to have cardiomyocyte architecture including sarcomeric organization of actin, myosin and other components, albeit immature.

In 2010, Moretti et al. characterized cells derived from a LQT1 patient who had the mutation R190Q in KCNQ1. They showed that these cells exhibited a prolonged APD due to reduced I_{Ks} current density (Moretti et al., 2010). Itzhaki et al. derived cardiomyocytes from a patient with an LQT2 mutation in HERG (A614V); these cells also showed a prolonged APD and reduction in I_{Kr} (Itzhaki et al., 2011). They used microelectrode arrays to record from groups of mutant cells and showed an increased incidence of EADs. To study mutations in the calcium channel, the group of Yazawa and colleagues were able to derive iPSCs from patients with LQT8 (Timothy syndrome) (Yazawa et al., 2011). They found that the mutation-carrying cells contracted slowly and irregularly, had exaggerated calcium influx with prolonged APD in ventricular type cells. These studies were able to confirm previous findings of channel dysfunction in a more native setting.

Two notable caveats with the iPSC approach are that the differentiated cells are immature and may not express the full complement of ion channels and accessory or regulatory proteins and cellular architecture as does an adult cardiomyocyte and that the differentiated cells may be heterogeneous. A challenge is to develop a selection method or purification scheme to isolate the induced cardiomyocytes in larger and more uniform quantities.

The iPSC system holds particular promise in determining the effect of potentially deleterious mutations in proteins other than ion channels such as regulatory or scaffolding proteins. This system may also be of particular utility in analysis of mutations non-coding areas (introns promoters, splice-sites and untranslated mRNA sequences). For therapeutics, iPSCs may provide a platform to test new potential pharmacologic approaches in a more native and genotype-specific setting prior to testing in animals and humans.

7. Mechanisms of deleterious mutations

LQTS mutations cause alterations in cardiac ionic currents that result in delayed action potential repolarization. The delay can be caused by sustained inward sodium or calcium currents, or impaired outward K^+ current. Mutations to ion channels or their regulatory proteins alter channel function such that an increase or decrease in current occurs; the mechanism by which the mutation causes these functional changes can be categorized into several classes: (1) changes in biophysical properties, (2) changes in channel synthesis and processing, and (3) changes channel regulation.

7.1 Biophysical

Biophysical defects are caused by mutation to channel subunits that result in channel gain or loss of function. Several biophysical parameters affect how much current a channel carries: the structure of the channel pore, channel gating, and the stability of the channel in the open versus closed states.

To discuss the effects on channel structure, we will focus on voltage-gated K^+ channels as an example (see Figure 1). The pore of a K^+ channel subunit is composed of two transmembrane helices (S5 and S6) and an intervening loop; when tetramerized, the loops form the K^+ selectivity filter that extends into the ion conduction pathway, while the helices line the remainder of the pore (Doyle, Morais Cabral et al., 1998; Jiang, Lee et al., 2002; Jiang, Lee et al., 2003; Long, Campbell et al., 2005). The structure of the selectivity filter is rigid as ion selectivity is based on size; it holds the same conformation regardless of whether the channel is open or closed. The pore-lining transmembrane helices though, move in response to changes in membrane voltage; when the channel is closed, the intracellular end of the helices prevent ions from accessing the selectivity filter, and when the channel is open, the helices are positioned such that ions can enter the pore. Deleterious mutations have been identified in the pore region. They presumably alter the structure of this sensitive region such that ions cannot access the selectivity filter, or cannot pass through the selectivity filter. A second region that may be affected is the voltage sensor. The S4 transmembrane domain of a K^+ channel is lined with positively charged amino acids. A change in the membrane potential causes movement in the voltage-sensor, and subsequently the pore region to which it is linked. Mutation to the voltage-sensing domain can result in delayed or impaired channel opening. Analysis of several LQT1 mutations in the S4 domain revealed a depolarizing shift in voltage-dependent activation of the channel, which indicates that a larger driving force was required to open the mutant channels (Henrion et al., 2009). Though

S4 contains the voltage-sensing residues, transmembrane domains S1 to S4 are structurally clustered together as the voltage-sensing domain; thus, mutations to residues in S1, S2, and S3 have also been associated with LQTS.

