Genetic aspects of cardiac arrythmia syndromes

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Ithaque

Quand tu partiras pour Ithaque, souhaite que le chemin soit long, riche en péripéties et en expériences. Ne crains ni les Lestrygons, ni les Cyclopes, ni la colère de Neptune. Tu ne verras rien de pareil sur ta route si tes pensées restent hautes, si ton corps et ton âme ne se laissent effleurer que par des emotions sans bassese. Tu ne recontreras ne les Lestrygons, ni les Cyclopes, ni le farouche Neptune, si tu ne les portes pas en toi-même, si ton coeur ne les dresse pas devant toi.

Souhaite que le chemin soit long, que nombreux soient les matins d'été, où (avec quelles déelices!) tu pénétreras dans des ports vus pour la premiere fois. Fais escale à des comptoirs phéniciens, et acquiers de belles marchandises: nacre et corail, ambre et ébène, et mille sortes d'entêtants parfums. Acquiers le plus possible de ces entêtans parfums. Visite de nombreuses cités égyptiennes, et instruits-toi avidement auprès de leurs sages.

Garde sans cesse Ithaque présente à ton esprit. Ton but final est d'y parvenir, mais n'écourte pas ton voyage: mieux vaut qu'il dure de longues années et que tu abordes enfin dans to île aux jours de ta vieillesse, riche qu'Ithaque t'enrichisse.

Ithaque t'a donné le beau voyage: sans elle, tu ne te serais pas mis en route. Elle n'a plus rien d'autre à te donner.

Si tu la trouves pauvre, Ithaque ne t'a pas trompé. Sage comme tu l'es devenue à la suite de tant d'experiences, tu as enfin compris ce que signifient les Ithaques.

Constantin Cavafy

Voor mijn ouders

CONTENTS

Chapter **1**

General Introduction

Introduction

The human heart consists of four chambers which function to continuously pump blood throughout the body, undergoing roughly two thousand million cycles (2.3 x 10e^9) in a typical lifetime. This continuous cycling is necessary to supply the whole body and all of its organs with oxygen and nutrients. To achieve this, the heart must relax so that its chambers (the atria and ventricles) can fill with blood, and then contract to propel the blood throughout the body. This is broken down in a two-step process. The returning blood, collected from the body, ends up in the right atrium, passes into the right ventricle, and is pumped to the lungs in which red blood cells exchange carbon dioxide for oxygen. Subsequently, the oxygenated blood returning from the lungs enters the left atrium, passes into the left ventricle, and is then ejected into the body circulation. Valves prevent the backflow of blood from the aorta to the ventricle, atrium, and the veins. This continuous sequence of relaxation and contraction is fundamental to vertebrate life.

The cardiac action potential

A single heartbeat consists of a rhythmic contraction and relaxation of the whole heart muscle mass. Each heartbeat is initiated by a pulse of electrical excitation that begins in a group of specialized pacemaker cells, called the sinus node, and spreads throughout the heart. This electrical impulse is made possible by the electrochemical gradient that exists across the surface membrane of each heart muscle cell or cardiomyocyte (Table 1). The contraction of each cardiomyocyte, brought on by this pulse of electrical excitation, is associated with an action potential (AP) in that cell. A prototypical action potential from a ventricular myocyte is shown in figure 1. The shape is distinctive and results from a delicate balance of depolarizing and repolarizing ionic currents. The membrane potential or voltage difference across the cell membrane of cardiomyocytes is due to difference in ionic composition between intra and extra-cellular fluids and the relative changes in membrane permeability to certain ions. Cardiac APs begin with a sharp depolarizing upstroke that results from a large and rapid increase in sodium conductance. Repolarization of the cell membrane is delayed while the membrane remains in a sustained, slowly decaying plateau phase for hundreds of milliseconds. This long duration of the cardiac AP produces a prolonged contraction, so that an entire chamber can fully contract before any portion begins to relax, a process that is essential for efficient pumping of blood. The prolonged plateau results from a maintained high calcium conductance and

TABLE 1. Intra- and extracellular concentrations of ions

Ions	Intracellular concentration (mM)	Extracellular concentration (mM)
Na^+	10	140
K^+	140	4
Ca^{2+}	10^{-4}	2
Cl^-	30	140

a delay in the subsequent increase in potassium conductance. Subsequently, a repolarization phase terminates the plateau caused by a fall in calcium conductance and an increase in potassium conductance. The high Ca^{2+} conductance during the plateau phase allows Ca^{2+} to flow into the cardiomyocyte as required for muscle contraction. This process, when it happens simultaneously, causes the atria and ventricles to contract and expel blood in a rhythmic fashion. The wave of depolarization is self-limiting; as a negative membrane potential is restored, the heart relaxes and fills with blood for the next cycle.

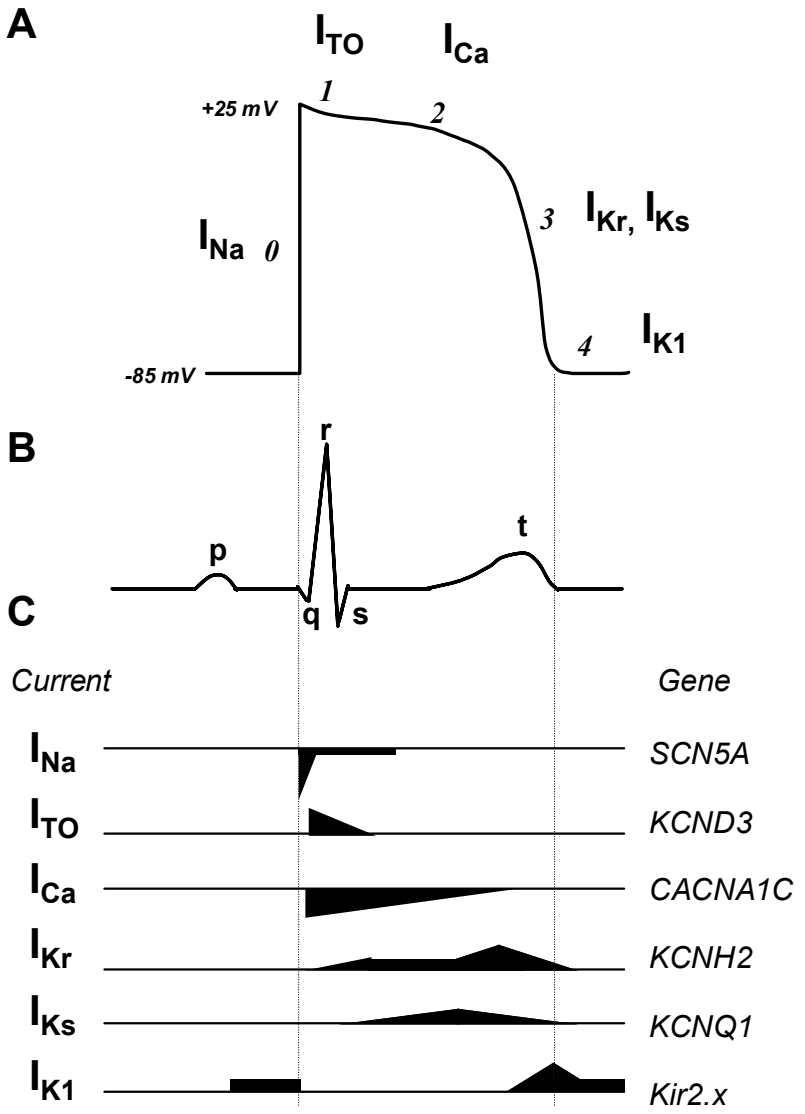

Figure 1. A. The ventricular action potential, divided into its 5 distinct phases. Main currents for each phase are depicted. B. Surface ECG aligned on the ventricular action potential, labelled for each peak. C. Diagram of the timing, the direction, and the intensity of the various currents aligned to the ventricular action potential, and their respective coding genes.

Ion channels

To facilitate this rapid cycling of currents across the cardiomyocyte membrane, specialized molecules are needed that can rapidly transport the ions underlying the various currents. Like all cells, the membrane of cardiac myocytes consists of a lipid bilayer structure that is highly impermeable to charged molecules or ions such as sodium, Na^+, calcium, Ca^{2+}, chloride, Cl^-, and potassium, K^+. However, at certain sites protein complexes span the cell membrane forming narrow water-filled pores, thereby providing a transmembrane corridor for ions to move in and out of the cell (figure 2). These protein complexes have evolved to mediate the selective flux of ions across biological membranes; those that contain a high-throughput pore (more than 100,000 ions per second per molecule) are known as ion channels[1,2]. Ion channels are generally selective for

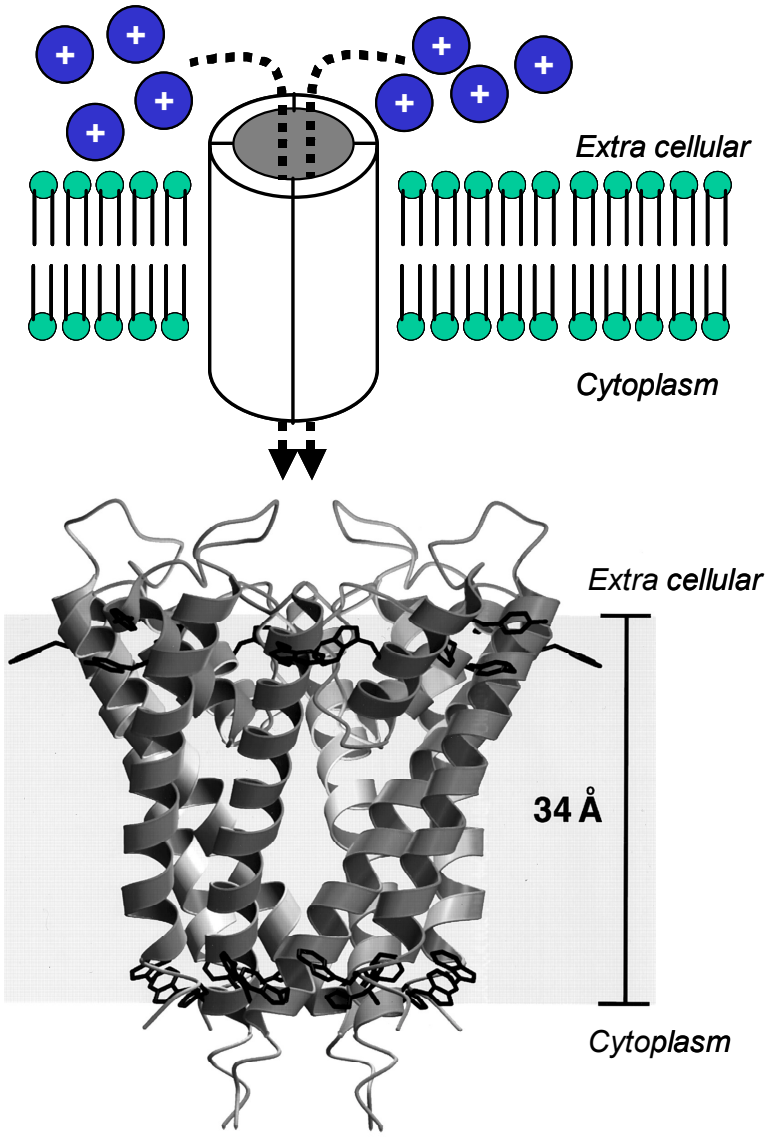

Figure 2. A. Schematic representation of an ion channel forming a pore in the membrane. The concentration of ions is higher outside the cell than inside. The ion channel enables rapid transport of ions down its electro-chemical path. The ion channel consists of four identical domains, which fold around the central pore. B. 3D representation of a bacterial K^+ ion channel, adapted from Doyle, D et al. The Structure of the Potassium Channel: Molecular Basis of K+ Conduction and Selectivity Science 1998 280: 69-77

one type of ion; based on ion size and charge, as well as channel properties, one particular type of ion can flow through one single ion channel complex. Therefore, one can distinguish between Na^+, K^+, Ca^{2+} and Cl^- channels. The opening and closing of these ion channels ultimately determines the membrane permeability. Since ions do not need to bind to the ion channel, they can simply diffuse through the ion channel pore. Ions can thus be transported through these channels at a very fast rate. Furthermore, ion transport is passive and is mediated by both the concentration gradient of the relevant ion and the electrical potential difference across the membrane; the net driving force or electrochemical gradient for each separate ion determines its transport efficiency. Ion channels can be opened briefly and then closed again in response to various specific stimuli; so-called voltage-gated channels respond to changes in the electrical voltage across the membrane, mechanically-gated channels react to changes in mechanical stretch, and ligand-gated channels act on the binding of their specific ligand. Individiual ion channels are characterised by their ionic selectivity, conductance (ease of flow of current through a channel), gating properties (regulation of channel opening and closing), kinetics (rate at which channels open or close) and pharmacology.

Ion channels and the cardiac action potential

The interplay between ion channels, ion concentrations and the electrical potential difference across the cardiomyocyte membrane ultimately determines the shape of the cardiac action potential. The cardiac action potential is divided up into 5 distinct phases (as mentioned above) each specific to a particular set of ion channels (Figure 1). When the cardiomyocyte is stimulated, voltage-gated sodium Na^+ channels open and allow Na^+ ions to flow into the cell. Na^+ channels inactivate quickly and completely, and very rarely re-open[3]. The resultant inward current shifts the membrane potential to a less negative value, thereby opening more Na^+ channels, and so on. This upstroke of the action potential is called phase 0, which is followed by phase 1, a short period also known as the early repolarisation phase, during which K^+ flows outward through rapidly activating and inactivating K^+ channels and thereby contributes to the initial repolarisation of the action potential prior to the onset of the plateau phase. This phase produces a distinct 'notch' in the ventricular action potential[4]. Subsequently, in phase 2 or the so-called plateau phase of the action potential, calcium channels that inactivate less rapidly and less completely, feature prominently in maintaining plateau depolarisation and the succeeding Ca^{2+} influx is ultimately responsible for the contraction of the cardiomyocyte[5]. Next in phase 3, the membrane potential returns to its resting value by the repolarisation of K^+ ions through several voltage-gated K^+ channels, until the next depolarisation triggers a new action potential. The resting phase of the AP is called phase 4 and is maintained by K^+ channels

that keep the membrane potential close the K^+ equilibrium potential, where few Na^+ or Ca^{2+} channels will be open, and thus further away from the action potential threshold. In these channels, current is much larger in the inward than in the outward direction. Its distinctive permeation properties cause these K^+ channels to shut-off as soon as depolarization progresses[1]. Therefore they are ideally suited to anchor the 'resting' potential, and provide little opposition to depolarization once the balance has tilted in that direction. In this fashion they have a stabilising effect on cardiac electrical activity. Thus in summary, K^+ channels open during repolarization, and K^+ exits the cell. While, during depolarization Na^+ and Ca^{2+} enter the cell through their respective channels.

Molecular characterization of ion channels

Na^+ channels

Myocardial cells typically express more than 100.000 Na^+ channels. They mediate the fast depolarisation in both nerve and muscle, and in the heart they contribute solely to the initial depolarisation or phase 0, as described above. The Na^+ channel is made up of the alpha subunit forming the pore of the channel, and various beta-subunits that subtly modify the alpha-channel behaviour. The alpha-subunit has a modular architecture consisting of four internally homologous domains (named I-IV; see figure 3). Each of these domains contains six transmembrane segments and resembles a single alpha subunit of the voltage dependent K^+ channels (see below). The four domains fold together to create the central pore, whose structure ultimately determines the selectivity and conductance properties of the channel. Several regions are evolutionary highly conserved indicating that they are important to the Na^+ channel's function. These are the pore-lining regions and a special segment of the transmembrane structure, the 4th transmembrane segment, or S4, which contains multiple positively charged residues and is thought to be the voltage sensor of the protein. The auxiliary beta-subunits are modulators of the Na^+ channel function, but appear to play a minor role in cardiac Na^+ channel. Na^+ channels activate and inactivate quickly and, as stated above, play a primary role in the initiation of the cardiac AP. Electrophysiological measurements have shown that co-expression of the alpha and beta-subunits of the cardiac Na^+ channel results in a current closely resembling the native $I_{na,}$ however the exact stoichiometry resembling the *in vivo* situation is unknown. The gene encoding the alpha-subunit is called SCN5A, it is located on chromosome 3p21-24, and contains 28 exons, roughly spanning 80kb of the human genome.

Ca^{2+} channels

Cardiomyocytes contain at least four different types of Ca^{2+} channels, two of which (the L- and T-type) are expressed on the cell surface membrane, and two (RYR2 and IP-3) on the sarcoplasmic reticulum membrane. The most important one, which is active during

the plateau phase (phase 2) of the action potential, is the L-type Ca^{2+} channel. L-type Ca^{2+} channels consist of five subunits, the alpha1, alpha2, delta, beta and gamma subunit. Analogous to other ion channels, the alpha1 subunit is the actual pore forming protein, while the other subunits modulate function in various ways. The structure and sequence of the cardiac L-type alpha subunit gene, or CACNA1C, shows remarkable homology to the alpha subunit of the Na^{+} channel. It also has four homologous domains composed of six transmembrane segments each. As in the Na^{+} channel the S4 segment in each domain functions as a voltage sensor. Ca^{2+} channels are very selective; their permeability to Ca^{2+} is a thousand fold higher than to other ions. The gene encoding the cardiac specific CACNA1C is located on chromosome 12; it spans an estimated 150 kb of the human genome and is composed of 44 invariant and 6 alternative exons.

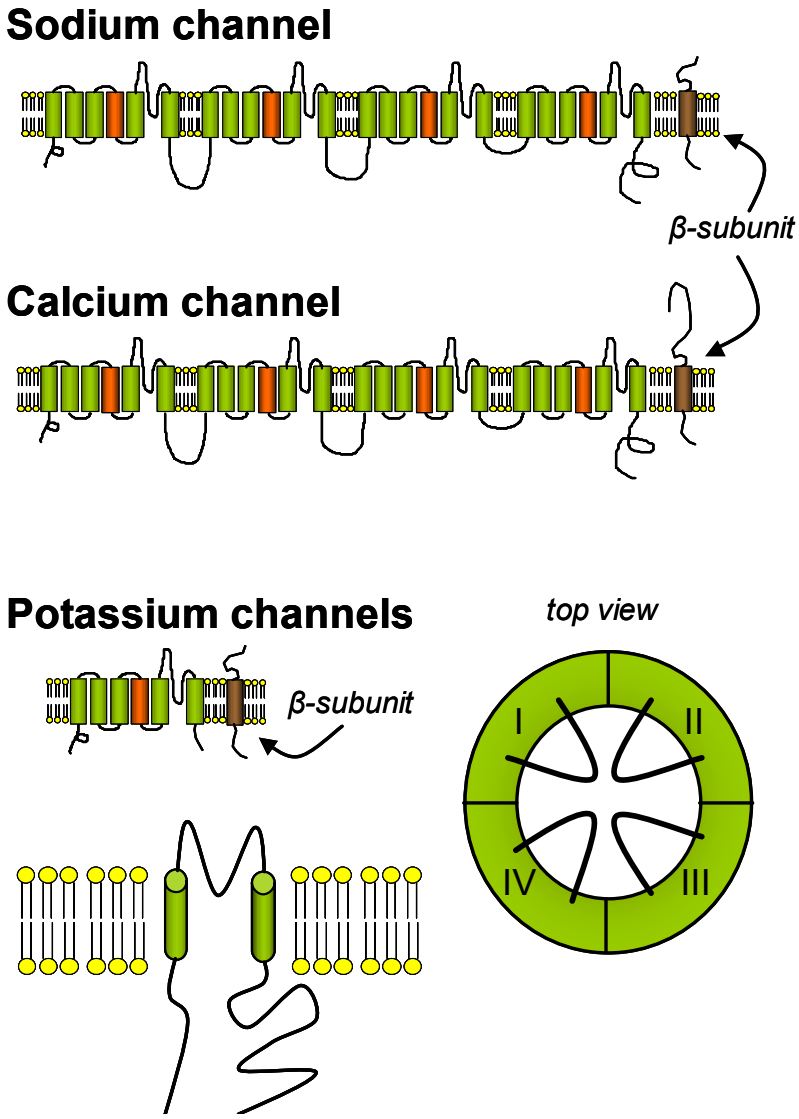

Figure 3. Schematic representation of sodium, calcium, and potassium ion channels. Sodium and calcium ion channels share a common structure, they have four domains of six transmembrane regions, whereby one protein forms one ion channel. Top of each diagram represents the extra-cellular environment, while the bottom represents the cytoplasm. Beta-subunits are shown on the right side of each channel. Potassium channels consists of four proteins forming one functional ion channel. Their structure is similar to that of sodium and calcium channels, the beta-subunit is shown to the right of the channel. Inward rectifiers have a two transmembrane structure (lower panel), which sets them apart from other ion channels.

K^+ channels

Myocardial cells express multiple types of potassium channels and their distribution and density varies throughout the heart. The various types of potassium channels can be divided into two main groups according to their function and electrophysiological effects. The first group consists of the depolarisation-activated or voltage-dependent potassium channels which function to control the amplitude and duration of the action potential. The second group of potassium channels comprises the inwardly rectifying channels, which are voltage-independent and contribute to the regulation of the membrane potential of the myocardial cell. The pore-forming alpha-subunit of voltage dependent K^+ channels contains six membrane-spanning segments, and the amino and carboxy end are located intracellularly. Assembly of four alpha subunits into a tetrameric structure is needed to create a single functional K^+ channel. In concurrence with the Na^+ and Ca^{2+} alpha subunits, the K^+ protein contains a voltage sensor located in the 4^{th} transmembrane segment (S4). In addition, K^+ beta-subunits exist which bind to highly conserved domains immediately preceding the S1 segment.