Unlike in K$^+$ channels, where loss-of-function mutations are the pathophysiologial defect, in Na$^+$ channels, gain-of-function mutations lead to an increased Na$^+$ current that maintain the cell in a depolarized state. Na$^+$ channels are responsible for the rapid influx of Na$^+$ ions in phase 0 of the action potential; this phase is extremely short-lived (milliseconds) as Na$^+$ channels normally rapidly inactivate. Mutations that alter Na$^+$ channel inactivation (rather than activation or deactivation) account for the majority of LQT3 . A defect in inactivation leads to a persistent Na$^+$ current throughout the action potential duration, which delays repolarization. Several cytoplasmic regions of the Na$^+$ channel are responsible for inactivation, and mutations in these regions lead to persistent current (Jones & Ruben, 2008). Biophysical mutations can act in a dominant-negative manner in patients with one wild-type allele and one mutant allele. Because K$^+$ channels are composed of four separate, identical channel subunits, wild-type and mutant subunits randomly combine together. Mutations that act in a dominant-negative manner may affect the function of channels that contain even one mutant subunit; less severe mutations may result in heteromeric channels with normal function or a partial defect. Sodium and calcium channels, however, are encoded such that the entire pore-forming channel is translated into a single polypeptide. Therefore, a patient who inherits a single mutant allele will have roughly 50% normal and 50% mutant channels. The mechanisms for dominant phenotype in these cases resides in the fact that LQTS mutations in sodium and calcium channels are "gain-of-function" which cannot be overcome by expression of the normal allele.
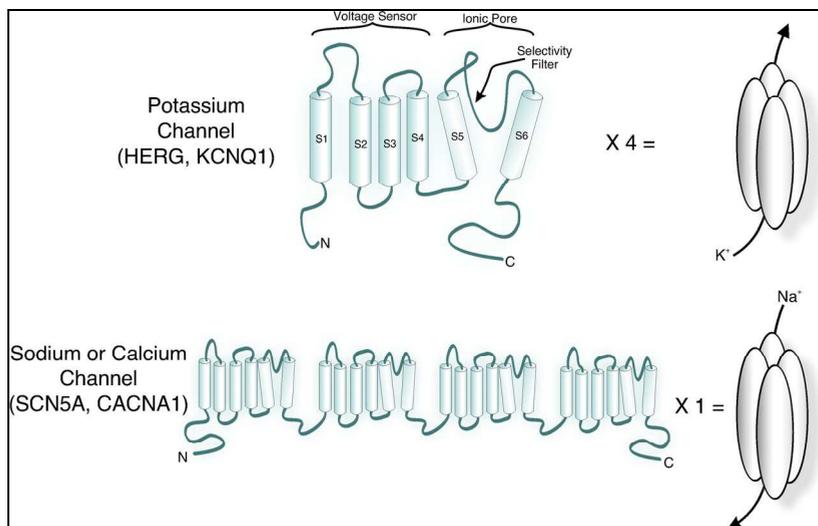

Fig. 1. Schematic representation of K$^+$ (top) and sodium or calcium (bottom) channel proteins. Note that four identical subunits combine to form a single unit for K$^+$ channels. Sodium and calcium channels are encoded as a singe polypeptide that is compised of four domains that are homologous the single subunit of a K$^+$ channel. S1 through S6 signify the transmembrane helices

7.2 Cell biological processes
7.2.1 Errors in synthesis

The first steps in channel synthesis include transcription of RNA in the nucleus and post-transcriptional modifications (capping, addition of a poly-A tail, splicing, and editing). The mature mRNA is then targeted to ribosomes on the endoplasmic reticulum to begin protein translation. Several classes of mutations can change mRNA stability and negatively affect abundance of functional protein. These include frame-shift and premature termination codons. These types of mutations may cause mRNA instability and subsequent degradation, a process called nonsense-mediated decay (NMD). NMD has been shown to be an underlying mechanism in other diseases. This phenomenon has been implicated as a cause recurrent intrauterine fetal death or LQTS in mutations of HERG (Bhuiyan et al., 2008) (Zarraga et al., 2011). Similarly NMD has been implicated to play a role in LQT3 mutation of the Na^+ channel SCN5A (Teng et al., 2009). It is certainly conceivable that mutations, yet to be described, may also introduce new binding motifs for micro-RNAs that would alter stability and the mRNA and hence, protein synthesis.

7.2.2 Errors in trafficking

In general, defective protein trafficking is emerging as an important disease mechanism that concerns a variety of cell types. A newly synthesized channel goes through numerous processing steps before it ultimately reaches the membrane and is functional. At the earliest stage, some signaling systems may affect channel synthesis itself (Chen et al., 2009; Chen et al., 2010; Sroubek & McDonald, 2011). After the channel is synthesized at the endoplasmic reticulum (ER) it must fold to attain its tertiary structure and then assemble with other subunits to form the functional macromolecular complex. Folding is a complex process involving helper proteins called chaperones, which work in iterations to achieve the final proper conformation. Once the protein is properly folded it may be glycosylated and it leaves the ER through vesicle transport to arrive at the Golgi. At the Golgi the glycoslyations may be further modified and finally the channel leaves the Golgi in vesicles bound for the plasma membrane.

Mutations may cause channel proteins to fold improperly; these mis-folded proteins may be recognized by the quality-control system and marked for degradation by the proteasome. This causes a trafficking error, and mis-folded protein may accumulate in the ER or Golgi membrane. Though severe mis-folding results in a non-functional channel (for example, mutations that prevent tetramerization of channel subunits), milder mutations may allow for a functional channel to fold, yet still be retained intracellularly. This is in theory possible, but under most circumstances it is difficult to test because functional experiments such as electrophysiology require proper trafficking. Mutations in HERG and KCNQ1 that affect trafficking can be loss-of-function and many of them can act in a dominant-negative fashion interfering with associated normal allele subunits. While tetramerization has been studied for these channels, the mechanisms are incompletely understood. Given a situation where wild-type and mutant subunits are co-expressed, the heterogeneous pool of tetrameric channels may express a range of current density from zero to the full wild-type amount. This could explain why some trafficking mutations have a more severe phenotype than others.

It is worth considering LQTS mutation-associated trafficking errors in HERG. A trafficking defect is the most common cellular phenotype for LQT2 mutants (Anderson et al., 2006). Particular attention has been paid to the HERG cytoplasmic C-terminal portion where

analysis of various LQT2 mutations has revealed that this segment is critical for tetrameric assembly and proper trafficking. While many of these studies have focused on the C-terminus of HERG, it is important to note that trafficking defective mutants have also been found throughout the N-terminus as well as the transmembrane domains (Balijepalli et al., 2010). Complex mechanisms for the forward trafficking (from ER to Golgi) of HERG have been suggested. Recently, Delisle et al. showed that HEK cell expressed HERG undergoes COPII-dependent ER export and also endosomal trafficking which determine its plasma membrane expression (Delisle et al., 2009). They also showed that this atypical trafficking route is mediated by small GTPases such as Sar1 and Rab11b. More recent trafficking studies show that LQT2 mutants may be subjected to quality-control in the ER-Golgi intermediate compartment (ERGIC) (Smith et al., 2011). It has also been shown that trafficking defective LQT2 mutants are subsequently degraded by the ER-associated degradation pathway (ERAD) and the ubiquitin proteasome pathway (Kagan et al., 2000; Gong et al., 2005). While this picture is incomplete (studies rely on heterologous expression), it does give us insight into the points during synthesis where HERG is particularly susceptible and how mutations affect its maturation.