The current active during phase 1 of the action potential, is called the transient outward current or 'I_{TO}'. It actually consists of two components: I_{to1} which activates and inactivates rapidly, in a conventional voltage-dependent manner[4,6] and I_{TO2}, which is less well characterized and varies from species to species. Available evidence suggests that it is activated by changes in the intracellular concentration of free Ca^{2+} [7,8]. The *KCND/Kv4* family of channels underlies I_{TO1} [9], but the molecular identity of I_{TO2} is not yet known. In human the *KCND3/Kv4.3* gene is the main component underlying I_{TO1}. Initial reports indicated that it is mostly expressed in brain and heart and is not detectable in kidney, liver, lung, pancreas, spleen, or skeletal muscle. Further investigations revealed that *KCND3* undergoes alternative splicing, and that only the so-called long variant or KCND3L, is expressed in heart. However, there are no differences in the splice variants in terms of their voltage dependence or inactivation kinetics in the basal state[10,11]. The *KCND3* gene lies on chromosome 1p13.2 and consists of 7 exons.

The delayed rectifier K^+ current is activated during phases 2 and 3 of the action potential. It opposes the inward calcium current and therefore determines the duration of the plateau and thus of the action potential itself. In many species the delayed rectifier consists of two components, the rapid (I_{kr}) and the slow (I_{ks}) delayed rectifier. The gene encoding the rapid delayed rectifier is *HERG* or *KCNH2*, its genomic organization consists of 16 exons spanning approximately 19 kb on chromosome 7q35. Several isoforms have been identified in human heart, with different channel kinetics. The global structure is very similar to other K^+ channels including 6 transmembrane domains. In

analogy with other alpha-subunits, a specific beta subunit called KCNE2 binds to KCNH2/HERG protein and together they produce a current closely resembling the native I_{kr}.

At first it was unclear which gene encoded the slow rectifier current I_{ks}. Positional cloning of the KvLQT1 gene (KCNQ1), and subsequent expression experiments showed that the KvLQT1 protein could represent some aspects of the native I_{ks} current. The KvLQT1 gene contains 16 exons and spans over 400 kb of genomic DNA on chromosome 11p15.5. It also has the classical 6 transmembrane regions, and includes the signature S4 voltage sensor and the S5-pore-S6 sequences. However, the expressed KvLQT1 protein alone did not display a complete delayed rectifier phenotype resembling the I_{ks} current. The co-expression with a beta-subunit called KCNE1 (minK) showed the fully recapitulated native I_{ks} current, suggesting that *in vivo* KCNE1 and KvLQT1 form the functional I_{ks} channel.

The second group of potassium channels comprises the inwardly rectifying channels, which are voltage-independent and contribute to the regulation of the membrane potential of the myocardial cell, and are mostly active during the final repolarisation or phase 4 of the action potential. Genes underlying this I_{k1} current are members of the Kir2.x gene family, located on various parts of the human genome. All members of the Kir2.x family are found in a wide range of tissues, though the heart and the brain show the strongest expression. All of the Kir2.x genes consist of a single coding exon. These exons codes for ion channel proteins that only contain two membrane-spanning segments, called M1 and M2, which is in contrast to the basic structural design characteristic of all the above-mentioned ion channels. Nevertheless, the pore-forming segment of Kir2.x channels shows a high degree of similarity with the pore of the 6 transmembrane-spanning ion channels, implicating a shared common ancestor.

Sudden cardiac death

Sudden cardiac death (SCD) is defined as natural death due to cardiac causes involving the abrupt loss of consciousness within one hour of the onset of acute symptoms, and while pre-existing heart disease may or may not have been known to be present, the time and mode of death are unexpected[12]. SCD accounts for 250000-400000 deaths per year in the United States depending on the definition used[13]. It is responsible for 50% of the mortality from cardiovascular disease in the US and it is the most common cause of death in adults in the United States[14]. Sudden cardiac death is three times more common in men than in women. It is often the first presentation of cardiac disease (25-50% of cases). It

occurs most commonly in the presence of coronary artery disease (CAD) and in 13-20% of patients with CAD, SCD is the initial symptom[15]. In congestive heart failure as many as 47% of the deaths are categorised as sudden death. SCD is also associated with valvular disease, although its incidence in patients with symptomatic aortic valve stenosis is low in asymptomatic patients[14]. In hyperthrophic cardiomyopathy (HCM), which occurs in about 2 out of every 1000 adults, the yearly incidence of SCD is about 2-4% in adults and 4-6% in children[14]. Finally, congenital heart defects such as aortic stenosis, tetralogy of Fallot, and transposition of the great arteries are all associated with SCD[12]. In addition, in patients surgically corrected for these congenital lesions, an increased risk of SCD is observed as a late complication of the surgery. In contrast to the above mentioned diseases, sudden cardiac death solely due to primary ventricular fibrillation, i.e., without apparent evidence of structural heart disease (see above), occurs in ±5% of victims of sudden cardiac death[14,16]. Preliminary data suggest that these patients have a 30% recurrence rate within 3 years of ventricular fibrillation, syncope, and cardiac arrest[17], and it is important to stress that their survival is largely related to potentially controllable or reversible electrophysiological disturbances rather than death due to advanced heart disease. ICDs should be particularly useful in these patients[18]. These patients were often termed idiopathic, because the cause of the VF was unknown. Recently, many of the idiopathic VFs were shown to be caused by mutations in ion channels, see below. With the discovery of single mutations in ion channels leading to arrhythmias it was clear that the heartbeat is wholly dependent on the proper movement of the ions across the surface membrane, and disorders of ion channels have since been named 'ion channelopathies' and now make up a key group of heart diseases[19]. Ion channelopathies predispose individuals to disturbances of normal cardiac rhythm. If the heart beats too slowly (bradycardias), or so rapidly that it cannot fill adequately (tachycardias), this leads to circulatory collapse and, in the extreme case, death. Although many individuals have anatomic and functional substrates that can develop into a life-threatening arrhythmia, sudden cardiac death can be considered an electrical accident, as only a relatively small number of patients actually do develop sudden cardiac death. It is the interaction between the anatomic and functional substrates, modulated by transient events or underlying genetic causes, that perturbs the balance leading to sudden cardiac death[12,20].

Mechanisms of sudden cardiac death

A major, if not the major, electrophysiological feature responsible for the initiation of ventricular fibrillation, underlying most SCD cases, appears to be *electrical heterogeneity*. In the normal heart electrical heterogeneity is common, as various cell types, e.g. ventricular muscle, Purkinje fibres, His bundles etc, exhibit different action potential characteristics,

refractoriness, and conduction velocities. However, when heterogeneity becomes pronounced, for instance, if one region of the myocardium exhibits ischemia-induced conduction delay and/or block that is different from neighboring regions, or another condition exists that can produce pronounced regional electrophysiological alterations, this can lead to ventricular fibrillation. Such alterations can be provoked by anatomic/functional substrates and by transient initiating events, and can modulate basic arrhythmia mechanisms of re-entry and triggered activity to provoke ventricular arrhythmias (Figure 4) leading to sudden cardiac death.

Common arrhythmias

As described above, sudden cardiac death accounts for approximately 50% of all cardiac deaths. The majority of SCDs are caused by acute fatal arrhythmias, such as ventricular fibrillation and ventricular tachycardia. Thus it is of interest to recognize and understand the various arrhythmias in order to treat and even prevent them. The majority of the arrhythmias occur in common diseases such as heart failure, ischemia and atrial fibrillation. Unfortunately, while the mechanistic background is becoming clearer, the (various) underlying electrochemical, molecular and genetic changes are often difficult to isolate. Some progress has been made in understanding the pathophysiological background of the arrhythmias in congestive heart failure and the pathophysiological background of atrial fibrillation.

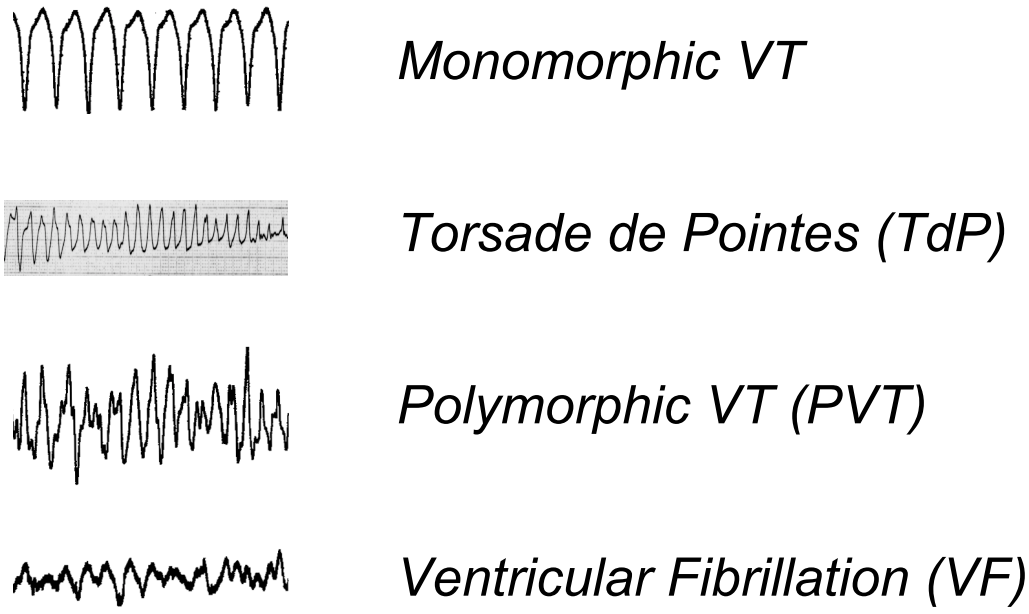

Figure 4. The various types of ventricular tachycardia. Tachycardia are depicted from mostly benign to highly lethal: monomorphic VT, Torsade de Pointes, polymorphic VT, and ventricular fibrillation.

Arrhythmias in congestive heart failure

Sudden death in patients with congestive heart failure is a common clinical occurrence. Often, the associated arrhythmia is a polymorphic ventricular tachycardia (PVT) leading to ventricular fibrillation and death. There is some resemblance in the kind of tachycardia seen in congestive heart failure patients and patients with the long QT syndrome, see below. Studies of animal models have documented the similarities between these two diseases on a tissue and cellular level. In both conditions heterogeneous increases in the action potential duration (APD) have been a consistent finding. In heart failure the APD prolongation correlates with down regulation of several potassium currents: the transient outward current I_{to}, the inward current I_{k1}, and the delayed rectifier current I_{ks} and I_{kr} , and an increase in I_{ca}(reviewed by Tomaselli and Marban[21]). As discussed above the plateau of the cardiac action potential is an unstable equilibrium; even small changes of inward or outward current can markedly delay repolarization. The reduction in potassium currents found in heart failure could therefore explain the prolongation of the APD and the increase in surface ECG repolarization time. The similarities between heart failure and the long QT syndrome suggest that alterations in the potassium current underlie the arrhythmic risk, and therefore that correcting the potassium channel deficit may reduce the incidence of sudden death in heart failure.

Atrial fibrillation

Unlike PVT associated with heart failure, where the rhythm quickly causes hemodynamic instability and death, atrial fibrillation (AF) can be sustained for long periods of time. Atrial fibrillation is characterized by rapid and irregular activation of the atrium, for example, 400-600 pulses of the atrium muscular wall per minute. The occurrence of AF increases with age with a prevalence rising from 0.5% of people in their 50s to nearly 10% of the octogenarian population. Several cardiac disorders predispose to AF, including coronary artery disease, pericarditis, mitral valve disease, congenital heart diseases, congestive heart failure and hypertension. Many of these are thought to promote AF by increasing the atrial pressure and or by causing atrial dilation; however, the precise mechanistic links are incompletely defined. AF also occurs in individuals without any other evidence of heart or systemic disease, a condition known as lone AF. AF can be sustained for long periods of time, and cellular adaptive processes reflect this. During sustained AF electrical remodelling occurs and causes a shortening of the APD and refractory period. Clinical and experimental studies have shown a 70% down regulation of the Ca^{2+} current I_{ca} and the transient outward current I_{to}, account for the observed changes in AP morphology. The inward rectifier and adenosine/acetylcholine activated

potassium currents (I_{k1} and I_{kach}) are both up regulated. The end result of this electrical remodelling is an improved ability of the atrial myocytes to sustain the rapid and chaotic impulses characteristic of atrial fibrillation (reviewed by Bosch et al.[22]). This situation creates a cycle where the rapid rate causes a shortened refractory period that allows the continuation of the rapid rate, an idea that has been termed 'AF begets AF'. The maladaptive nature of the channel alteration suggests that interrupting these changes on a molecular level is a potential treatment for AF.

Familial arrhythmias

In contrast to the above-mentioned common arrhythmias, it has recently become clear that a group of cardiac disorders exists, now termed ion channelopathies, that are relatively rare, but nonetheless lead to severe cardiac arrhythmias and carry a significant risk of sudden cardiac death. Moreover, many of these ion channelopathies are familial, that is, there is usually a positive family history of syncope and sudden cardiac death. This implies that these ion channelopathies have an underlying genetic background. By combining accurate clinical categorizations and molecular genetics many of these ion channelopathies have recently been described in detail and the underlying causes identified.

Long QT syndrome

Introduction

Long QT syndrome (LQTS) is a cardiovascular disorder characterized by an abnormality in ventricular repolarization, leading to a prolonged QT interval on the surface electrocardiogram (Figure 5). The syndrome is associated with syncope and sudden death due to episodic cardiac arrhythmias, particularly Torsade de pointes and VF. Current estimates suggest that between 1/5000 and 1/10000 persons are gene carriers and that the congenital long QT syndrome causes 3000 to 4000 sudden deaths in children and young adults each year in the United States[23]. The long QT syndrome was the first arrhythmic disorder to be characterized at a genetic level. The concentrated effort to identify genetic defects associated with the long QT syndrome has led to the discovery of several new potassium channel subunits and provided a better understanding of the interaction of the various components of the action potential. The long QT syndrome is actually a combination of two clinical syndromes that share QT prolongation as their major feature. The difference between these two variants is based on the genetic transmission pattern, the more common autosomal-dominant form (the Romano–Ward syndrome) is associated with a pure cardiac phenotype[24,25] and the rarer autosomal-

recessive form (the Jervell and Lange–Nielsen syndrome) is also characterized by the association with congenital neuronal deafness[26], although exceptions are described. As a consequence of the genetic information, it became clear that what has been classified under the unifying name "long QT syndrome" actually represents many different diseases caused by different ion channel mutations (see below); these mutations all produce alterations in ionic currents, leading to the same result: a prolonged ventricular repolarization[27].

Clinical characteristics

The hallmark of a LQTS patient is the markedly prolonged QTc interval on an ECG; the majority of patients with LQTS have a QTc interval that is well above 440 milliseconds (Figure 5). Other characteristic ECG features are larger, prolonged, and bizarre looking T waves that may display a notched, bifid, biphasic, or alternans appearance, abnormal U waves, and occasionally sinus bradycardia with sinus pauses[28,29]. Recently it was suggested that the different genotypes of LQTS (see below) may even display specific ECG phenotypes[30,31]. The clinical features of the individual LQTS patient may range from minor symptoms, such as dizziness, to seizure, syncope, and sudden death. Among immediate family members of the LQTS patient there is often a history of syncope and unexplained sudden cardiac death. These symptoms are almost always due to Torsade de Pointes arrhythmias, as described earlier. An important characteristic of Torsade de Pointes is its potential for self-termination or deterioration into ventricular fibrillation. Patients with the diverse variants of the LQTS (see below) can often be characterised according to certain specific triggers. LQT1 patients seem to be characterized by exercise- and stress-related events that are adrenergically stimulated. Especially triggers as diving

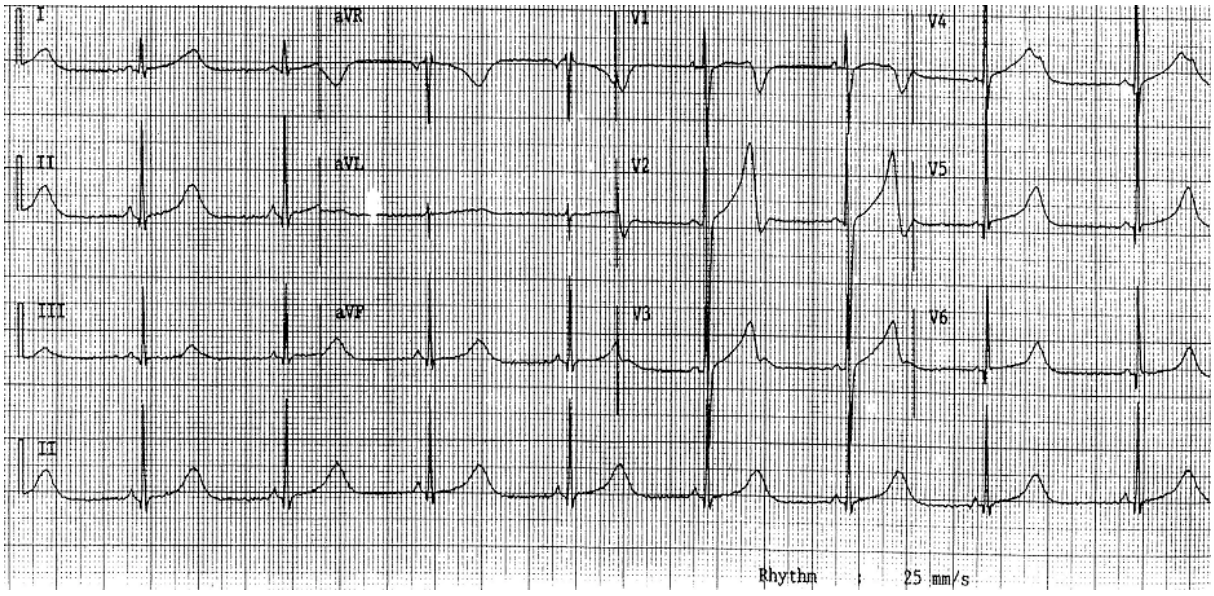

Figure 5. Typical Long QT type 1 ECG, note the high amplitude T-wave with prolonged duration. The rhythm is sinus rhythm 50/min.

and swimming are almost exclusively found in LQT1 patients[32,33]. Patients with LQT3 are at particularly high risk at rest or asleep because their QT interval is excessively prolonged at slow heart rates[34]. This clear distinction between LQT1 and LQT3 with respect to exercise-related triggers does not hold for patients with LQT2, who tend to display events both at rest and during exercise[35]. However, events provoked by auditory stimuli, such as an alarm clock or telephone ringing, almost exclusively occur in patients with LQT2[32,33,35]. For the other variants of LQTS, LQT4, LQT5, and LQT6 it is less clear what triggers underlie the disease. The LQTS usually manifests before the age of 40 years, mainly in childhood and adolescence. The age at which the disease manifests the first time in an individual patient depends on the genotype of the family. According to the International Long QT Syndrome Registry data, the median age at which the first cardiac event occurred was 9, 12, and 16 years in subjects with LQT1, LQT2, and LQT3, respectively[36]. Adult males are less prone to the development of cardiac events because of shorter QT intervals compared to women, boys, and girls, especially in LQT1 and LQT2 groups[33,37]. The risk of torsades and sudden death is highest at the early waking hours, especially for LQT2 patients, which correlates with the diurnal peak of the QT interval at that time. The clinical course of the congenital long QT syndrome is largely influenced by the gene affected[36]. By age 15 years, almost 60% of patients with LQT1 have had a cardiac event (syncope, cardiac arrest, or sudden cardiac death) compared with fewer than 10% of patients with LQT3[33]. The number of cardiac events that occurred up to the age of 40 years was also higher in patients with LQT1 (63%) or LQT2 (46%) than in patients with LQT3 (18%). In contrast, the likelihood of death during a cardiac event was much higher in patients with LQT3 (20%) than in those with LQT1 and LQT2 (4%)[36]. Because of the small number of patients, no data are yet available for patients with LQT4, LQT5, and LQT6.

Treatment

Symptomatic LQTS patients require treatment given the high risk of torsades and sudden death[38]. It is important to inform patients and their parents about the importance of adherence, as nonadherence exposes patients to increased risk for these cardiac events. Administration of β-blocking drugs is very effective in preventing ventricular arrhythmias in the long QT syndrome, although the response is genotype dependent (see below). The 10-year mortality rates among symptomatic patients decreased from approximately 50% in patients with the congenital long QT syndrome who were not treated to less than 5% after these patients were routinely prescribed β-blocker therapy[38]. However, in patients with recurrent cardiac symptoms or aborted cardiac arrest, β-blocker therapy might not completely prevent sudden cardiac death, and an implantable cardioverter defibrillator (ICD) is warranted for these high-risk patients. The identification of underlying gene mutations in the LQTS has led to genotype-specific therapy. While beta-blockers are

efficient in LQT1 and LQT2, mexiletine, a sodium channel blocker, is treatment of choice in LQT3[31,38]. Moreover, pacing is important in LQT3 to avoid tachyarrhythmic events induced by bradycardia. Furthermore, in the presence of certain disease genes, certain modifications in life-style to avoid specific triggers are possible. About 10% of LQTS mutation carriers present with a normal ECG[30], as yet the value of β-blocker treatment in these patients is unclear.