Recent studies have examined the role of extracellular potassium in the endocytosis and degradation of HERG. Recently, the work of Guo and colleagues has provided a biochemical basis and mechanistic approach to study the behavior of HERG in low-potassium conditions. The 155 kDa form of HERG undergoes endocytic internalization from the plasma membrane and proteasomal degradation through a mechanism involving caveolin (Massaeli et al., 2010). Further work was done by Massaeli and colleagues who studied the behavior of pore-lining mutations in HERG under zero-potassium conditions. They found that alanine mutants at certain positions in the pore helix and selectivity filters abolished the low-potassium induced degradation. This is an interesting mechanism since arrhythmias are often precipitated by electrolyte disturbances such as hypokalemia (Berthet et al., 1999).

7.3 Regulation

In addition to intrinsic channel defects, there are many regulatory proteins that interact with channels to modulate their activity. Since LQT1 and LQT2 patients often have arrhythmias precipitated by physical or emotional stress, it is important to consider the human stress response affect these channels. The α- and β-adrenergic systems are activated during stress. The β-adrenergic system involves the β-adrenergic receptor, a hetero-trimeric G-protein, and cyclic adenosine monophosphate (cAMP), a second messenger that ultimately activates protein kinase A (PKA). HERG current is acutely reduced by PKA signaling due to direct phosphorylation of the channel. Furthermore, cAMP can interact with the HERG channel directly in a manner that partially abrogates the suppressive effects of phosphorylation. An added complexity to this signaling pathway is the interaction between 14-3-3, a scaffolding protein, and HERG (Kagan et al., 2002; Kagan & McDonald, 2005)). 14-3-3 dynamically binds proteins (including HERG) upon phosphorylation, primarily by PKA. When this occurs with HERG, channel activation is accelerated and current augmented. An LQT2 mutation has been described in which the deleterious effect is disruption of 14-3-3 binding (Choe et al., 2006). An A-kinase anchoring protein (AKAP) is likely involved in targeting PKA to HERG in a macromolecular complex, which may intensify current modulation (Li et al., 2008). The Kass group showed S27 in the KCNQ1 N-terminus is phosphorylated by PKA and this causes an increase in current. They also showed that a AKAP Yotiao targets PKA to the channel complex (Marx et al., 2002). These studies demonstrate an important, specific,

and tightly controlled form of regulation by the components of the β-adrenergic pathway in relation to the two K$^+$ channels.

In contrast to the β-adrenergic system, the α-adrenergic system involves phospholipase C, which hydrolyzes the membrane lipid phosphatidyl inositol-4,5-bisphosphate (PIP$_2$) into the signaling molecules inositol 1,4,5-trisphosphate (IP$_3$) and the second messenger diacylglycerol (DAG). DAG and calcium go on to activate protein kinase C (PKC) isoforms. An acute decrease in the PIP$_2$ concentration, which occurs upon α-adrenergic stimulation, reduces HERG currents (Bian et al., 2001)**.** This effect is dependent on consumption of PIP$_2$ at the membrane and direct binding of PIP$_2$ to HERG but occurs independently of calcium signaling or PKC activity (Bian et al., 2004; Bian & McDonald, 2007)). PKC regulation of HERG remains an active area of investigation where conclusive results await (Thomas, 2003) (Cockerill et al., 2007). For KCNQ1/KCNE1 and I$_{Ks}$, Varnum et al. showed that PKC stimulation decreased in I$_{Ks}$ due to KCNE1 phosphorylation at serine-102 (Varnum et al., 1993). The mechanism of this I$_{Ks}$ downregulation remained unclear until the Abbott group showed that PKC downregulates I$_{Ks}$ current through inducing endocytosis (Kanda et al., 2011). Another group studied the regulation of I$_{Ks}$ by PIP$_2$ and showed that application of PIP$_2$ delayed rundown of I$_{Ks}$ in excised patch recordings (Loussouarn et al., 2003).