Pathophysiological mechanism

The normal cardiomyocyte repolarization is the result of the net outward electric current that reflects the balance between inward currents, mainly through Ca^{2+} and Na^{+} channels, and outward currents, largely through K^{+} channels (as described earlier). This net balance between inward and outward currents ultimately determines the duration of AP (Figure

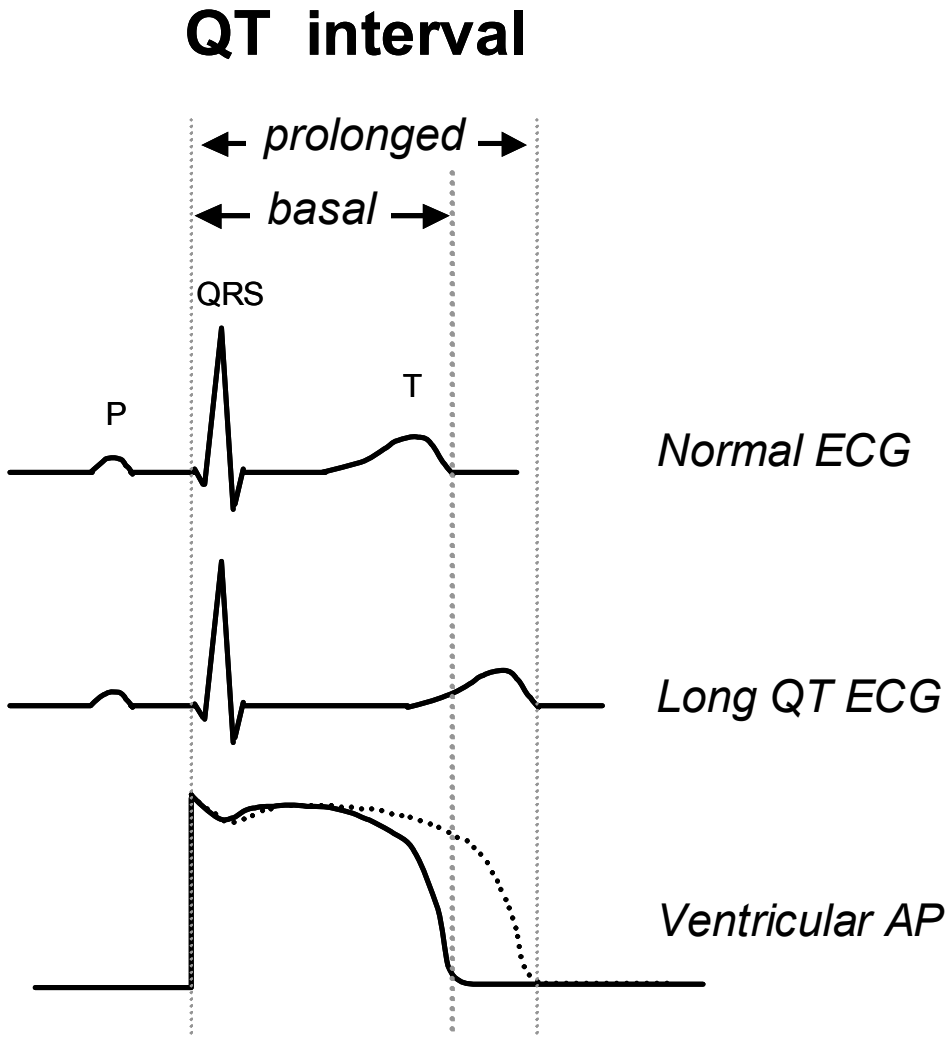

Figure 6. Pathophysiological mechanism of the Long QT syndrome. On top a normal ECG is depicted, in the middle panel a Long QT ECG. On the bottom the presumed ventricular action potentials thought to underlie the ECGs are depicted aligned with the two ECGs. The impaired action potential repolarization results in the prolongation of the QT-interval. Prolonged repolarization makes myocytes susceptible to so-called early after depolarizations (EADs), and life-threatening ventricular tachycardias.

6). LQTS is either caused by loss of function mutations in genes that encode the ion channel proteins responsible for rapid (I_{Kr}) or slow (I_{ks}) components of the "delayed rectifier" K^+ current, or by gain of function mutations in the gene that encodes the cardiac Na^+ channel. Both mechanisms lead to impaired AP repolarization, and result in the prolongation of the QT-interval. Prolonged repolarization makes the cardiomyocytes susceptible to early after depolarizations (EADs); these are oscillations in cell membrane potential that interrupt phase 2 or 3 repolarization. They arise during excessive action potential prolongation and are thought to trigger arrhythmias in pathologic conditions of action potential prolongation. If these EADs are of sufficient magnitude they can depolarize the cell membrane to the threshold potential and trigger repetitive APs, and this triggered activity is believed to be the initiating cause of the polymorphic ventricular tachycardia TdP characteristic of LQTS (Figure 4). An individual episode of the torsades is generally short lived and terminates spontaneously. However, it has a tendency to occur in rapid succession and therefore may cause syncope and death. This explains why affected patients often survive several syncopal attacks before succumbing to a fatal one.

Genetic basis

A family history of unexplained syncope or sudden death, especially in the young family members of a patient with unexplained syncope or sudden death, is often present in LQTS probands. Initially, the two well-described forms of the congenital LQT were the Jervell Lange-Nielsen syndrome and the Romano Ward syndrome. During the last decade, significant advancements have been made in determining the genetic basis of the congenital LQT, and on the basis of the ion channels and the genes involved, 6 subtypes of congenital LQT have been characterized. Mutations in the KCNQ1 (KVLQT1) gene, located on chromosome 11, cause LQT1. The KCNQ1 gene encodes the α-subunit of a cardiac potassium channel I_{Ks}, the slowly activating potassium delayed rectifier[39]. The LQT1 is the principal gene responsible for both Jervell Lange-Nielsen and Romano-Ward syndromes, and it accounts for approximately 40% of the genotyped LQT families. The mutations in the HERG gene, located on chromosome 7, cause LQT2. The HERG gene encodes for another cardiac potassium channel I_{Kr}, the rapidly activating potassium delayed rectifier[40]. The LQT2 accounts for approximately 45% of the genotyped LQT families. The mutations in the SCN5A gene, located on chromosome 3, cause LQT3. The SCN5A gene encodes a cardiac sodium channel I_{Na}, the cardiac voltage-dependent sodium channel[41]. The LQT3 accounts for approximately 5% of the genotyped LQT families. Interestingly, mutations in the same gene but at different loci result in Brugada syndrome[42] (see further down) and progressive conduction system disease (Lenegre-Lev disease)[43]. However, these mutations are loss-of-function type mutations in contrast to the gain-of-function type mutations in LQT3. The LQT4 locus has been identified at chromosome 4 where it associated with the LQTS in one large French kindred. Very

recently a loss-of-function mutation in ankyrin-B (also known as ankyrin 2), which is a member of a family of versatile membrane adapters, was shown to underlie LQT4[44]. The mutations in the KCNE1 (minK) gene, located on chromosome 21, cause LQT5. The KCNE1 gene encodes the β-subunit of the cardiac potassium channel I_{Ks}[45]. The KCNQ1 (LQT1) and KCNE1 (LQT5) gene products assemble to form a complete I_{Ks} channel protein. The LQT5 accounts for only a small number of the genotyped LQT families. Along similar lines, mutations in the KCNE2 (MiRP1) gene, located on chromosome 21 causes LQT6[46]. Likewise to LQT5, the KCNE2 (MiRP1) gene encodes a small membrane protein, which is considered a part of the I_{Kr} channel. The HERG (LQT2) and KCNE2 (LQT6) gene products assemble to form a complete I_{Kr} channel protein. Although currently more than 300 mutations have been discovered, the majority of them being missense mutations, not all the genes responsible for LQT have been identified, as in 30% of the LQT patients an underlying mutation could not be found. However, since sporadic cases of LQTS can occur as a result of spontaneous mutations, a lack of family history does not entirely preclude the diagnosis of congenital LQT.

Drug-acquired long QT syndrome

LQTS exists in both familial (congenital) and acquired forms. The acquired form is associated with the side effects of numerous common medications that cause in certain patients a prolonged QTc interval upon exposure to the drugs. Particularly certain types of antihistamines, antimicrobial agents, antidepressants, antiarrythmic drugs, and diuretics have been reported to be associated with acquired long QT syndrome[47,48]. Most of these medications block HERG subunits, leading to reduced repolarizing K^+ current and a subsequent delayed cardiomyocyte repolarization. Interestingly, the first ion channel gene mutations and polymorphisms that predispose people to drug-provoked QT interval prolongation and ventricular arrhythmias have been identified[49,50]. Recent studies have indicated that mutations in MiRP1, a subunit of I_{Kr} channels (also associated with LQT5), are associated with drug-induced long QT syndrome[50]. Acquired long QT syndrome can also occur secondary to electrolyte disturbances. However, most of the patients with drug induced LQTS do not have ion channel defects, but they seem to carry a predisposition for malignant arrhythmias[51]. At present the value of genetic diagnosis in these acquired long QT syndrome patients is unclear, but it is clear that they should avoid any LQTS-provoking drugs.

Brugada syndrome

Introduction

The Brugada syndrome is another inherited arrhythmia that is characterized by idiopathic ventricular fibrillation and sudden cardiac death. Brugada syndrome patients not only present with ventricular fibrillation, but also with a conduction disturbance most typically with a right bundle branch block pattern, and ST segment elevation in ECG leads V1-3 in structurally normal hearts. It is associated with a high mortality rate[52]; Brugada syndrome is the leading cause of natural deaths in males younger than 50 years in South Asia where it is closely related to the sudden unexpected death syndrome responsible for an annual mortality rate of 1/2500 in young male Thai patients up to a mortality of 1/1000 in countries like Laos[53]. Reports also suggest a high prevalence of this syndrome in the Japanese population. Overall estimates are that this syndrome is responsible for around 50% of sudden deaths in individuals without structural heart disease. The many familial cases display autosomal dominant inheritance with varying degrees of incomplete penetrance[42,54,55]. It mainly affects men, with the first arrhythmic event occurring during the fourth decade of life, but it can also occur in children even during the first months of life[54].

Clinical characteristics

The Brugada syndrome is characterized by an ECG resembling a right bundle branch block or less specific conduction disturbances with a ST segment elevation in the right precordial leads V1 to V3 (unrelated to ischemia, electrolyte abnormalities, or structural heart disease) (Figure 7). The ECG pattern is dynamic and varies presumably with varying

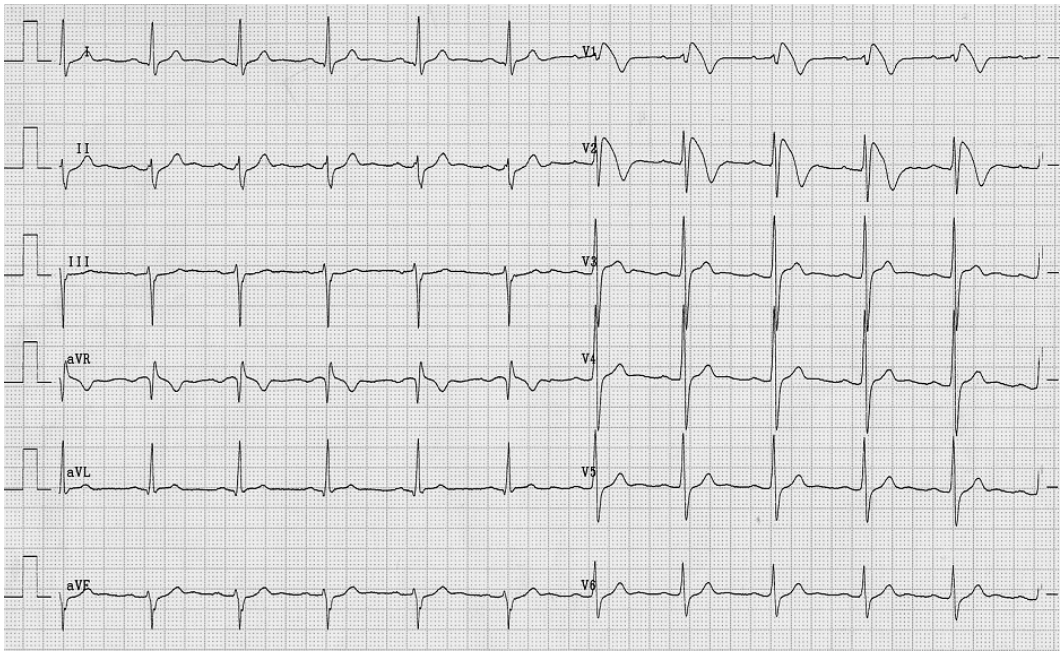

Figure 7. Typical Brugada ECG, note the ST-segment elevation in the right precordial leads V1-V2. Conduction is disturbed (PQ interval 200 msec., left axis, QRS width 120 msec.

risk of developing ventricular fibrillation[52]. The polymorphic venctricular arrhythmias cause syncope when self-terminating and sudden death when long lasting and not terminated by cardiopulmonary resuscitation. Additional features of the Brugada syndrome include left axis deviation and increased HV interval, indicative of conduction disturbances[52,56]. Brugada syndrome patients have a structurally normal heart as assessed by invasive and non-invasive investigations and cardiac biopsies. And finally a familial occurrence is present in about half of the patients showing, as mentioned, an autosomal mode of inheritance.

Treatment

Unfortunately in spite of the major advance in the diagnosis and understanding of the pathophysiology of the Brugada syndrome (see below), an implantable cardioverter-defibrillator (ICD) is currently the only effective therapy to prevent sudden cardiac death[57]. Beta-blockers, amiodarone and flecainide do not prevent recurrences of ventricular arrythmias or might even worsen it. Limited evidence suggests that EP-guided pharmacological treatment with Class IA drugs (quinidine in particular) seems to be safe and effective in previously resuscitated Brugada patients, as the ST elevation disappears upon quinidine administration[58]. Moreover, experimental data supports the concept that quinidine, by virtue of its I_{TO} blocking effect, might be effective in Brugada syndrome patients[59]. Treatment of symptomatic individuals with an implantable defibrillator is generally accepted, but the treatment of asymptomatic individuals or asymptomatic carriers of disease-related mutations (see below) with a normal ECG remains a major problem. However, given the similarly bad prognosis, ICD treatment is also advocated for asymptomatic patients with the typical Brugada ECG features[60].

Pathophysiological mechanism

Antzelevitch and his group suggested that a mechanism based on the transmural differences in action potential morphology underlies the Brugada syndrome (Figure 8)[61]. The endocardial action potential and the epicardial action potential are strikingly different in that the action potential of epicardial myocytes possesses a distinct 'spike-and-dome' morphology caused by a pronounced transient outward (I_{to}) current, which is absent in endocardial myocytes. This predisposes epicardial myocytes to 'all-or-none' repolarization. If the transient outward current would be amplified or the inward current at the end of phase 1 would be decreased, a loss of the epicardial action potential dome would ensue and result in a selective abbreviation of the epicardial action potential. Consequently, this would give rise to a voltage gradient across the myocardium generating the ST-segment elevation observed in the Brugada ECG. Therefore, arrhythmias could arise from phase 2 re-entry secondary to the increased epicardial and transmural dispersion of repolarization. The prominence of the ECG changes in the right precordial leads could be explained by

the fact that right ventricular epicardial cells display a larger I_{to} (and hence more pronounced `spike-and-dome') than left ventricular epicardial cells[62]. Moreover, it can be hypothesised that because of the thinness of the right ventricular wall, the relative contribution of epicardial action potentials to the surface ECG is more prominent in right than left precordial leads[56]. Experiments on canine right ventricle tissue, which involved augmentation of the outward current or suppression of inward current at the end of phase 1 of the action potential, have provided direct evidence that accentuation of epicardial–endocardial action potential heterogeneity underlies the development of ST-segment elevation and the genesis of arrhythmias[61].

Genetic basis

Since decreased Na^+ channel function was suggested as one of the most probable mechanisms underlying the Brugada syndrome, an approach to identify a candidate gene was directed to SCN5A, the cardiac Na^+ channel. Chen et al. initially found three mutations in SCN5A that co-segregated with the disorder in small families[42]. Since then

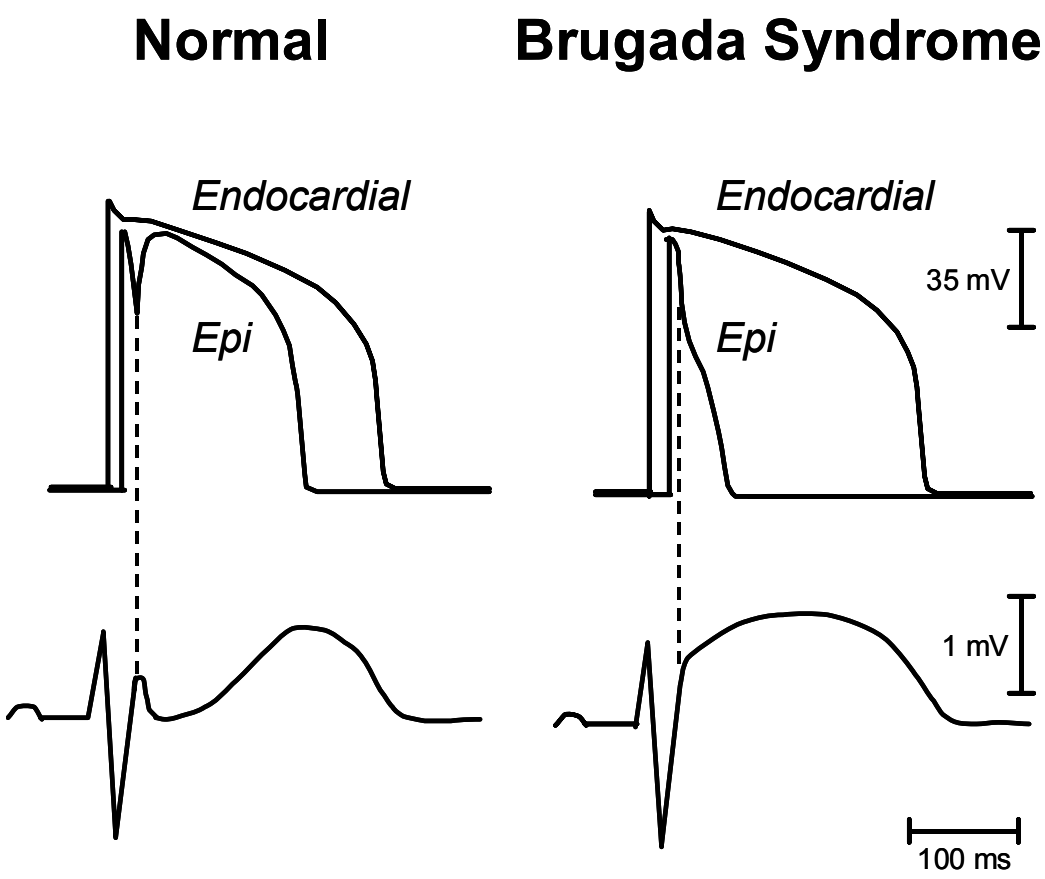

Figure 8. Differences between the epi- and endocardial action potentials are thought to underlie the Brugada syndrome. On the left the APs and ECG of a normal person is depicted, note the pronounce phase 1 of the AP. On the right Brugada patients APs and ECG are depicted, note the abbreviation of the epi cardial action potential in phase 1. This would lead to a transmural dispersion of repolarization, which could lead to phase 2 re-entry, and thus induce lethal arrythmias. Figure adapted from reference 55.

several other mutations responsible for the Brugada syndrome have also been identified in SCN5A and some of their biophysical properties have been clarified[63]. Overall two categories of mutations are described, either they decrease expression levels of the SCN5A channel at the sarcolemma or they lead to functional disruption of the channel due to altered biophysical properties. The former category of mutations includes an insertion of two nucleotides disrupting the 5' splice site of intron 7, a frame shift mutation at codon 1397 leading to the creation of an in-frame stop codon and the generation of a truncated SCN5A protein, and a missense mutation (R1432G) residing in the P-loop of DIII[64]. Injection of *SCN5A* cRNA encoding the frameshift mutation into *Xenopus* oocytes and expression of the R1432G channel in tsA-201 cells both failed to express Na^+ currents[42,64]. The effect of this mutation has been shown to be due to defective protein trafficking toward the plasma membrane. The other category of Brugada mutations results in various functional defects in activation and inactivation, most of which promote reduced Na^+ current density (loss-of-function). Functional analysis of several missense mutations found in Brugada patients show variable effects on channel functions, all resulting in loss-of-function[63]. However, it is becoming increasingly clear that caution should be exerted in the interpretation of characteristics of ion channels expressed in heterologous expression systems. For example, channel kinetics are dependent on the expression system used[65], the temperature at which the channels are analysed and the expression of the β1-subunit and its splice variants[66,67]. Nonetheless, it seems clear that Brugada mutations either lead to decreased expression levels of the channels or to a disruption of the channel functions.

It is striking that mutations of the same gene, SCN5A, can result into different phenotypes. SCN5A mutations that result in a gain-of-function are associated with the Long QT syndrome type 3, while SCN5A mutations leading to a loss-of-function seem to result in the Brugada syndrome and/or conduction disease. In that respect it is remarkable that recently a large 8-generation family was identified that was characterized by a high incidence of nocturnal sudden death and electrocardiographic features of both the LQTS and the BS in the same patients[68]. Subsequent screening of the SCN5A gene led to the identification of the 1795insD mutation, located in the acidic proximal portion of the cytoplasmic C-terminal domain, in all electrocardiographically affected individuals. Functional analysis revealed that the expressed currents of the mutant showed a disruption of fast inactivation with persistent current during continued depolarisation as well as a negative shift of steady-state inactivation curve and enhanced slow inactivation resulting in a decreased current expression. The two kinetic abnormalities in inactivation could bring different phenotypic expression of LQTS at slow heart rate and the Brugada-type ECG signs in fast heart with exercise. These findings were corroborated by ECG-

recordings from a patient carrying the 1795insD mutation during rest and with exercise. QT-interval was prolonged and ST-segment elevation was moderate at rest. With exercise there was an increase of ST-elevation that closely correlated with an increase in heart rate, and the QT-interval was shortened[69].

The overall rate of identified mutations in the SCN5A gene in Brugada syndrome patients lays around 20% in Europe and 10-20% in Japan. This suggests that either the screening techniques employed are not sensitive enough, or alternatively, that the Brugada syndrome is genetically heterogeneous. The latter possibility is emphasized by the description of a family with Brugada syndrome in whom there is absence of linkage to the *SCN5A* locus on chromosome 3[70]. This argues that as yet unidentified genes might underlie cases of the Brugada syndrome. Given that increased epicardial and transmural dispersion of repolarization underlie the Brugada syndrome, other genes involved in the 'spike-and-dome' morphology of epicardial myocytes are prime candidates.

Catecholaminergic polymorphic ventricular tachycardia

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disease characterised by adrenergic induced arrhythmias in the form of bi-directional and polymorphic ventricular tachycardia. Polymorphic ventricular tachycardia is a rapid ventricular tachycardia with changing morphology of the QRS complexes (Figure 9). A finding common to (young) patients with PVT is the clinical presentation with syncope and a high incidence of sudden cardiac death. Moreover, cardiac investigations show no evidence of structural heart disease and the resting 12-lead electrocardiogram *including* the QT interval is unremarkable.

Clinical characteristics

Clinically catecholaminergic polymorphic ventricular tachycardia can be defined by several unique characteristics. As described above, the key features of CPVT include syncope and collapse triggered by exercise, emotion or stress, and highly reproducible and inducible stress-related polymorphic and/or bi-directional ventricular tachycardia in the absence of structural heart disease (Figure 9)[71-73]. Cardiac investigations usually show no evidence of structural heart disease; i.e. clinical history, blood chemistry, electrocardiography and exercise testing (except for arrhythmias), echocardiography, and cardiac catheterization with coronary angiography are all unremarkable. Moreover, the resting 12-lead electrocardiogram, including the QTc interval, is normal. However, bradycardia may be common, especially in (young) children[74,75]. Young patients suffering from PVT are often misdiagnosed with epilepsia when the rapid PVT provokes seizures. The only

discriminating factor between CPVT and the long QT syndrome is a normal QTc interval in the former. In contrast to the reproducible provocation with stress or exercise, CPVT is generally not inducible with programmed electrical stimulation (PES), however the use of catecholamines (e.g. isoproterenol) may unmask the arrhythmogenic substrate. Overall, CPVT mainly affects children; the recent studies demonstrate that the majority of patients have an age of onset of CPVT symptoms (syncope or cardiac arrest during stress) around the age of 10 years. However, CPVT patients may present with a much later age of onset, as several CPVT patients are reported who were asymptomatic until their mid 30s[74,76,77]. Most importantly though, a clear correlation between the age of the first syncope and the severity of the disease exists; early occurrence of the first syncope worsens the prognosis[71]. Retrospective analysis shows that CPVT has a highly malignant course when untreated, with estimates of mortality ranging from 30 to 50 percent by the age of 20 to 30 years[72]. In general, the diagnosis of CPVT might be difficult since the resting ECG is usually normal. However, frequent syncopes in (young) patients in relation to exercise or emotion, a positive family history for syncope or SCD, absence of heart abnormalities, and possible presence of sinus bradycardia, are strong indications for CPVT. The diagnosis of CPVT can subsequently be confirmed by means of Holter recordings and especially exercise testing.

Treatment

The current treatment of choice for CPVT is β-blockers without sympathomimetic activity. This concept was initially based on an early retrospective analysis of 59 published CPVT cases[71], of which only 38 received β-blocker treatment. With this treatment there were four sudden deaths, or 10.5%. In contrast, 10 out of the 21 patients who did not

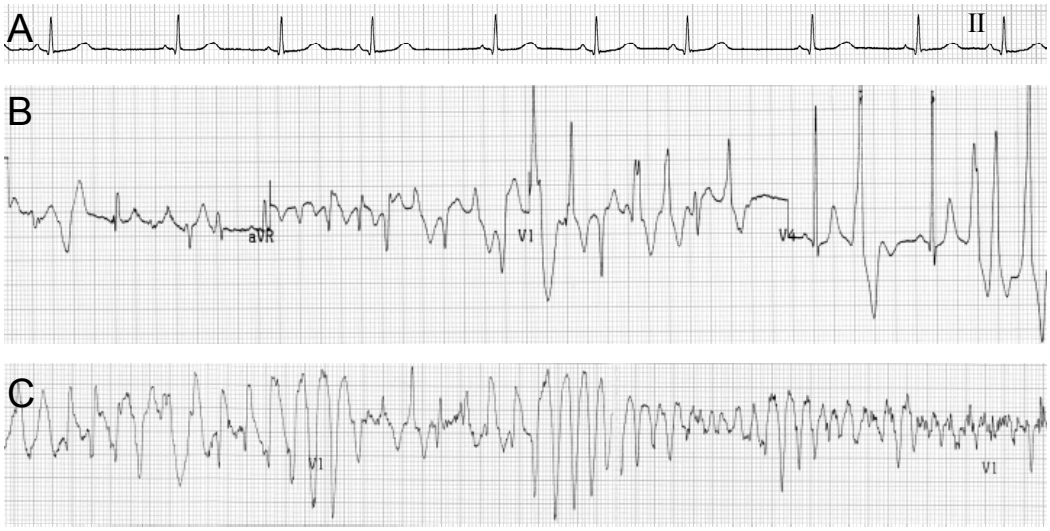

Figure 9. A. ECG of a CPVT patient showing a normal QTc interval (428ms) and a slow heart rate (70 bpm) at rest. B. Polymorphic ventricular premature beats at the beginning of the stress test. C. Self-terminating polymorphic salvos as the heart rate increases above 100 bpm. In panel B different leads are sequentially shown.

receive beta blockers, experienced sudden death at the mean age of 19.5 years, which corresponds to a 48% mortality rate before the age of 20. At present, the vast majority of the CPVT patients described in literature received β-blockers in various dosages and regimes. With a mean follow-up time of 6±2 years, 88% (198/225) are symptom free, though some patients continued to have VPBs at exercise tests. 12% of CPVT patients experienced additional episodes of VT/VF or syncopes while on β-blockers. Unfortunately, 4% CPVT patients on β-blockers died, though, the majority of these deaths were associated with non-compliance after appropriate diagnosis and treatment[71,74,76]. The occurrence of a small group of CPVT patients, who continue to have episodes of VT/VF or syncope on β-blockers, indicates that additional modes of therapy are needed, particularly considering the high mortality of the disease. Experience with the implantation of ICDs in non-responsive patients underlines the importance of this procedure as in 6 of the 12 CPVT patients in whom an ICD was implanted, an appropriate shock terminated ventricular tachyarrythmias over the follow-up period of 2 years. Nonetheless, an ICD can potentially have pro-arrhythmic effects in CPVT patients, as stress caused by appropriate or inappropriate discharges could prove disastrous by evoking a self-inducing stress circle of arrhythmia-shock-stress-arrhythmia-shock-stress etc. However, a combination therapy, involving both an ICD and β-blockers, should safeguard against any such adverse effects and provide ultimate protection in non-responsive patients. Limited evidence suggests that drugs such as amiodarone, verapamil, mexiletine and type I antiarrhythmic drugs are not effective in controlling the arrhythmic disorder and might even be harmful[71,76]. In conclusion, "individually adapted" β-blocking treatment has a favorable overall outcome in the majority of patients, but additional modes of therapy should be considered.

Pathophysiological background

The polymorphic ventricular tachyarrhythmias as seen in CPVT patients resemble the type of arrhythmias seen in experimental models with digitalis toxicity[78]. This has led to speculation that aberrant calcium handling might underlie the disease. The crucial link between cardiac excitation and subsequent cardiac muscle contraction, the so-called excitation-contraction coupling, is specifically regulated by calcium ions[79]. As mentioned above the CPVT arrhythmias resemble those seen in digitalis toxicity. Since the latter are caused by an electrophysiological mechanism known as delayed afterdepolarizations (DADs), it has been proposed that a similar mechanism underlies the polymorphic ventricular tachyarrhythmias seen in CPVT patients[78]. DADs are triggered impulses that result from sub-threshold membrane depolarisations after repolarisation of the previous action potential is complete, as described earlier. If the DAD produces an inward current sufficiently strong to exceed the threshold potential of the cardiomyocyte, depolarisations will occur. The abnormal impulses generated from this can cause a triggered arrhythmia if

surrounding polarised cells propagate the depolarisation wave and may give rise to extrasystoles and associated arrhythmias. Moreover, several factors increase the amplitude of DADs and hence the probability that the threshold potential will be reached. These include increasing the rate of the triggering action potential (corresponding to an increase in heart rate) and increasing intracellular calcium loading[80]. This former possibility supports the fact that polymorphic ventricular tachycardia occur as the sinus rate of CPVT patients increases.

Genetic basis

Many cases of CPVT are familial, and the first extensive report with a definite linkage analysis was published by Swan *et al.*, showing linkage of CPVT with chromosome 1q42-43[72]. This was followed by publications which associated mutations in a gene from the 1q42-43 region, the cardiac specific ryanodine receptor type 2 (RyR2), with CPVT[73,81,82]. Ryanodine receptor channels are intracellular Ca^{2+} release channels forming a homotetrameric membrane-spanning calcium channel on the sarcoplasmic reticulum (SR). They are characterized by a large cytosolic domain, which is probably involved in regulating the channel function, and a channel pore located at the carboxy terminal end, in the last 10% of the protein[83,84]. Three different isoforms of ryanodine receptors (RyR), each encoded by different genes, have been characterized so far. Isoform 1 (RyR1) is principally expressed in skeletal muscle, isoform 2 (RyR2) is the predominant form in cardiac muscle and isoform 3 (RyR3) is expressed at low levels in various tissues including the brain[85]. The majority of RyR2 channels in cardiomyocytes are distributed in areas of the SR membrane that lie in proximity to the T-tubule invaginations of the sarcolemmal membrane[86]. Here they are closely associated with the L-type voltage-dependent Ca^{2+} channel and this spatial association of the two channels is key to the signal amplification process that couples the excitation of myocardial cells to the actin/myosin contractile apparatus. The ryanodine receptors are not merely ion channels, they are considered macromolecular signaling complexes, as they contain binding sites for signaling proteins such as kinases, phosphatases and adaptor/anchoring proteins, which are specifically bound by several domains on the cytosolic side of the RyR channel[79]. The RyR2 gene is located on chromosome 1q42 and consists of 105 exons, which translate into 15kb of mRNA. It encodes a protein of 4967 amino acids, making it the largest known ion channel to date.

In contrast to the majority of CPVT patients with an autosomal dominant mode of inheritance, two studies have recently demonstrated a recessive form of CPVT in 4 families[87,88] (see also chapter 3). In the first study by Lahat *et al.* a large consanguineous Bedouin family was identified in which 9 children from 7 related families have died suddenly. Twelve other children suffered from recurrent syncope and seizures starting at

the age of 6±3 years. The parents of the affected individuals were asymptomatic and were all related. In contrast with the "classical" CPVT, segregation analysis suggested an autosomal recessive mode of inheritance. In concurrence with autosomal dominant CPVT variant, recessive symptomatic CPVT patients had a relative resting bradycardia, and polymorphic VT could be induced by either treadmill exercise or isoproterenol infusion. After performing a genome wide screen, a common disease locus of 16 megabases on chromosome 1p13-21 was identified, which segregated in all affected patients. They subsequently identified in all the affected patients a homozygous missense mutation in a highly conserved region of the cardiac calsequestrin gene (CASQ2), effectively defining the autosomal recessive form of CPVT[87]. The CASQ2 gene encodes a protein that serves as the major Ca^{2+} reservoir within the SR lumen of cardiac myocytes. CASQ2 protein is anchored in close proximity to RyR2 and has the ability to bind extremely large numbers of Ca^{2+} cations in the SR[89]. Strikingly, heterozygous carriers of the mutation were devoid of any clinical symptoms or ECG anomalies. The phenotypes of the CASQ2 CPVT patients seem more severe compared to RyR2 CPVT patients. In particular the age of onset is earlier than that of the reported RyR2 mutations. Since there is no compensation from wildtype protein, homozygous mutations probably produce a more severe phenotype and thus surface earlier. In conclusion, CASQ2 CPVT patients present with a more severe clinical phenotype including early age of onset. In addition, the CASQ2 mutations are mostly associated with consanguineous families and therefore diagnosis in CASQ2 CPVT patients is more difficult due to the absence of a positive family history. Finally, the identification of a second CPVT causative gene suggests that CPVT is genetically heterogeneous, similar to other arrhythmogenic disorders.

With the discovery of genetic heterogeneity for CPVT the question arises whether there are additional genes involved in the disease. Evidence for this comes from the fact that in at least 29 CPVT families no causative mutation has been found, although a familial history of juvenile sudden death was present in 52% of them, implying an underlying hereditary cause. It is likely that some mutations in RyR2 or CASQ2 might have been missed due to incomplete screening of the genes, or the lack of sensitivity of the screening techniques used. On the other hand, given the recent discovery of the CASQ2 mutations, other proteins could be involved in CPVT, implicating further genetic heterogeneity, in analogy with other arrhythmogenic disorders such as the long QT syndrome[90]. Given that Ca^{2+} overload seems to be the trigger that is specific in CPVT patients, other genes involved in the Ca^{2+} release and re-uptake pathway are attractive candidates.

Short-couple variant of Torsade de Pointes

Introduction

Short-couple variant of torsade de pointes (SCTP) is a rare distinct clinical entity characterized by rapid nonsustained tachycardia of twisting of the point's morphology in patients with strictly a normal QTc interval[91]. Moreover, like in most of the above described arrhythmias short-couple variant of torsade de pointes patients are young adults of either sex with no detectable structural heart disease. The typical SCTP ECG displays torsade de pointes, with an unusually short coupling interval (always less than 300 msec) of the first beat of the torsade of pointes or of the isolated VPBs, a characteristic that seems to be specific to this disease (Figure 10). The largest series of patients so far was described by Leenhardt et al., he reported on 14 patients, aged 34±10 years, all of whom experienced syncope related to the typical ECG of torsade de pointes for whom the QTc interval was normal, and all of them had the short-coupling interval of the first beat or of the isolated premature beats[91]. During the long-term follow-up (±7 years) 2 patients received beta-blockers and the other 12 were treated with high doses of verapamil (360 to 720 mg/d), moreover 3 patients received an ICD. In general SCTP is a malignant disease as 5 of the initial 14 patients died despite therapy; two of the patients on beta-blockers and three of the patients on verapamil died suddenly, moreover all three patients who received an ICD had appropriate shocks despite verapamil therapy. A recent report on a 41-year-old man, who presented with SCTP also indicates that an ICD and verapamil are the therapy of choice as the patient received several appropriate shocks within a follow-up period of 5 years[92]. Alternatively, amiodarone might be effective as

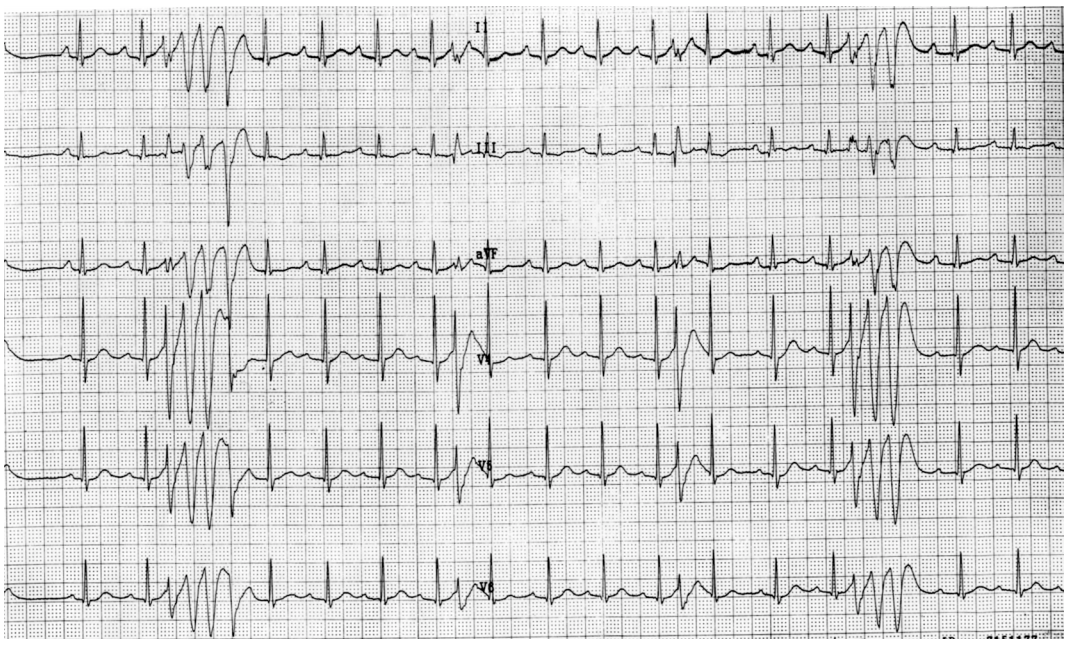

Figure 10. Typical ECG representing the short-coupled variant of Torsade de Pointes (leads II, III, aVF, V4, V5, V6). Note the extremely short coupling interval of initiating extrasystoles (250ms). After the initiation extrasystole, a shifting of the electrical axis is observed in short runs of Torsade de Pointes.

well, as another recent case report describes a Japanese patient with SCTP who remained free of syncope attack for over 5 years on amiodarone (200mg/day)[93]. However, limited evidence suggests that beta-blockers and quinidine are not clinically effective. Therefore, although the recurrence rate on verapamil is as high at 30%, it is the only therapy that consistently although not constantly suppresses the arrythmia. Amiodarone might be an effective alternative, but more clinical data are needed. Regardless, Leenhardt et al. stress that the combination of anti-arrhythmic drugs and an ICD is the only clinically effective therapy for SCTP in preventing sudden cardiac death[91].

Pathophysiological background

In concordance with the above-mentioned cardiac arrhythmias, SCTP also seems to have a genetic basis, as 4 of the 14 patients described by Leenhart et al. had a familial history of sudden cardiac death and syncope[91]. However, in contrast to the above-described arrhythmias, no genetic cause or underlying pathophysiological mechanism has been identified. Torsade de pointes, as they occur in SCTP, are often supposed to be secondary to early after depolarizations (EADs). However, EADs have the prerequisite of a prolonged cellular action potential and bradycardia dependence which doesn't fit with the short-coupling interval and the rarity of pause-induced arrythmias in SCTP. Though EADs are known to be slow-channel dependent, which fits with the effect of verapamil in SCTP, which inhibits the slow inward calcium current. Alternatively delayed afterdepolaritzations (DADs) might underlie SCTP, as DADs occur in the presence of intracellular calcium overload and are effectively surprised by verapamil. Therefore DADs might be also be proposed as the mechanism of the first beat of the torsade, although the short-coupling interval would not be easy to explain. A hint as to the underlying mechanism comes from the case report of a Japanese patient in whom the SCTP occurred in concurrence with (inducible) hypokalemia, subsequent restoration to normal potassium levels prevented SCTP[93]. Genes involved in the potassium transport and handling might therefore be prime candidates for this disease. However, taken together it is as yet unclear what the exact underlying mechanism is for SCTP, and therefore it is hard to speculate on candidate genes. Prime candidates are genes associated with the above-mentioned arrhythmias, such as those involved in the Long-QT syndrome, the Brugada syndrome and CPVT.

Atrial fibrillation

As explained earlier, atrial fibrillation is characterized by rapid and irregular activation of the atrium. It is a common arrhythmia whose molecular aetiology is poorly understood. However, a new study has shed light on the molecular background of atrial fibrillation and suggests that atrial fibrillation is, in some cases, also associated with an underlying familial component.

Genetic basis

Recently, a family with hereditary persistent AF was identified and a causative mutation in the KCNQ1 gene was found[94]. The KCNQ1 gene encodes the pore-forming α subunit of the cardiac I_{ks} potassium channel. Functional analysis of the mutant revealed a gain-of-function effect on I_{ks} currents, which contrasts with the dominant negative or loss-of-function effects of the KCNQ1 mutations previously identified in patients with long QT syndrome (see earlier). Thus, the mutation is likely to initiate and maintain AF by reducing action potential duration and effective refractory period in atrial myocytes. It is thus conceivable that other mutations or common polymorphisms might underlie further cases of AF in the general population. Systematic screening of KCNQ1 in AF families and in AF patients without systemic (heart) disease could substantiate this.