7.4 Correlation of mutational mechanisms with clinical phenotype and the approach to genetic testing results

Different channel mutations cause a range of clinical phenotypes, from very mild (asymptomatic) to severe (sudden cardiac death at a young age). Though some generalizations can be made correlating the mechanism by which a mutation acts and severity of clinical phenotype, the task is made difficult by the extensive list of implicated residues and their broad distribution across each gene. As one may expect, mutations to channel pore loops are generally severe, since they directly impact on channel conductance. A study of 858 LQT2 patients in 2009 revealed that patients with mutation to the pore region of HERG (S5 – pore loop – S6) had significantly higher rates of cardiac events than patients with mutations in the S1 – S4 transmembrane domains or the N- or C-termini, with the difference increasing with increasing age. The study also explored possible differences between types of mutations and found that in the C-terminus, patients with non-missense mutations were at significantly higher risk than those with missense mutations (Shimizu et al., 2009).

It is still difficult, though, to predict what type of cellular defect a certain mutation may case. Mutations that affect trafficking are not clustered in any particular region, and mutations that cause biophysical defects can also affect trafficking. K$^+$ channel mutations are complicated by the ability to form wild-type/mutant heteromultimeric channels that exhibit different levels of defect depending on the number of mutant subunits. Functional analysis by in vitro expression of mutant channels is the only way to fully assess the cellular phenotype of a mutation. Additional genetic and environmental influences exist such that two patients with the same mutation may differ in clinical presentation. We do not yet know all the different factors that may affect the relative expression of mutant versus wild-type channels in a heterozygous patient, such that the distribution is not a 50/50 mix. One patient may express significantly differing amounts of normal or mutant allele subunits, and therefore have a variable clinical phenotype. For K$^+$ channels, there is an overlap between the I$_{Kr}$ and I$_{Ks}$ currents in their role during repolarization (known as "repolarization reserve"), so the clinical presentation of a patient may be mild unless the unaffected current is also compromised by environmental factors.

A computational prediction tool called KvSNP for voltage-gated K^+ channel genes to predict the severity of possible disease-causing mutations has been published (Stead et al., 2011). Two recent case studies illustrate the complexity of patient presentation and how prediction databases, although initially valuable, have limitations. Each case involved patients with QT prolongation noted on ECG, yet mild clinical history until presentation with sudden cardiac death in early adulthood. One patient had the LQT1 mutation KCNQ1-S277L, located in the S5 pore helix just proximal to the pore loop, predicted by KvSNP to be a severe mutation. The location suggests a biophysical defect, but thorough analysis revealed a combination of trafficking defect with a partially dominant-negative biophysical effect on heteromultimeric channels that managed to traffic properly to the membrane. The second patient had the LQT2 mutation HERG-G816V, located in the C-terminal region adjacent to a cyclic-nucleotide binding domain important for HERG regulation. This mutation was not predicted to be severe, yet functional analysis showed abnormal trafficking and significantly reduced current. Given the severe cellular defects, one would not predict a generally mild clinical phenotype. Both patients presented with sudden death when they experienced a second exogenous insult such as drug-induced blockade or electrolyte disturbance that reduced their remaining repolarizing current (Chen et al., 2011; Krishnan et al., 2011).
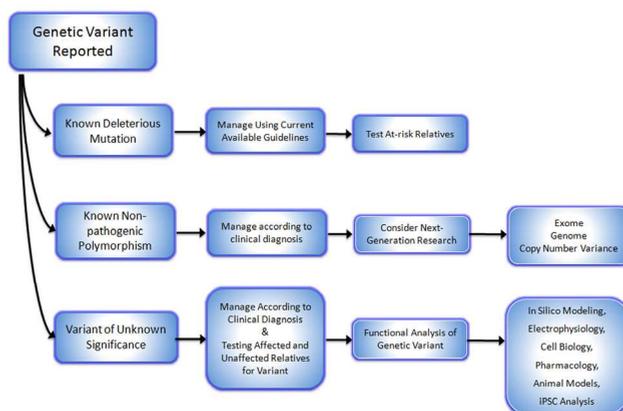

Fig. 2. A general approach to managing families with suspected hereditary arrhythmia syndromes after receiving genetic testing results.