Summary

Sudden cardiac death is a major health problem associated with significant mortality and morbidity. It accounts for approximately 250000-400000 deaths per year in the United States and is the most common cause of death in adults in the United States. It is associated with the occurrence of lethal disturbances of the cardiac rhythm, so called ventricular tachyarrhythmias. Most of these sudden cardiac deaths occur in the setting of structural heart diseases such as coronary artery disease, congestive heart failure, and ischemia. In contrast, sudden cardiac death solely due to primary ventricular fibrillation, i.e., without apparent evidence of structural heart disease, occurs in ±5% of victims of sudden cardiac death. Moreover, survivors of SCD in this category have a very high recurrence rate of 30% within 3 years for VF, syncope, and cardiac arrest. Recently, many of these so-called idiopathic SCDs were shown to be familial and shown to have an underlying genetic cause. These heritable cardiac arrhythmia diseases have since been named 'ion channelopathies'. The morbidity and mortality of these ion channelopathies are significant and research has started to define the problems at a clinical and molecular level. Options for treatment include pharmacotherapy, and implanted devices. Although antiarrhythmic medications can greatly reduce the arrhythmic events in the ion channelopathies, their systemic effects are sometimes poorly tolerated; in addition, their

paradoxical ability to make some arrhythmias worse while treating others can actually increase mortality in some specific situations. Implanted devices (such as pacemakers and defibrillators), although palliative, are nonetheless very effective. However, this strategy does not prevent tachyarrhythmias and is associated with a lifetime commitment to repeated procedures, significant expense and potentially complications. Therefore, given the high risk of sudden cardiac death, the imperfect therapeutic options at hand, and the many familial occurrences of ion channelopathies, it is of utmost importance to uncover the various molecular and genetic backgrounds of these lethal inheritable cardiac arrhythmias; with detailed genetic information, it would be possible to understand the underlying mechanisms, develop new therapeutic options, carry out preventive strategies, establish early diagnoses, and perform pre-symptomatic genetic screenings.

Scope of the thesis

The scope of the thesis is to uncover the various genetic and clinical aspects of inheritable cardiac arrhythmias in order to establish a genotype-phenotype relation.

To uncover the genetic and clinical aspects of cardiac arrhythmia syndromes we have used an array of molecular and mostly genetic techniques, including sequence analysis, PCR, SSCP, RFLP, cloning, linkage analysis, RT-PCR, genome walking, radiation hybrid mapping, genotyping, extended PCR, inter-sequence comparisons, statistics, functional expression, and phylogenetic comparisons.

In *chapter 2,* we describe a large 8-generation kindred characterized by a high incidence of nocturnal sudden death, and QT-interval prolongation and the "Brugada ECG" occurring in the same subjects. We identified a mutation in the *SCN5A* gene, 1795insD, which is causally linked to both the QT-interval prolongation and the "Brugada ECG", indicating that the Long QT syndrome and the Brugada syndrome are more closely related than heretofore appreciated and can even be caused by the same mutation. As we were unable to find a causal mutation in the majority of Brugada patients, we followed a candidate gene approach for the involvement of *KCND2* and *KCND3* ion channels in the pathogenesis of LQTS and Brugada syndrome. We describe the genomic and chromosomal localization and the mutational screening of these genes in *chapter 3*. In *chapter 4,* we have aimed to (1) summarize the clinical data of the various recent publications on catecholaminergic polymorphic ventricular tachycardia (CPVT), (2) describe the underlying molecular causes and pathophysiological mechanism(s), and (3) propose diagnostic guidelines. In *chapter 5,* we report on a study of the autosomal recessive form of CPVT and the identification of the first nonsense mutations in the

cardiac calsequestrin gene, *CASQ*2, causally linked to CPVT. In *chapter 6,* we describe the assessment of underlying genetic causes, clinical features, and response to therapy in 24 CPVT probands, and report on the identification of missense mutations in the cardiac ryanodine receptor (*RYR2*) in 13 of them. In *chapter 7,* we describe the screening of a group of 30 CPVT patients, in 12 of whom we were unable to find an underlying mutation in either the *RYR2* or the *CASQ2* gene, for mutations in the *Kir2* gene family. Systematic screening of all the four members of the *Kir2* gene family led to the identification of an autosomal dominant missense mutation in the *Kir2.1* gene, R67W, which associates solely with CPVT without any features of Andersen syndrome, and the identification of multiple *Kir2.2* pseudo-genes.

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Chapter **2**

A Single Na^+ Channel Mutation Causing Both Long-QT and Brugada Syndromes

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Abstract

Mutations in *SCN5A*, the gene encoding the cardiac Na^+ channel, have been identified in 2 distinct diseases associated with sudden death: one form of the long-QT syndrome (LQT_3) and the Brugada syndrome. We have screened *SCN5A* in a large 8-generation kindred characterized by a high incidence of nocturnal sudden death, and QT-interval prolongation and the "Brugada ECG" occurring in the same subjects. An insertion of 3 nucleotides (TGA) at position 5537, predicted to cause an insertion of aspartic acid (1795insD) in the C-terminal domain of the protein, was linked to the phenotype and was identified in all electrocardiographically affected family members. ECGs were obtained from 79 adults with a defined genetic status (carriers, n=43; noncarriers, n=36). In affected individuals, PR and QRS durations and QT intervals are prolonged ($P<0.0001$ for all parameters). ST segment elevation in the right precordial leads is present as well ($P<0.0001$). Twenty-five family members died suddenly, 16 of them during the night. Expression of wild-type and mutant Na^+ channels in *Xenopus* oocytes revealed that the 1795insD mutation gives rise to a 7.3-mV negative shift of the steady-state inactivation curve and an 8.1-mV positive shift of the steady-state activation curve. The functional consequence of both shifts is likely to be a reduced Na^+ current during the upstroke of the action potential. LQT_3 and Brugada syndrome are allelic disorders but may also share a common genotype.

Key Words

Long-QT syndrome, Brugada syndrome, *SCN5A*, arrhythmia, Na^+ channel

Introduction

SCN5A, the gene that encodes the human cardiac Na^+ channel α subunit,[1] is mutated in one form of the long-QT syndrome (LQT_3) and in Brugada syndrome.[2,3] There are characteristic and readily distinguishable ECG patterns in these 2 syndromes. In LQT_3 patients, a long isoelectric ST segment precedes a peaked T wave.[4] Brugada syndrome is diagnosed on the basis of characteristic ECG features in the absence of structural heart disease; these features include right precordial ST-segment elevation, which may be intermittent, and which is exacerbated by Na^+ channel block and ameliorated by isoproterenol.[5],[6] QT intervals have been reported to be normal in patients with Brugada syndrome.[5] Clinically, there appears to be some overlap between the 2 syndromes, as both exhibit a relatively high incidence of nocturnal sudden cardiac death without prior symptoms.[6-8]

The prolonged QT interval in LQT_3 results from persistent inward Na^+ current during the plateau phase of the action potential, secondary to incomplete inactivation of mutated channels.[9] Changes in the α- and β_1 subunit interaction have also been implicated.[10] Although functional abnormalities have been described for Brugada syndrome–related *SCN5A* mutant channels,[3,11] the mechanism(s) whereby these explain the Brugada phenotype are less clear.

In this study we present clinical and genetic data of a single large *SCN5A*-linked family, phenotypically characterized by nocturnal death and electrocardiographically by both LQT_3 and Brugada syndrome features in the same patients. We show that LQT_3 and the Brugada syndrome are more closely related than heretofore appreciated and can even be caused by the same mutation. We also report on the functional consequences of the Na^+ channel mutation involved, as revealed by measuring Na^+ channel activity in the *Xenopus* oocyte expression system.

Material & Methods

Patients

The study was performed according to a protocol approved by the local ethics committees. Informed consent was obtained from all patients, who were all screened by one of us. For purposes of linkage analysis, the phenotype was assigned in a subset of the family according to the criteria described in the online Materials and Methods (see http://www.circresaha.org).

After the mutation was identified (see below), a careful history, an ECG at rest and in the supine position, and peripheral blood samples for genotype analysis were taken from as many other family members as possible. Patients with specific reasons for prolongation of the QT interval were excluded from further (electrocardiographic) analysis. We analyzed 12-lead ECGs (paper speed, 25 mm/s), only of patients ≥16 years of age, with particular reference to rate and to PR, QRS, and QT interval (the longest interval in any lead was taken). QTc was calculated according to the Bazett formula. In the final analysis, mutation carriers either are obligate carriers (by pedigree analysis) or have a proven genetic status (see below). Individuals within this family from which no DNA or ECGs were available were defined as mutation carriers when they died suddenly and unexpectedly under typical circumstances (see below).

Signal-averaged electrocardiography (SAECG) was performed in the supine position according to the method previously described for a subset of these patients.[12] We considered the SAECG abnormal according to the predefined criteria.[12] In 5 individuals, an invasive electrophysiological study by standard procedures was performed.

Linkage analysis

Genotyping of microsatellite markers around the known LQT (*SCN5A*, LQT_4, *HERG*, *KCNQ1*, and *KCNE1*) and Brugada syndrome (*SCN5A*) loci was performed by standard, semiautomated methods. Linkage analyses were carried out using the MLINK program from the LINKAGE 5.1 package.

Mutation analysis of SCN5A

Mutation analysis of *SCN5A* was done by single-strand conformation polymorphism (SSCP) analysis followed by direct sequence analysis of aberrant conformers. All 28 exons of the *SCN5A* gene were amplified using intronic primers designed by Wang et al[13] and analyzed in this way. Independent of the outcome of SSCP analysis, 12 exons (2, 3, 12, 17–21, 24, 26–28) were also analysed by direct sequence analysis.

Functional expression

Mutant Na^+ channel cDNA was prepared by mutagenesis on the double-stranded pSP64T-hH1(sp) plasmid.[1,14] Wild-type (WT) and mutant constructs were linearized and cRNAs were synthesized. cRNA concentration was determined spectrophotometrically at a 260-nm wavelength.

Electrophysiology

Stage V through VI *Xenopus* oocytes were isolated and injected with 5 to 20 ng of cRNA according to standard methods.[15] Voltage-clamp experiments were performed 2 to 4 days after injection, using a Geneclamp 500 2-electrode voltage clamp amplifier (Axon Instruments). Na^+ currents were corrected for leakage current using Geneclamp leak subtraction. Steady-state activation and inactivation parameters were determined using protocols similar to those published previously by Wang et al.[16] Electrophysiological experiments were performed at room temperature (21°C).

Statistical analysis

Differences between groups (mutation carriers and noncarriers) were compared by the Fischer exact test or unpaired Student *t* test, where appropriate. A 2-tailed probability value of <0.05 was considered statistically significant. In electrophysiological studies, differences between WT Na^+ current and mutant Na^+ current were compared using the unpaired Student *t* test.

Results

The anonymized pedigree of the family is presented in Figure 1. Linkage analysis in a subset of the family (11 affected and 12 unaffected) revealed linkage to SCN5A (Table 1), whereas no evidence of linkage was detected to the LQT4, HERG, KCNQ1, and KCNE1 loci. Subsequent SSCP analysis of the coding region of SCN5A using primers

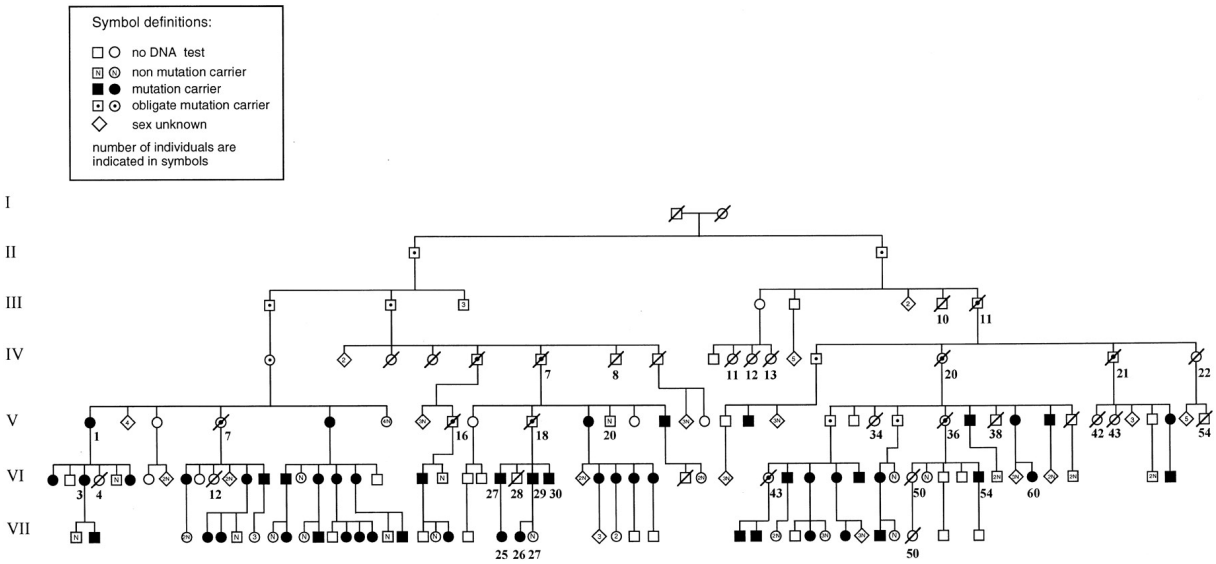

Figure 1. Pedigree of the family. Seven generations are presented (for reasons of privacy, the eighth generation is not depicted). Persons identified by genotype analysis as carriers of the 1795insD mutation are indicated by filled circles (females) or squares (males). Further explanation is given in the figure. The pedigree has been altered to protect confidentiality.

TABLE 1. Linkage Analysis of Chromosome 3p21 Markers

	Lod score at θ =				
Marker	0	0.1	0.2	0.3	0.4
D3S1211	-∞	2.26	1.72	1.01	0.32
SCNI16	3.68	2.82	2	1.2	0.52
SCN5A 1795InsD	6.5	5.41	4.2	2.85	1.35
D3S1298	2.6	2.26	1.78	1.19	0.54
D3S1100	5.78	4.69	3.48	2.16	0.85

flanking the exon-intron boundaries identified an aberrant conformer in exon 28 in affected family members (Figure 2; n=53, including children). The aberrant conformer was not present in unaffected family members nor in 100 alleles from unrelated control individuals. DNA sequencing of exon 28 of affected family members revealed heterozygosity for a TGA insertion at position 5537. This insertion results in the insertion of aspartate after tyrosine 1795 (1795insD) within the highly negatively charged region of the C-terminal domain of the protein. SSCP analysis of all the other exons and direct sequence analysis of a large part of the coding region (see Materials and Methods) in 3 affected individuals (VI-27, VI-29, and VI-30) revealed no further abnormalities.

We were able to trace the history of 203 family members in 8 generations (Figure 1). ECGs were obtained from 119 individuals, of whom 79 adults had a defined genetic status (mutation carriers, n=43; noncarriers, n=36). Figure 3 demonstrates an example

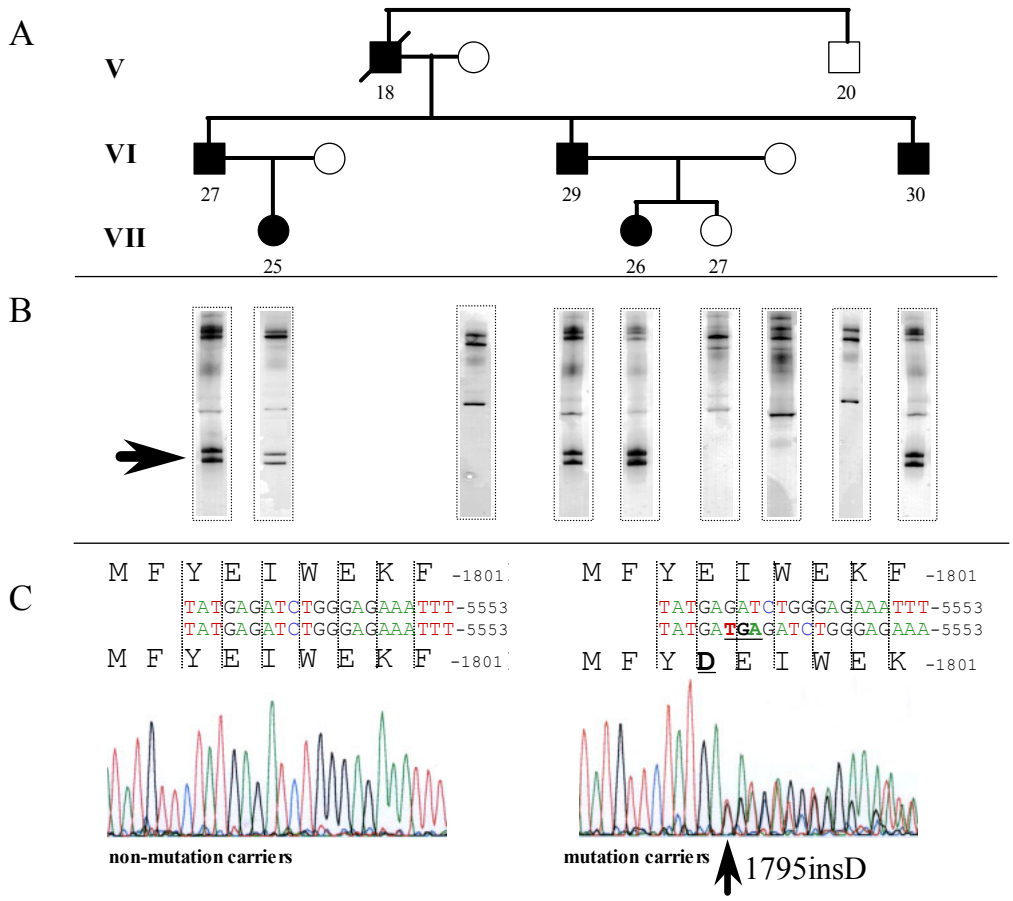

Figure 2. Insertion of 3 nucleotides (TGA) at position 5537, resulting in the addition of aspartic acid in the *SCN5A* protein of affected individuals. A, Representative part of the pedigree (for symbols see Figure 1). B, SSCP analysis of exon 28 of *SCN5A*, with a distinct shift (arrow) in mutation carriers. C, Sequence analysis of exon 28 of *SCN5A* in mutation carriers and non–mutation carriers, showing the insertion of TGA resulting in the addition of aspartic acid after the tyrosine at position 1795 of the *SCN5A* protein (arrow).

(patient VI-27). Heart rate is relatively slow, PR and QRS durations are slightly prolonged, and the QT interval is markedly prolonged (Figure 3B). In the right precordial leads, ST-segment elevation is apparent (Figure 3A). Table 2 summarizes basic demographic and electrocardiographic data of the 79 genotyped family members. Whereas sex and age are similar in affected and nonaffected members, mean heart rate is slightly lower (P<0.02), and conduction parameters (PR and QRS intervals) are slightly prolonged in mutation carriers (for both parameters; P<0.0001). In addition, HV interval was prolonged in 4 of the 5 carriers in whom an invasive electrophysiological study was performed: 58, 78, 75, and 80 ms in V-1, VI-27, VI-54, and VI-60 respectively, and 50 in VI-3. SAECG was abnormal in 23 of 29 mutation carriers tested (79%) and abnormal in 2 of 14 noncarriers (14%; P<0.001). Figure 4 depicts normalized QT intervals (QTc) versus heart rate in analyzed patients. QTc was clearly prolonged in the vast majority of mutation carriers, in particular in those in whom heart rate is slow. PR and QRS prolongation was concomitantly present in 14 carriers, whereas only PR or only QRS prolongation was seen in 10 carriers (and in 6 noncarriers) and 10 carriers (and in 1 noncarrier), respectively (Figure 5A). ST-segment elevation was present in 21 of the 43 carriers versus 3 of 36 noncarriers (P<0.001; mean values in Table 2). Figure 5B shows QTc intervals versus ST segment elevation in individuals. In 16 carriers, both QTc is prolonged and right precordial ST segments are elevated. In 13 carriers, only QTc was prolonged, whereas in 5 carriers (and 3 noncarriers) only ST-segment elevation was apparent. In all 3 carriers (VI-27, VI-29, and VI-

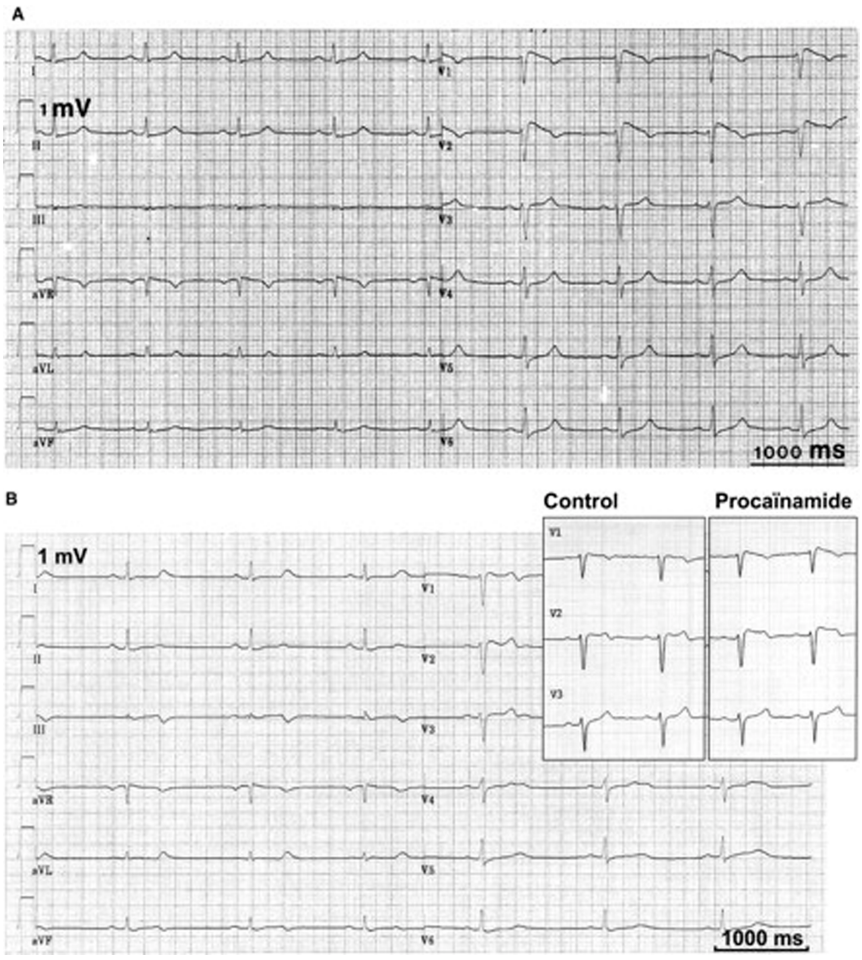

Figure 3. ECG recordings of patient VI-27. A, ECG at first visit. Note the prolonged PR interval and the marked ST-segment elevation in lead V_{1-3}. QTc is slightly prolonged (465 ms). B, During sinus bradycardia (cycle length 1600 ms), QTc prolongs further (QT 670 ms, QTc 530 ms). Inset, Selected recordings (V_{1-3}) are depicted shortly before and after 250 mg of procainamide. Note the increase in ST-segment elevation after drug exposure. Calibrations are standard.

TABLE 2. Patient Basic Demographic and ECG Characteristics (mean±SD)

	Number	Male/Female %	Age, y	Rate, bpm	QTc, ms	PQ, ms	QRS, ms	STelev, mm
Carriers	43	47/53	41.0±14.31	67.5±16.41	489.4 ±44.09	201.0±19.71	117.6±14.36	1.58±1.04
Noncarriers	36	47/53	39.4±18.37	74.5 ±13.50	403.7±24.82	156.9±31.71	93.4±13.25	0.36 ±0.56
Significance, *P* <			NS	0.02	0.0001	0.0001	0.0001	0.0001

PR and QRS are the PR interval, measured from the onset of the P-wave to the onset of the QRS complex, and the duration of the QRS complex, respectively. For measurement of QTc and ST segment elevation (STelev)

30) who received a bolus procainamide (250 mg IV), ST-segment elevation was increased further (see inset, Figure 3B). There were no echocardiographic abnormalities in 29 mutation carriers.

Unexpected nocturnal sudden cardiac death was the only symptom in this family, occurring in 16 family members since 1905 (10 female, 6 male; see online Table, available at http://www.circresaha.org). Eight patients died suddenly under unknown circumstances. One died in the chair of the barber while being shaved (IV-8). Death was witnessed in 5 cases, occurring between 4:00 and 7:00 AM, and the episodes were characterized by sudden onset of gurgling and gasping, and moaning respiration. Patients were unconscious and could not be awakened. No electrocardiographic recordings are available from these episodes. Previous ECGs were available in 4, all demonstrating bradycardia with significant QT-segment prolongation. Nine victims were obligate carriers of the aberrant gene. Three clinically affected individuals have been evaluated in hospital, and sudden arousal during the early morning hours did not reveal any (additional) electrocardiographic abnormality. The mean age (±SD) of sudden cardiac death victims was 32.3±14.63 (n=22), with 19 individuals ≤40 years (male/female ratio, 9/10).

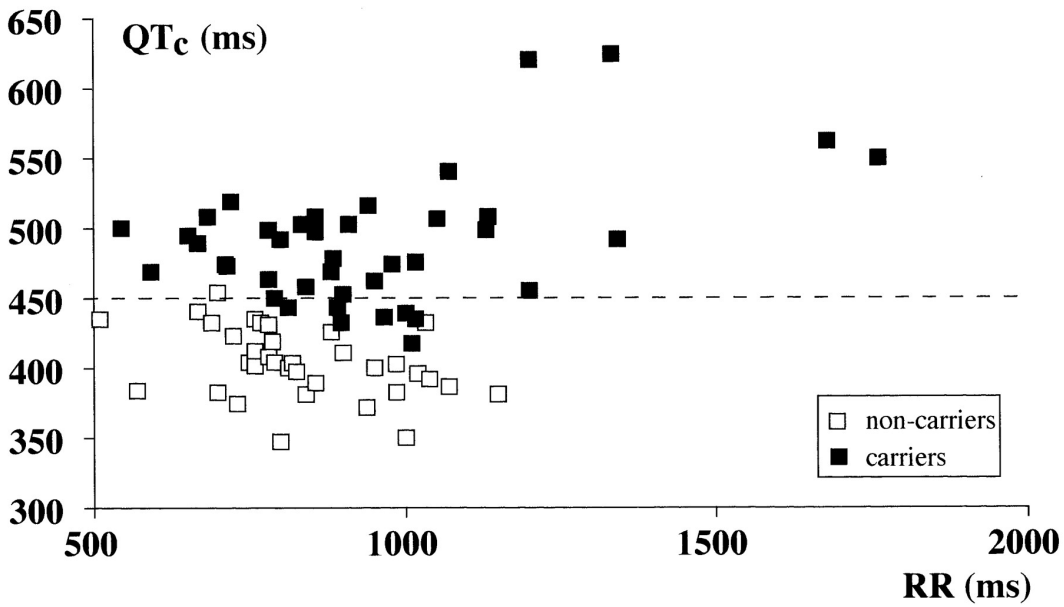

Figure 4. QTc vs RR interval in all individuals analyzed (43 mutation carriers [■] and 36 noncarriers [□]). QT intervals are particularly prolonged at long RR intervals. However, also at normal heart rate (RR 600 to 1000 ms), QT interval is prolonged in most carriers

To establish the consequences of the 1795insD insertion on the electrophysiological properties of the Na^+ channel, macroscopic Na^+ currents (I_{Na}) were recorded in oocytes injected with cRNA encoding either the WT or the 1795insD mutant Na^+ channel α subunit. Figure 6A shows typical families of Na^+ current traces elicited by 5-mV depolarizing steps between -90 and +40 mV from a holding potential of -100 mV. There was a striking difference in peak amplitudes between the WT Na^+ current (I_{Na},WT, Figure 6A, left) and the 1795insD mutant Na^+ current (I_{Na},1795, Figure 6A, right), despite the fact that similar amounts of cRNA were injected. The average (±SEM) current-voltage relations in Figure 6B show that the maximal I_{Na} amplitude was 9.9±1.7 μA (n=14) and 2.2±0.5 (n=22) for I_{Na}, WT and I_{Na},1795 respectively. The averaged data were obtained from 6 different batches of oocytes. The much larger peak amplitude of WT Na^+ currents compared with 1795insD Na^+ currents was a consistent finding. In addition, the voltage for both the threshold of activation and the maximum peak current was shifted by +5 mV for 1795insD channels. We also determined the steady-state voltage dependence of activation and inactivation for I_{Na},WT and I_{Na},1795, as illustrated in Figure 6C. The averaged data points of the inactivation curve were fitted with a Boltzmann function with V1/2 of -78.7 mV and a k of -4.5 for the WT Na^+ channel (n=21) and a V1/2 of -86.0 mV and a k of -5.0 for the 1795insD mutant Na^+ channel (n=22). These results indicate a negative shift of the inactivation curve of the 1795insD mutant by 7.3 mV. The averaged data points of the activation curve were fitted with a Boltzmann function with a V1/2 of -40.2 mV and a k of 5.3 for the WT Na^+ channel (n=20) and a V1/2 of -32.1 mV and a k of 5.7 for the 1795insD mutant Na^+ channel (n=22), resulting in an 8.1-mV positive shift of the activation curve of the 1795insD mutant. Both shifts will result in a reduced Na^+ current during the upstroke of the action potential and a reduced Na^+ window

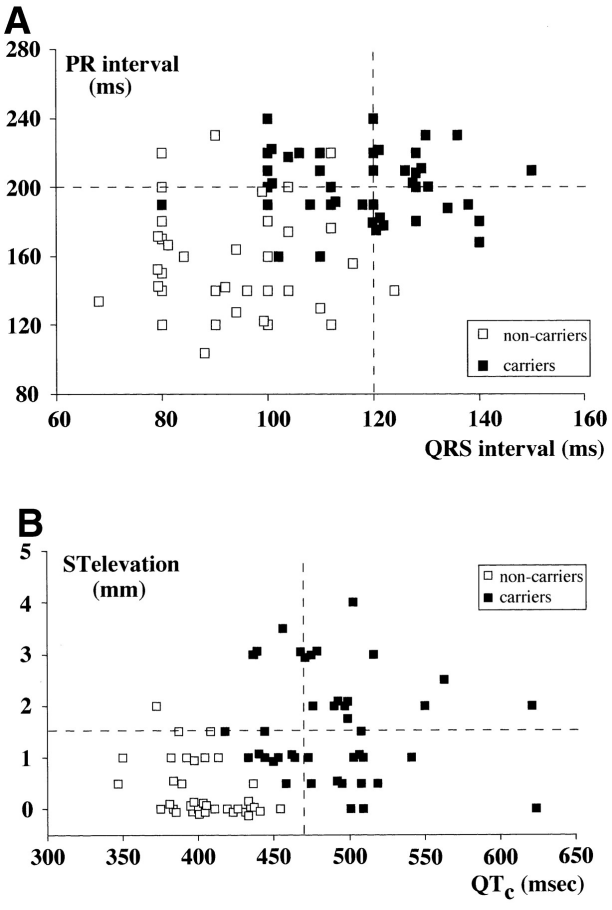

Figure 5. PR vs QRS interval (A) and ST-segment elevation vs QTc (B). A, Upper limit of normal values is indicated by dotted lines. B, Values indicated by dotted lines are those used for the linkage study. Note that exclusively mutation carriers have combined PR and QRS interval prolongation (A) and combined ST-segment elevation and QTc prolongation (B). See text for further discussion.

current. Recovery from inactivation (Figure 6D) was slightly, but significantly, slower for the 1795insD mutant channel. When the data were fitted with a single exponential function, mean time constants (±SEM) were 12.2±0.6 ms (n=22) and 14.7±0.7 ms (n=22) for the WT and the 1795insD mutant Na^+ channel, respectively.

Because LQT3 has been associated with incomplete inactivation of the Na^+ channel, resulting in a persistent Na+ current, we sought to determine whether a reduced rate of inactivation or incomplete inactivation was also present in our 1795insD mutant Na^+ channel. I_{Na},WT and I_{Na},1795 were recorded at -20 mV, and current decay was fitted with either a single- or double-exponential function (not shown). The results showed that both the fast and the slow time constant of inactivation were only slightly, and not significantly, larger for the 1795insD Na^+ channel (mean±SEM, WT: τ fast=0.98±0.06, τ slow=7.1±0.7 [n=20]; 1795insD: τ fast=1.15±0.06, τ slow=10.49±2.9 [n=22]). Also, the study of procainamide- and tetrodotoxin-sensitive 1795insD Na^+ currents did not reveal the presence of a persistent inward current. Because 1795insD Na^+ currents were of very small amplitude, we considered the possibility that a persistent inward current, usually

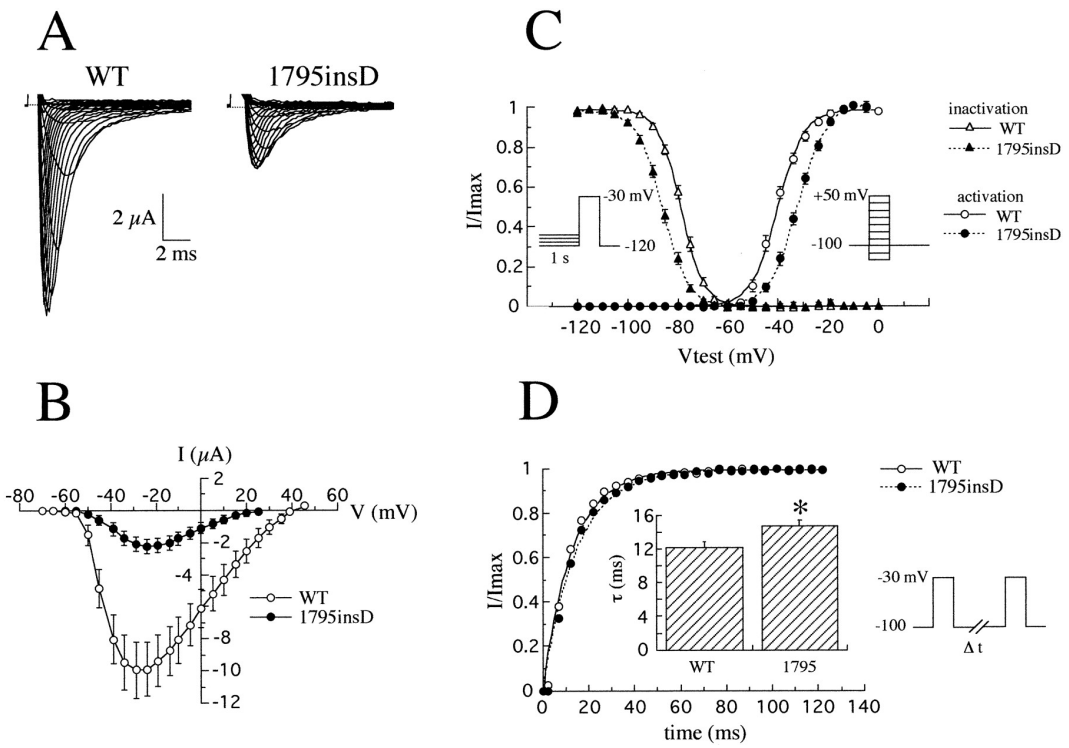

Figure 6. Comparison of electrophysiological characteristics of WT and 1795insD mutant Na^+ channels expressed in *Xenopus* oocytes. A, Examples of Na^+ current traces elicited by 5-mV progressive step depolarizations between -90 and 40 mV from a holding potential of -100 mV. Left, WT Na^+ currents. Right, 1795insD mutant Na^+ currents. Note the differences in peak current amplitudes. B, Average current-voltage relationship for the WT (○, n=14) and the 1795insD mutant (•, n=22) Na^+ channel. Data are mean±SEM. C, Steady-state voltage dependence of activation for WT (○, n=20) and 1795insD mutant (•, n=22) Na^+ channels, and of inactivation for WT (△, n=21) and 1795insD mutant (▲, n=22) Na^+ channels. See text for further discussion. D, Recovery from inactivation of WT (○) and 1795insD mutant (•) Na^+ channels at –100 mV. Inset, Bar histogram showing average time constants for WT (n=22) and 1795insD mutant (n=22) Na^+ channels. See text for further discussion. *P<0.05.

<2% of the peak current, was too small to distinguish. Unfortunately, attempts to increase the expression level by injecting 5 to 10 times higher amounts of cRNA increased peak 1795insD Na^+ currents no further than 3 μA.

Discussion

We describe a single, large *SCN5A*-linked family with phenotypic characteristics of both LQT_3 and Brugada syndrome in the same patients. QT-interval prolongation and abnormal T-wave configuration are seen particularly at slow heart rates, and as shown previously in individual patients in this family, normalization occurs as rate increases.[12] Similar steep APD- and QT-rate relationships have been observed in LQT_3 patients[7] and in experimental models mimicking LQT_3.[17] It is likely that normal K^+ currents result in physiological or supraphysiological cardiac repolarization during fast rates in these patients. In contrast, incomplete inactivation of I_{Na} (as a result of mutation in the *SCN5A* gene) results in abnormal repolarization at slow rates.[7,9] Further compounding the QT abnormality is the bradycardia seen in affected patients (Figure 4), as has been reported for other LQT_3 patients.[10] It is likely that these bradycardia-induced QT-interval abnormalities and resultant torsade de pointes underlie the high incidence of nocturnal sudden death in this and other LQT_3 families.[7,8] Alternatively, a high vagal tone, likely of importance in Brugada syndrome–related ventricular fibrillation (see below),[18] may be causally related to nocturnal death in this family.

The Brugada syndrome is characterized by right precordial ST-segment elevation with or without apparent right ventricular conduction delay.[5,6,19] More general, mild, intraventricular conduction defects are usually also present, manifested by prolonged HV intervals and abnormal QRS axes.[8] The ST abnormalities can be transient and can be modified by pharmacological interventions. Brugada syndrome patients present with syncope or out-of-hospital cardiac arrest and may have a family history of sudden cardiac death. Asymptomatic individuals appear to have a poor prognosis.[5] Sudden cardiac death and documented episodes of ventricular fibrillation without antecedent QT abnormalities occur preferentially at night. This is especially the case in the syndrome of sudden unexplained death in young South-Asian men, which appears to be part of the clinical spectrum.[20] Causally related enhancement of vagal tone, just before the (fatal) arrhythmia, has been suggested,[18] and acetylcholine augments the ST-segment changes.[21] Note that 1 of our patients died suddenly while being shaved (potentially a carotis sinus massage, which may increase vagal tone). In the family, significant ST-segment elevation was present in 49% of patients. Procainamide exaggerated the effects in the 3 patients in

which it was tested (Figure 3). In addition, intraventricular conduction defects (prolonged QRS and HV interval, late potentials) were generally present. Although the conduction delay is mild, the concomitant presence of both PR and QRS conduction delay in a significant subset of affected patients (Figure 5A) suggests a hampered conduction in different cardiac compartments. These features are all compatible with the Brugada syndrome diagnosis. Importantly, 16 affected subjects displayed both right precordial ST-segment elevation and QT-prolongation (Figures 3 and 5B).

In affected family members, 3 nucleotides (TGA) were inserted at nucleotide position 5537. This mutation gives rise to the insertion of a charged amino acid (Asp) after residue 1795 in the C-terminal end of the α subunit of the Na^+ channel (1795insD). No further abnormalities were found in *SCN5A*. LQT_3 (by definition) and Brugada syndrome are allelic disorders with involvement of *SCN5A*.[2,3] Mutations in the C-terminal end have been linked to both syndromes in individual families or patients.[10,11] The typical ECG features associated with the Brugada syndrome, as well as the ventricular and atrial conduction delays in 1795insD-affected patients, suggest a reduction in I_{Na} amplitude. It can be postulated that the balance between inward and outward currents during the characteristic phase 1 of the epicardial action potential may be shifted toward (enhanced) repolarization by the reduction in I_{Na} amplitude. Loss of the epicardial action potential plateau phase may ensue and cause transmural heterogeneity and ST elevation as a result of transmural current flow from endocardium to epicardium.[6,22] The functional consequences of 1795insD may particularly affect the contribution of the right ventricle, where epicardial action potentials are proportionately well represented and exhibit a particularly well established "spike and dome morphology".[23] Indeed, in our and other patients with the Brugada syndrome,[6,21] reduction of I_{Na} by Na^+ channel blockers augments the ST-segment abnormalities (Figure 3). The results from our expression study are in line with a reduced Na^+ current. We found a 7.3-mV negative shift of the steady-state inactivation curve and an 8.1-mV positive shift of the steady-state activation curve of the 1795insD mutant channels. The functional consequence of both shifts is likely to be a reduced Na^+ current during the upstroke and phase 1 of the action potential. Moreover, 1795insD Na^+ currents had 5-fold smaller amplitudes than WT currents, which is less than expected on ground of the shifts in activation- and inactivation curves alone. It suggests the presence of additional differences, such as a reduced Na^+ channel density or conductance. Our findings are different from functional characterization of the Brugada syndrome *SCN5A* mutations described so far. These included faster recovery from inactivation and a negative shift of the steady-state activation curve.[3,11]

It is difficult to link the prolonged QT interval in these patients to the observed kinetic characteristics of the 1795insD mutant channel. In general, prolongation of the repolarization process suggests an increase in net inward current during the plateau phase of the action potential. Indeed, it has been shown that persistent inward Na^+ current, secondary to incomplete inactivation, underlies LQT_3.[9] Analysis of procainamide- and tetrodotoxin-sensitive currents did not reveal such a persistent Na^+ current in our study. The observed small increase in inactivation time constants is probably not sufficient to account for the prolonged QT interval, certainly not in view of the overall reduction in I_{Na} amplitude.

To ultimately decide on the presence (or absence) of a persistent inward current, further experiments are needed. Several factors may have hampered its detection in the present study. First, experiments were performed at room temperature. It has been shown that the kinetics of both WT and ΔKPQ Na^+ channels are highly sensitive to temperature, having 2-fold faster activation and inactivation kinetics at 33°C compared with 23°C and a positive shift of the activation and steady-state inactivation at the higher temperature.[24] Second, ion channel properties may be dependent on the expression system.[25] In the present study Na^+ channels were expressed in a nonmammalian system. Third, it has been shown that the nearby D1790G and E1784K mutations affect the voltage dependence of I_{Na} inactivation by altered interaction between the α- and β_1 subunit.[10,26] Interaction of the β_1 subunit with the α subunit also significantly affects I_{Na} amplitude.[27] Hence, it is conceivable that co-expression of the β_1 subunit with the 1795insD mutant αsubunit uncovers kinetic properties of the channel that may give rise to prolongation of the repolarization process. In this respect it should be noted, however, that co-expression of the α subunit with the β_1 subunit did not affect the persistent inward current in case of the E1784K mutant.[26] Finally, single-channel measurements in multichannel patches may be used to reveal late openings indicative for the presence of a persistent current.

In summary, we describe a large *SCN5A*-linked family, characterized by QT prolongation, in particular during bradycardia; discrete conduction disturbances throughout the heart; and nocturnal sudden cardiac death. Electrocardiographic features of LQT_3 and Brugada syndrome are present in the same (affected) individuals, demonstrating that LQT_3 and Brugada syndrome are more closely related than heretofore appreciated. Both syndromes can even be caused by the same mutation.