These reports highlight the challenge that clinicians face upon receiving genetic testing results for patients suspected of having a hereditary cause of cardiac arrhythmia. The results from clinical laboratories may be given as clear-cut pathogenic deleterious mutations that have been reported in the literature. Such cases are relatively straightforward and further testing of at-risk family members is indicated with treatment and management dictated by the clinical presentation and recommendations for the documented mutation. Alternatively, the testing result may be read as a known non-pathogenic polymorphism that has been documented in normal populations. In this instance the clinician must guide therapy to the clinical diagnosis and consider whether the patient warrants further investigation such as analysis of copy number variance or various "omics" studies (whole exome or genome sequencing), which presently comprise investigative research studies. The third possibility is that genetic testing results are given as possible deleterious mutation or variants of

unknown significance. This is a difficult puzzle for the clinician to solve. An initial step might be to search mutational or polymorphism genetic databases for reports of the given variant, but the commercial laboratories usually perform this task. Another is to submit the reported variant to in silico analysis as described above (KvSNP), but remaining aware of the potential inaccuracies. More desirable is to perform one or more of the several functional analyses outlined above. Although this will entail collaboration with an academic laboratory, it will provide more solid evidence for, or against the variant being deleterious. Figure 2 illustrates a suggested algorithm for the approach of genetic testing results.

7.5 Exploring novel therapeutic modalities

One of the greatest challenges in LQTS is developing new therapeutic modalities aimed at the root cause of the defect instead of managing or preventing arrhythmias. One example is designing methods that correct the trafficking defective phenotype in many LQT2 cases. Work by January and colleagues have sought to use pharmacological methods to rescue trafficking deficient HERG mutants (Gong, 2006). In some LQT2 mutations trafficking can be partially rescued in heterologous systems by lower temperature, glycerol or DMSO, which act as non-specific chaperones. HERG channel blocking drugs E-4031, astemizole and cisapride have also been shown to rescue some mutant-related trafficking defects, but functionality was abolished since the channel pore was blocked. As is the case for many in-vitro studies, the results are hard to translate into clinical therapies at present. Similar therapeutic models are in development for cystic fibrosis and rescue of CFTR trafficking mutants, but the same difficulties prevail (Becq et al., 2011). The ideal goal is to achieve a trafficking rescue without pore blockage. Encouraging results have been reported by Rajamani et al. who showed that the antihistamine fexofenadine was able to rescue some trafficking-deficient HERG mutants without channel block (Rajamani et al., 2002).

Other efforts have utilized functional screens to discover small molecules that would suppress the long-QT phenotype irrespective of mechanism. An interesting approach has been reported using the *breakdance* (see section 4.3) mutant to screen for molecules that would rescue the phenotype (Peal et al., 2010). The investigators isolated 2 compounds that shortened the APD. The mechanisms by which these drugs work remain unclear as does the application of these drugs to mammals or later to humans. Nevertheless, this provides a good starting point and shows the utility of zebrafish as a genetic model in a high-throughput screen.

8. Conclusion

Modern medical genetics has advanced the diagnosis and treatment of hereditary arrhythmia syndromes greatly in the past 15 years. Future advances will include recognition of modifying genetic and environmental factors that influence penetrance and severity. There is also hope for novel gene- and mutation-specific therapies. An achievable goal in the sort-term will be clear delineation of genetic mutations and variants that presently reported to clinicians that patients and families with possible hereditary arrhythmias.

9. Acknowledgment

10. References

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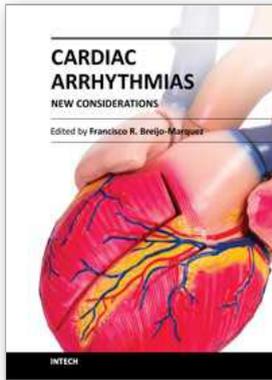
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