Acknowledgments

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Chapter **3**

Genomic Organisation and Chromosomal Localisation of Two Members of the KCND Ion Channel Family, KCND2 and KCND3

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Abstract

To follow a candidate gene approach for the involvement of the *KCND2* and *KCND3* genes (*Kv4.2* and *Kv4.3*) in the pathogenesis of the long QT syndrome (LQTS) and Brugada syndrome, it is necessary to determine the genomic organisation of *KCND2* and *KCND3*. We therefore resolved the intron-exon boundaries and flanking intronic sequences and found that *KCND2* consisted of six exons and *KCND3* of seven exons. Subsequently, we designed the oligonucleotide primers needed for amplifying the coding exons of both *KCND2* and *KCND3* and established conditions for polymerase chain reaction amplification of each exon from genomic DNA. Furthermore, the chromosomal localisation of *KCND2* and *KCND3* was determined as 7q31 and 1p13.2, respectively. Subsequent screening of 21 Brugada patients, who did not have mutations in the *SCN5A* gene (our data), resulted in four polymorphisms, none of which could be linked to the disease. This information should facilitate the systematic screening of *KCND2* and *KCND3* exons for mutations in (inherited) arrhythmia syndromes, such as LQTS and Brugada.

Introduction

The cardiac action potential is generated through the concerted action of many ion channels, pumps and transporters. Voltage-gated K^+ channels, in particular, underlie the repolarisation phase of the action potential. The transient outward current (I_{TO}) is especially important during the early phase of repolarisation in many species, including human, as it sets the plateau voltage of both the atrial and ventricular action potential[1-3]. Alterations in the amplitude or kinetics of I_{TO} may influence cardiac functions such as contractility[4] and impulse propagation[5] and can contribute to arrhythmogenesis under specific conditions. A mouse model presenting a functional knockout of I_{TO} shows marked increases in action potential durations in ventricular myocytes and prolongation of the QT interval in surface electrocardiogram (ECG) recordings[6].

Prolongation of the action potential has been linked to the long QT syndrome (LQTS)[7,8]. Brugada syndrome is characterised by right precordial ST-segment elevation and right bundle branch block in the absence of structural heart disease[9,10]; recently, a theory has been proposed in which an increase in I_{TO} might explain the ST-segment elevation observed in Brugada patients[11]. Both syndromes are cardiac disorders that predispose individuals to syncope, seizures and sudden death from ventricular tachyarrhythmias. Mutations in genes encoding ion channels involved in the generation of the cardiac action potential are causally related to both syndromes[12-14]. We have recently demonstrated that the LQT-type 3 and Brugada syndromes are more closely related than previously appreciated[15]. Furthermore, both diseases show genetic heterogeneity and there are descriptions of families and cohorts of patients who do not show linkage to any of the known disease related genes[16]. Thus, it is of importance to understand the molecular basis of the cardiac I_{TO} K^+ channel.

The gene underlying I_{TO} has been characterised as a member of the *KCND* subfamily (Kv4). The current consensus is that *KCND2* (*Kv4.2*) and/or *KCND3* (*Kv4.3*) code for the primary alpha subunits responsible for I_{TO}[17]. In the human ventricle, *KCND3* is the gene shown to encode the K^+ channel that underlies I_{TO}[18]. In the mouse and rat, KCND2 has been shown to be the major contributor to cardiac I_{TO}[19]. The cDNA of the human *KCND3* channel has been described and is abundantly expressed in both heart and brain[20,21]. Human *KCND3* has alternative splicing of a 19 amino acid insert that harbours a consensus protein kinase C phosphorylation site[20]. Experiments have shown that both the long and short splice variants are expressed in the human brain but that only the long form is found in the heart[21]; this is similar to findings regarding the two alternatively spliced forms of the rat KCND3 channel[22,23]. Recently, the human cDNA for *KCND2*

has been cloned and functionally characterised; no evidence of alternative splicing has been found[24].

It is necessary to determine the genomic organization of *KCND2* and *KCND3* to follow a candidate gene approach for the involvement of these genes in the pathogenesis of LQTS and Brugada syndrome. The identification of exon boundaries and the determination of intronic sequences flanking these boundaries should enable investigators to screen *KCND2* and *KCND3* systematically for mutations. Therefore, we have studied the genomic structure of *KCND2* and *KCND3*; in addition, we have localised the *KCND3* gene to 1p13.2.

Materials and methods

Polymerase chain reaction amplification of genomic DNA

Genomic DNA was extracted from peripheral blood lymphocytes by using standard protocols. A typical polymerase chain reaction (PCR) consisted of 10x PCR buffer (Perkin Elmer), 0.125 mM dNTPs (BM), 4 pmol primers, 1 U SuperTaq (HT Biotechnology), and 90 ng genomic DNA final concentration, in a total volume of 25 ml. PCR was performed in a PTC 200 (MJ Research) with a cycle sequence of 94°C for 60 s, 35 cycles of 94°C for 30 s, 55–68°C for 30 s (depending on the primer, see Table 1) and extension at 70°C for 45 s, and 70°C for 300 s. Subsequently, the products were analysed on a 1% ethidium bromide (EtBr) agarose gel.

Modified PCR amplification of genomic DNA

XT-PCR is a modification of the normal PCR process to accommodate for larger PCR products (>2 kb); two different polymerase enzymes are mixed in this reaction. A typical XT-PCR contained 0.275 mM dNTPs, 1 pmol primers, 5x PCR buffer (250 mM TRIS/HCl pH 9.2, 70 mM (NH)4 SO4 , 10% v/v dimethylsuphoxide and 1.5% v/v Tween 20), 2.25 mM MgCl2 , 1.75 U SuperTaq (HT Biotechnology), 0.0175 U PWO (a polymerase from *Pyrococcus woesei*; Boehringer Mannheim), 1 M betaine and 500 ng genomic target DNA (final concentrations) in a total volume of 25 ml. The XT-PCR was hotstarted and the following cycle sequence was used: 93°C for 1 min 15 s, 10 times 93°C for 15 s, 68°C for 30 s and 68°C for 4 min, followed by 20 cycles where the extension at 68°C was extended by 20 s each cycle. Subsequently, the products were analysed on a 1% EtBr agarose gel. PCR was performed in a PTC-200 (MJ Research).

Sequencing

PCR or XT-PCR products amplified from genomic DNA were purified by QiaQuick (Qiagen) microspin chromatography and sequenced in both directions by the fluorescent dideoxy chain termination method on an ABI 310 (Applied Biosystems) DNA sequencer with BigDye terminator (Perkin Elmer).

Genome walking

The human GenomeWalker kit (Clontech) was used to walk downstream and upstream of exons of KCND3 and KCND2. Primers complementary to KCND3 cDNA were used in combination with an outer adapter primer in primary PCRs by using five adapter ligated human genomic libraries as templates, as described by the manufacturer. Samples of the primary PCRs were used in secondary PCRs with a nested KCND3 primer and a nested adapter primer. Products of the secondary PCRs were resolved by agarose gel electrophoresis, purified by using the QiaQuick Gel extraction kit (Qiagen) and sequenced as described. Radiation hybrid mapping To establish the chromosomal location of KCND3, the Stanford G3 panel (Research Genetics) was used for radiation hybrid (RH) mapping[25]. PCRs for the RH assay were performed as described above with two KCND3 cDNA specific primers. Reactions were carried out in duplicate in two 96-well PCR plates with genomic DNA from two positive control samples (human genomic DNA), two negative control samples (hamster genomic DNA), and 83 RHs (Stanford G3 panel). The remaining nine wells in each plate contained water instead of genomic DNA. Each set of 96 samples was electrophoresed and gel images were captured. Each gel from the

Table 1. Forward and reverse PCR primers for KCND3 and KCND2

Gene/Exon	Forward primer	Reverse primer	Annealing
KCND3			
1	TGC TGA ACT AAC TCC AAG CTG G	CTC CTT GGT GTC CTC GTT GAA	60
1	GGT GGC CAA CTG CCC CAT GCC C	GTT CTC CCT CTT GCG GTC CTT	60
1	TGC TGC TAC GAG GAG TAC AAG	AGA AGA AGG CCA CCG AGT AG	60
1	TGG TGG AGA CGG TGC CGT GCG	CGG AGG CAC AGC TCT TCA GT	60
1	ACT GAA GAG CTG TGC CTC CG	GGC TGG TCT GCC CTC CAA CCT	60
2	AAC AGG TGA ATG ATT GGC AGG	AGC TCT AGT CCT GGC TCC CT	60
3	GGA AGC CAG CCT CAC AGC TTC	TGG TGA GAG TGC TGG TGT CCC	62
4	GCC CTT TGA CCT TTA GTG GAG A	GGC CCA GAG TGA AGA TGT GAG	60
5	AGG GGT GGA ATG TTT GAC TCA	AGA AGA ATC AGC AGC ACA TGC	60
6	CAG AAC AGA GAC AGG CAG CCA	GAT GAT TCG AGC CTT TGC GGG	62
7	CTC CTA GTT ACC ACG AGC AAG	AGT GAC CAC CCA CCA ACA TG	62
KCND2			
1	TGA CTT TTG GCT GCT TCG GT	GCT TCC CAG TGC GGT AGA AA	62
1	ATC TTC CGC CAC ATC CTG AA	GAT CCG CAC GGC ACT GTT TC	62
1	TTC ATT GCC GTC TCT GTC ATC	GAT CCT GAA GAC CCG GAA GAC	60
1	CAG CGG AGC CTT TGT CAC ACT	TGG GTC CAC AAT CGC CTC AC	62
2	GGA AAA AAT TCT TAG TTT CAG GTA G	CCA GAG CAC TGA ATA ACC ATT AAG	60
3	GGA TTA TAC AAG GGT TCA TTC ACT G	GGA TAG CCA TTG TCA TAA TTA GCC	60
4	GAA ATA TGT AGC CGA GGT TCG A	CGG AAG CAT TAA GTA TGC ACA A	58
5	ATT TCG TTT TTA AAA ATG TGC TTC T	CAG AAG ACA CAC CCT GGG AAC	60
6	GCA TTA CAA CAC ATA TTC TTC AGT TG	GTT TCT TCT TTC TTC TAT GTT GAC AT	58

duplicate pair was scored independently for the presence or absence of PCR products of the expected size. A hybrid was scored as positive only if a band of the expected size was present on each of the duplicate gels. Discrepancies between the two gels were designated as unknown or R in subsequent analysis. The resulting score was submitted to the RH mapping server at Stanford (http://www-shgc.stanford.edu/RH/rhserverformnew.html).

Results

Gene structure of *KCND3*

As a starting point for determining the genomic organisation of *KCND3*, the full length cDNA as published in Genbank was used (accession no. AF048713). Comparison between *KCND3*, *KCND2* and *KCND1* revealed some areas of homology and possible intron/exon boundaries. Primers were designed around these possible boundaries. A search of the high throughput genomic sequences (HTGS) and genome survey sequence (GSS) subdivisions of Genbank revealed two bacterial artificial chromosomes (BACs; CIT-HSP-2172p21.TR accession no. B94456; RPCI-11–99M15 accession no. AQ322365) that possibly contained an exon of KCND3. Upon sequencing of BAC CIT-HSP-2172p21.TR with exonic primers, we could establish the intron/exon boundaries of exon 4 of KCND3. These sequences were confirmed by designing intronic primers, amplifying the exon and subsequent sequencing from human genomic DNA. Furthermore, published GSS sequences of both BACs revealed intronic sequences around exons 2 and 7. The other exons were amplified by XT-PCR with exonic primers around presumed introns and by genome walking from known cDNA into introns. Using these techniques, we were able to determine that KCND3 consisted of seven exons, the largest being 1102

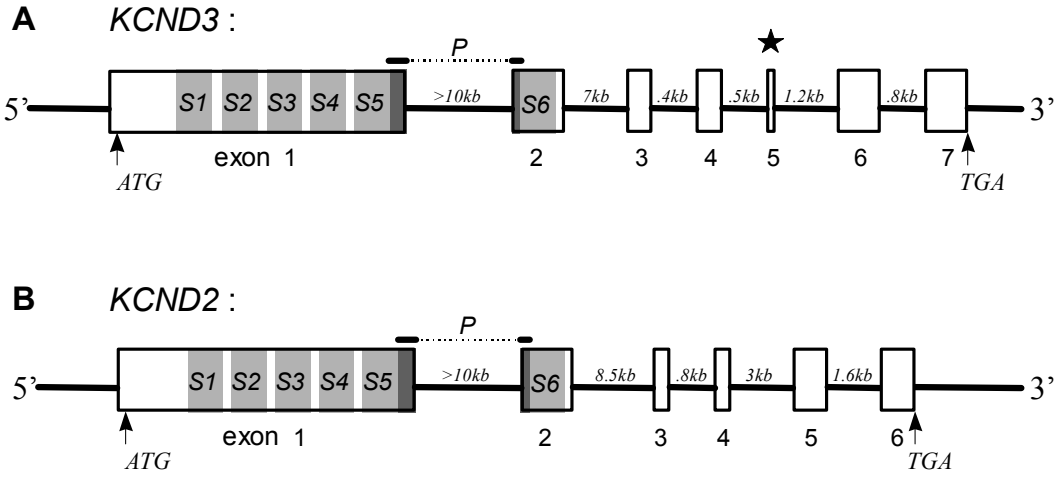

Figure 1. A Genomic structure of KCND3. Exons are represented as *boxes* and introns as *thick lines*, transmembrane segments are designated *S1-S6*, and *P* defines the pore region of the protein. *Asterisk* indicates the alternatively spliced exon 5, which has a putative phosphorylation site. Lengths of introns are roughly given based upon XT-PCR products. B Genomic structure of KCND2; lengths of introns are given based upon BAC sequence analysis

Table 2. Intron-Exon boundaries of KCND3

Acceptor site			Donor site		Codon
Intron	Exon	Exon (bp)	Exon	Intron	
5'-UTR	ATG GCG GCC	1 (1107)	CAC ACT GGG	gta agt cag	1-369
ctc tct cag	GTA CGG AGA	2 (162)	GCA CAA AAG	gta agc ctc	370-423
caa tca cag	AAG GCC CGC	3 (102)	GAG CTG ACG	gta ggt gcc	424-457
ttt tac tag	GGC ACC CCA	4 (90)	AAA ACC ACT	gtg agt ccc	458-487
gtc ctg tag	GGG TTG TCC	5 (57)	ACC ATC AAG	gta taa ctt	488-506
ctc ccg cag	AAC CAC GAG	6 (248)	CAC AAC CAG	gtg ggt ggc	507-589
cta tcc cag	TCG CTC CAG	7 (202)	GTC TTG TAA	3'-UTR	590-656

Table 3. Intron-Exon boundaries of KCND2

Acceptor site			Donor site		Codon
Intron	Exon	Exon (bp)	Exon	Intron	
5'-UTR	ATG GCG GCG	1 (1114)	AAC ACT AGG	gta ggt gcc	1 - 371
tta act cag	GTA TGG TGA	2 (162)	GCA CAA AAG	gtg cgt att	372 - 425
ctc cta tag	AAA GCT AGA	3 (95)	CAG CTG CAG	gta caa tca	426 - 457
tgt taa cag	TCC TCA GAG	4 (92)	AAA ACC ACG	gta agg aga	458 - 487
gtg gaa cag	AAT CAC GAG	5 (247)	GTC TAA CAG	gta cct gag	488 - 570
ttc tat cag	CCG ATC CAG	6 (177)	GCT TTG TAA	3'-UTR	571 - 629

bp and the smallest 57 bp (Fig. 1A,Table 2). We also determined the intron lengths between the exons. It was striking that nearly all the transmembrane segments were encoded by exon 1 and that the pore region of *KCND3* was divided by an intron. We established the difference between the long and short splice variant of KCND3 to be an inclusion of exon 5. By establishing all the intron/exon boundaries, plus the sequencing of parts of the introns, we were able to design intronic primers for the amplification of all the seven exons of KCND3 (Table 1).

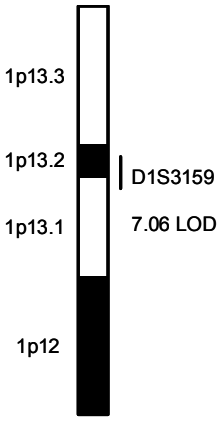

Figure 2. Mapping of KCND3, by using radiation hybrid mapping, to marker D1S3159

Chromosomal location of KCND3

To establish the chromosomal location of *KCND3*, RH mapping was performed as described. A 167-bp PCR product was generated by using exonic primers located in exon 6 to screen the RH map. This resulted in the linkage of exon 6 of *KCND3* with marker SHGC-9127 with a highly significant LOD score of 7.06. SHGC-9127 is a pseudonym for marker D1S3159; this marker maps on the genome database (GDB) comprehensive map to 1p13.2 (Fig. 2). Thus, *KCND3* is located at 1p13.2 within the same binary identification number as D1S3159 on the human genome.

Gene structure and chromosomal localisation of *KCND2*

Full length cDNA of *KCND2* (accession no. AJ010969) was used to search Genbank divisions of non-redundant Genbank coding sequence (NR), GSS and HTGS. Two BACs were found that contained all the exons of KCND2 (accession nos. AC004888 and AC004946). By designing exonic and intronic primers for *KCND2*, amplifying parts of exons and introns and subsequently sequencing from human genomic DNA, we were able to confirm the presence of all the exons of *KCND2* as described by both BACs. One missing part of the intronic sequence was obtained by genome walking. In this way, we could establish all the intron/exon boundaries and were able to design intronic primers for the amplification of all of the six exons of KCND2 (Table 1). We established that *KCND2* consists of six exons, the largest being 1116 bp and the smallest 93 bp (Fig. 1B, Table 3). We were also able precisely to determine the intron lengths between the exons, except for the first intron. Both BACs containing *KCND2* are reported to be localised on chromosome 7q31 according to Genbank.

Screening of *KCND2*/*KCND3* in Brugada patients

Using the intronic primers (Table 1) we screened 21 Brugada patients, for whom SCN5A mutations were excluded, for *KCND2* and *KCND3*. In these 21 patients we found four polymorphisms (Table 4), none of which could be linked to the disease, as the frequency of the polymorphisms between controls and Brugada patients was not statistically significant (data not shown).

Table 4. KCND3 polymorphisms in 21 Brugada patients

Hom Major Allele	Het	Hom Minor Allele	Codon/Nucleotide change	Amino acid change	Exon/Intron	Region
0.9	0.1	0	CCC/CCT	P87P	exon 1	N-terminal domain
0.96	0.04	0	TCC/TCG	S223S	exon 1	S1-S2 linker
0.73	0.21	0.06	C/A	IVS2 +15	intron 2	IVS2
0.69	0.29	0.02	A/T	IVS5 +31	intron 5	IVS5

Discussion

We have determined the genomic structures of two members of the human *KCND* subfamily, *KCND2* and *KCND3*, which are believed to be responsible for the I_{TO} current. We have synthesised oligonucleotide primers based on flanking intronic sequences for the seven *KCND3* coding exons and six *KCND2* coding exons and have established conditions for PCR amplification of each exon from genomic DNA. Furthermore, the chromosomal location of *KCND3* has been resolved by using RH mapping; BAC

localisation has provided the chromosomal location of *KCND2*. This information should facilitate the systemic screening of *KCND2* and *KCND3* exons for mutations in (inherited) arrhythmia syndromes, such as LQTS and Brugada.

Insight into the link between I_{TO} and LQTS is given by dominant negative KCND2 expressing transgenic mice, which present a functional knockout of I_{TO}. These mice show marked increases in action potential durations in ventricular myocytes and prolongation of the QT interval in surface ECG, indicating the importance of KCND2 in the repolarisation of the action potential in mice[6]. In humans, I_{TO} seems less important for repolarisation of the majority of ventricular myocytes. A possible link between I_{TO} and Brugada syndrome is the mechanism, as proposed by Antzelevitch and co-workers, based on transmural differences in action potential morphology[11,26]. Unlike the endocardial action potential, the epicardial action potential possesses a "spike-and-dome" morphology caused by a pronounced transient outward current mediated phase 1[27,28], which predisposes these cells to "all-or-none" repolarisation. Augmentation of the outward current at the end of phase 1 would lead to loss of the epicardial action potential dome and selective abbreviation of the epicardial action potential giving rise to a voltage gradient across the myocardium generating the ST-segment elevation observed in the ECGs of Brugada patients. In this setting, arrhythmias could arise from phase 2 re-entry secondary to the increased transepicardial and transmural dispersion of repolarisation[29].

To date, it is unclear whether both KCND2 and KCND3 contribute to I_{TO} in the human heart. The expression patterns of human and rat differ considerably. It is known that *KCND2* is highly expressed in rat myocytes and its distribution displays a similar heterogeneity to that of the I_{TO} current in rat[18]. In contrast, KCND3 seems to be uniformly expressed between the endocardium and the epicardium. In the human heart, *KCND3* mRNA levels are readily detectable; the presence of *KCND2* mRNA is not detectable in the atria or ventricles but it is prominently found in the human brain[24,30]. However, both homomultimers of rat or human KCND2 or KCND3 fail to reproduce all aspects of I_{TO} behavior in either mammalian cell or *Xenopus* oocyte expression systems[31,32]. A possible explanation is that I_{TO} is generated by heteromultimers of KCND2 and KCND3. The gating properties of KCND2 and KCND3 ion channels are similar but not identical[31]. Gating properties and subunit stoichiometry of a heteromultimeric assembly of KCND2 and KCND3 are not yet clear. Moreover, the short splice variant of KCND3 differs in its steady state inactivation from the KCND3 long splice variant[21]. Furthermore, it has been shown for rat KCND2 and KCND3 that co-expression in *Xenopus* oocytes with a polyA RNA of 2–4 kb isolated from rat brain induces changes in channelfunction, whereas the polyA RNA does not induce channel activities by itself[33]. This indicates a possible co-assembly of KCND ion channels with

auxiliary beta subunits. It is likely that a combination of splice variants, heteromultimers of KCND2 and KCND3 ion channels, and auxiliary beta subunits produces the native I_{TO} current in humans.

The genomic structure of the two members of the *KCND* family as reported in this study is comparable. Both have a large first exon and a large first intron, followed by multiple smaller ones. However, they share only 66% identity at the amino acid level. The first four exons of both genes are similar but the remaining exons differ. Given that the alternative splicing of *KCND3* resides in the last part of its protein, it is not surprising that we have been unable to find an alternatively spliced exon for *KCND2* as we have for *KCND3*. This is supported by there being no alternative splicing forms of *KCND2* presently known. For *KCND3*, only two isoforms have been reported[20]; since we started working from known cDNA of these two splice forms, we cannot rule out the possibility that other alternative spliced exons of *KCND3* exist. However, a search of the Genbank expressed sequence tag division has not revealed further alternatively spliced exons for *KCND3*. Consequently, since no other isoforms are currently known, we consider that we have established the complete genomic organisation of both *KCND2* and *KCND3*.

Our chromosomal location of *KCND3*, viz. 1p13.2, is in accordance with that published by Kong et al., who used fluorescence in situ hybridisation with 4,6-di-amidino-2-phenylindole banding and determined the border of 1p13.2 and 1p13.3 as the location of KCND3 by using a P1 artificial chromosome[20]. We have used RH mapping to physically link KCND3 to marker D1S3159, which has previously been mapped to 1p13.2. Taking these data together, we believe that *KCND3* is probably located at 1p13.2.

In summary, we describe the genomic organisation of human *KCND2* and *KCND3*, their location and intron-exon boundaries, and the primers and conditions necessary for the amplification of their exons from genomic DNA. This information should facilitate the screening for mutations in *KCND2* and *KCND3* in (inherited) arrhythmia syndromes such as LQTS and Brugada.

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Chapter **4**

Catecholaminergic Polymorphic Ventricular Tachycardia: Clinical Data, Therapy and Molecular Biological Backgrounds

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To be submitted

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disease characterised by adrenergic induced arrhythmias in the form of bi-directional and polymorphic ventricular tachycardia (PVT). Polymorphic ventricular tachycardia are rapid ventricular tachycardia with changing morphology of the QRS complexes. A finding common to (young) patients with PVT is the clinical presentation with syncope and a high familial incidence of sudden cardiac death (SCD) with no evidence of heart disease. Young patients suffering from PVT are often misdiagnosed with epilepsia when the rapid PVT provokes seizures. The many familial occurrences of these PVTs have helped uncover mechanisms and etiology behind some forms of polymorphic ventricular tachycardia. In particular a lot progress has been made in the congenital long QT and Brugada syndromes. Recent studies have also placed CPVT in that small group of inherited arrhythmogenic disorders. CPVT is a distinct clinical entity associated with a high mortality rate of up to 50% by the age of 30 years. Its key features include childhood onset of syncope and collapse triggered by exercise and other stressful scenarios, with reproducible polymorphic and/or bi-directional ventricular tachycardia demonstrated during exercise testing and catecholamine infusion. Cardiac investigations show no evidence of structural heart disease and the resting 12-lead electrocardiogram is unremarkable including the QT interval. Cases have been reported throughout the world and both sexes appear to be susceptible. This review aims to (1) summarize the clinical data of the various recent publications on CPVT and (2) describe the underlying molecular causes and pathophysiological mechanism(s). Finally, diagnostic guidelines will be proposed.

Historical overview

CPVT was first reported as a case of exercise induced bi-directional tachycardia in the 1970s by Coumel[1], numerous short series and case reports followed yet it was not until Leenhart *et al.* reported a 10 year follow-up of 21 children with the disorder in 1995[2] that it became established as a distinct clinical entity. A retrospective analysis of CPVT-like cases from literature show that a number of authors have used different terminology in reporting the serious syncopal ventricular tachyarrhythmia triggered by stress or emotion occurring in children with no patent heart disease or long QT[1,3-23]. Terms including syncopal paroxysmal tachycardia, malignant paroxysmal ventricular tachycardia, multifocal ventricular premature beats, paroxysmal ventricular fibrillation, bi-directional tachycardia, double tachycardia induced by catecholamines, and syncopal tachyarrhythmia have been

used to describe the various electrocardiographic aspects of this rare form of catecholaminergic arrhythmia.

Epidemiology

For this review we have combined all the recent CPVT publications[2,24-33], resulting in a group of 244 symptomatic patients from 127 families, with clear clinical and phenotypic descriptions of CPVT including the characteristic ECG consisting of polymorphic ventricular tachycardia upon exercise testing and the absence of structural heart disease. In addition, 29 asymptomatic patients have been found by mutational analysis, see below (Table 2). No gender difference is observed in the symptomatic patients (121 males, 123 females), however in the asymptomatic patient group females (n=21) outnumber males (n=8). Age at first arhythmic event varied from 3 to 53 years, with an average age of onset of 10±2 years. A family history of juvenile sudden cardiac death was present in 43% of the patients (Table 1).

Clinical characteristics and diagnosis

As stated earlier, catecholaminergic polymorphic ventricular tachycardia can clinically be defined by several unique characteristics. The key features of CPVT include syncope and collapse triggered by exercise, emotion or stress, and highly reproducible and inducible stress-related polymorphic and/or bi-directional ventricular tachycardia in the absence of structural heart disease[2,31,34]. Cardiac investigations usually show no evidence of structural

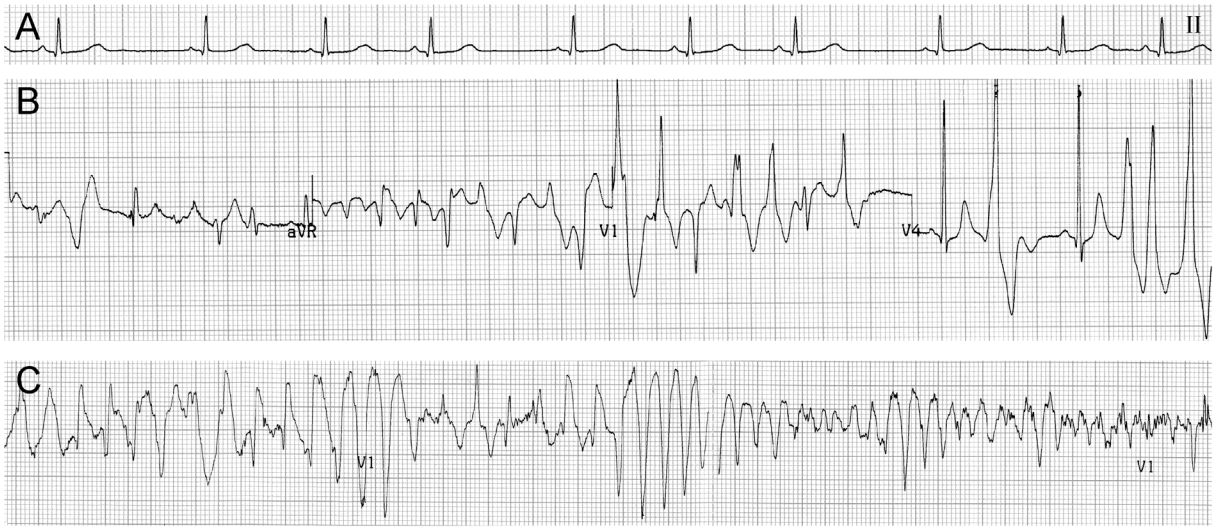

Figure 1. ECG recording in lead II of a CPVT patient showing a normal QTc interval (428ms) and a slow heart rate (70 bpm) at admission (A). Examples of polymorphic ventricular premature beats at the beginning of the stress test (B) followed by self-terminating polymorphic salvos as the heart rate increases above 100 bpm (C). Recorded at 25mm/s and 10mm/mV.

heart disease; i.e. clinical history, blood chemistry, electrocardiography and exercise testing (except for arrhythmias and specific abnormalities of the QRS, ST and QT segments), echocardiography, and cardiac catheterization with coronary angiography are all unremarkable. Moreover, the resting 12-lead electrocardiogram, including the QTc interval, is normal (Table 1). However, bradycardia may be common, especially in (young) children[28,33]; the average resting heart rate of the identified CPVT patients was 66 bpm (Table 1). During exercise, a reproducible sequence of events occurs; as sinus rate increases, atrial arrhythmias and ventricular extrasystoles appear. If the effort continues salvos of monomorphic or bi-directional VT eventually lead to bursts of polymorphic VT (see Figure 1). Depending on the intensity of the adrenergic stimulation, the events disappear in reverse order. In contrast to this reproducible provocation with stress or exercise, CPVT is generally not inducible with programmed electrical stimulation (PES); only 8% (2/25) of the CPVT patients tested were inducible (Table 1). The use of catecholamines (e.g. isoproterenol) may unmask the arrhythmogenic substrate, as 54% (20/37) of the patients tested were inducible by catecholamines[24,26]. Overall, CPVT mainly affects children; the recent studies demonstrate that the majority of patients have an age of onset of CPVT symptoms (syncope or cardiac arrest during stress) around the age of 10 years (Table 1). However, CPVT patients may present with a much later age of onset, as several CPVT patients are reported who were asymptomatic until their mid 30s[24,26,33]. Most importantly though, a clear correlation between the age of the first syncope and the severity of the disease exists; early occurrence of the first syncope worsens the prognosis[2]. Retrospective analysis shows that CPVT has a highly malignant course when untreated, with estimates of mortality ranging from 30 to 50 percent by the age of 20 to 30 years[34].

Several characteristics are specific to CPVT and should be considered when making a diagnosis. The family history of CPVT patients is often positive for SCD in relation to specific triggers (emotion, stress, exercise); 43% (55/127) of the CPVT families had occurrences of preceding SCD (Table 1). The patient's history is characterized by syncope in response to similar triggers, indeed 77% (178/232, of 12 CPVT patients this information was not reported[25]) of the described CPVT patients had previous episodes of syncope. The hallmark of CPVT is the reproducible occurrence of polymorphic ventricular tachycardia during exercise or stress at heart rates above 110 beats per minute (with or without syncope). In addition, 92% (223/242, 2 CPVT patients were not tested[24]) of the clinically affected CPVT patients were inducible by exercise testing (Table 1). Moreover, sinus bradycardia should be an important aspect in the consideration of a CPVT diagnosis, especially in young children with unexplainable syncopes. A typical response during exercise includes the occurrence of isolated supra-ventricular

extrasystoles (with a typical morphology, see below), followed by doublets with a bidirectional pattern, followed by more complex ventricular arrhythmias (ie. Polymorphic VT). The absence of structural heart abnormalities has to be confirmed using the above-mentioned parameters. And finally, the only discriminating factor between CPVT and the long QT syndrome is a normal QTc interval in the former.

Regrettably, there is a small group of CPVT patients with an apparently normal phenotype, even after exercise testing. In 29 individuals the diagnosis of CPVT could only be made after identification of underlying mutations in Ca^{2+} handling genes (see below), as they didn't display any CPVT phenotype. Interestingly, 73% (21/29) of these patients were female, hinting to a possible gender bias. This is in line with a recent study that suggests that male gender is a risk factor for syncope in genotyped CPVT patients[26]. In contrast, another study did however not observe a significant gender based difference in syncope free survival in genotyped CPVT patients[33], thus it is unclear if gender has an influence on the phenotype of CPVT patients. The presence of different mutations (see below) leading to different phenotypes in the various patients of both groups could underlie the discrepancy between the studies. Regardless of the underlying mechanism, some of these phenotypically normal CPVT patients with normal exercise tests, did experience syncope and sudden cardiac death[27], implying that an asymptomatic phenotype doesn't guarantee protection from CPVT.

The diagnosis of CPVT might be difficult since the resting ECG is usually normal. However, frequent syncopes in (young) patients in relation to exercise or emotion, a positive family history for syncope or SCD, absence of heart abnormalities, and possible presence of sinus bradycardia, are strong indications for CPVT. The diagnosis of CPVT can subsequently be confirmed by means of Holter recordings and especially exercise testing.

Clinical course and therapy

The current treatment of choice for CPVT is β-blockers without sympathomimetic activity. This concept was initially based on an early retrospective analysis of 59 published CPVT cases[2], of which only 38 received β-blocker treatment. With this treatment there were four sudden deaths, or 10.5%. In contrast, 10 out of the 21 patients who did not receive beta blockers, experienced sudden death at the mean age of 19.5 years, which corresponds to a 48% mortality rate before the age of 20. At present, most CPVT patients described in literature (225/254, including 10 silent gene carriers) received β-blockers in various dosages and regimes. With a mean follow-up time of 6±2 years, 88%

Table 1. Overview of clinical data from CPVT publications

Reference	Clinically affected patients	Number Families	Gender affected M/F	Age of onset, y	Exercise related syncope	Juvenile SD Families	Rest ECG pre med, bpm	QTc, ms	Inducible by exercise test	Inducible by catecholamine	Inducible by PES
Non-genotyped											
Leenhardt 1995	21	21	12/9	7.8±4	20/21	7/21	60±9	404±25	21/21	…	0/6
Sumitomo 2003	29	29	13/16	10±6	23/29	4/29	59±11	400±20	27/27	12/16	…
	50	**50**	**25/25**	**9±5**	**43/50**	**11/50**	**60±10**	**402±23**	**48/48**	**12/16**	**0/6**
RYR2											
Laitinen 2001	26	4	12/14	>10	9/14[3]	4/4	73±14	430±40	26/26	…	…
Priori 2002[1]	43	30	28/15	8±2	29/43	10/30	…	normal	39/43	6/19	2/19
Bauce 2002[2]	43	8	21/22	>11	28/43	8/8	…	normal	28/43	…	…
Postma 2003	65	24	29/36	13±2	52/65	16/24	79±2	397±5	65/65	…	…
	177	**66**	**90/87**	**11±1**	**118/165**	**38/66**	**76±8**	**414±23**	**158/187**	**6/19**	**2/19**
CASQ2											
Lahat 2001	13	1 (7)[4]	4/9	7±4	13/13	6/7	64±13	400±20	13/13	2/2	…
Postma 2002	4	3	2/2	8±3	4/4	0/4	60±12	424±19	4/4	…	…
	17	**4**	**6/11**	**8**	**17/17**	**6/11**	**62±3**	**412±17**	**17/17**	**2/2**	**…**
Total	**244**	**120 (127)**	**121/123**	**9±2**	**178/232**	**55/127**	**66±8**	**409±14**	**223/242**	**20/37**	**2/25**

[1]includes Priori 2001 data; [2]includes Tiso 2001 data; [3]only 2/4 families reported; [4]families share common haplotype; … not available

(198/225) are symptom free, though some patients continued to have VPBs at exercise tests (Table 2). 27 out of 225 of CPVT patients (12%) experienced additional episodes of VT/VF or syncopes while on β-blockers (Table 2). Moreover, 9 of the 225 CPVT patients on β-blockers died. Though, at least 3 of these deaths were associated with non-compliance after appropriate diagnosis and treatment[2,24,33]. The occurrence of a small group of CPVT patients, who continue to have episodes of VT/VF or syncope on β-blockers, indicates that additional modes of therapy are needed, particularly considering the high mortality of the disease. Experience with the implantation of ICDs in non-responsive patients comes from the study by Priori et al.[26]. They reported, in contrast to the majority of the CPVT studies, that 46% (18/39) of their patients treated with β-blockers had cardiac arrhythmias in spite of therapy. In 12 of these 18 patients an ICD was implanted and over the follow-up period of 2 years 6 of them received an appropriate shock to terminate ventricular tachyarrythmias. Nonetheless, an ICD can potentially have pro-arrhythmic effects in CPVT patients, as stress caused by appropriate or inappropriate discharges could prove disastrous by evoking a self-inducing stress circle. However, a combination therapy, involving both an ICD and β-blockers, should safeguard against any such adverse effects and provide ultimate protection in non-responsive patients. The apparent discrepancy in efficacy of β-blocker treatment between the various studies probably reflects the many underlying mutations, a difference in β-blocker dosage, or a bias in the selection of (severely affected) CPVT families. Alternatively, this discrepancy in β-blocker efficacy could also be due to the presence of different polymorphisms influencing β-blocker metabolism. Obviously, larger groups of CPVT probands are needed to address the issue of β-blocker efficacy in CPVT. Meanwhile, the maximal well-tolerated dosages should be prescribed and Holter recordings and exercise tests should be repeated periodically to assure that the degree of sinus tachycardia that precedes onset of arrhythmias is never reached. Moreover, once the diagnosis is established it is crucial to make the patients aware of the necessity of faultless compliance

Table 2. Overview of treatment, follow-up and genetic data from CPVT publications

Reference	Patients on beta-blockers	Followup, y	VT/VF/syncope on beta-blockers	SCD on beta-blockers	Mutation identified[3]	Silent Gene Carriers M/F	Inheritance
Non-genotyped							
Leenhardt 1995	21/21	7±6	3/21	2/21	…	…	…
Sumitmo 2003	21/29	6±8	0/21	4/21	…	…	…
	42/50	**7±7**	**3/42**	**6/42**			
RYR2							
Laitinen 2001	26/26	8±6	2/26	0/26	3/4 (1)	0	dominant
Priori 2002[1]	39/43	4±3	18/39	0/39	14/30 (5)	1/3	dominant
Bauce 2002[2]	26/43	6.5	0/26	0/26	8/8	5/10	dominant
Postma 2003	75/75	7±4	2/75	3/75	12/24 (9)	2/8	dominant
	166/187	**6±4**	**22/166**	**3/166**	**37/66 (15)**	**8/21**	
CASQ2							
Lahat 2001	13/13	1.6	1/13	0/13	1/1	7/7	recessive
Postma 2002	4/4	4	1/4	0/4	3/3	12/4	recessive
	17/17	**3±2**	**2/17**	**0/17**	**4/4**	**19/11**	
Total	**225/254**	**6±2**	**27/225**	**9/225**	**41/70**	**27/32**	

[1]includes Priori 2001 data; [2]includes Tiso 2001 data; … not available; [3](..) number of families with juvenile SCD and absence of mutation

to the beta-blocking therapy given the number of non-compliance related sudden cardiac deaths. Limited evidence suggests that drugs such as amiodarone, verapamil, mexiletine and type I antiarrhythmic drugs are not effective in controlling the arrhythmic disorder and might even be harmful[2,24].

In conclusion, "individually adapted" β-blocking treatment has a favorable overall outcome in the majority of patients, but additional modes of therapy should be considered.

Pathophysiological background

The polymorphic ventricular tachyarrhythmias as seen in CPVT patients resemble the type of arrhythmias seen in experimental models with digitalis toxicity[35]. This has led to speculation that aberrant calcium handling might underlie the disease. In general, the in- and efflux of various ions through specific ion channels tightly control the amplitude and duration of the myocardial action potential. The crucial link between cardiac excitation and subsequent cardiac muscle contraction, the so-called excitation-contraction coupling, is specifically regulated by calcium ions[36]. The calcium concentration in the cytosol of the myocyte is very low during rest (100nmol/L). Excitation of the myocyte by neighboring myocytes cause the influx of Ca^{2+} ions through voltage gated L-type ion channels. This in turn activates the cardiac ryanodine receptors (RyR2), which open briefly and raise the intra-cellular Ca^{2+} concentration to 1μmol/L, by releasing Ca^{2+} ions from the internal stores of the sarcoplasmic reticulum (SR). This abrupt elevation of the cytosolic Ca^{2+} concentration, leads to activation of Ca^{2+} sensitive contractile proteins (eg troponin C),

which trigger muscle contraction. After its contraction, the cardiac muscle must go into relaxation. Consequently, the bound Ca^{2+} needs to dissociate from the troponin C, which requires a reduction in cytosolic Ca^{2+}. The excess Ca^{2+} is removed from the cytosol by the sarcolemmal Na^{+}/Ca^{2+} exchanger, which transports Ca^{2+} out of the cell, and the SR Ca^{2+}/ATPase pump, which actively replenishes the SR Ca^{2+} stores[37].

As mentioned above the CPVT arrhythmias resemble those seen in digitalis toxicity. Since the latter are caused by an electrophysiological mechanism known as delayed afterdepolarizations (DADs), it has been proposed that a similar mechanism underlies the polymorphic ventricular tachyarrhythmias seen in CPVT patients[35]. DADs are triggered impulses that result from sub-threshold membrane depolarisations after repolarisation of the previous action potential is complete. Interestingly, they are caused by aberrant Ca^{2+} handling, either inappropriate sarcoplasmic Ca^{2+} release during diastole (myocyte relaxation) or excessive Ca^{2+} influx through the L-type calcium channel[36]. Both these mechanisms activate inward depolarizing currents through the Na^{+}/Ca^{2+} exchanger[38]. If this inward current is sufficient to cause a DAD whose amplitude exceeds the threshold potential of the myocyte, depolarisations will occur. The abnormal impulses generated from this can cause a triggered arrhythmia if surrounding polarised cells propagate the depolarisation wave and may give rise to extrasystoles and associated arrhythmias. Moreover, several factors increase the amplitude of DADs and hence the probability that the threshold potential will be reached. These include increasing the rate of the triggering action potential (corresponding to an increase in heart rate) and increasing intracellular calcium loading[39]. This former possibility supports the fact that polymorphic ventricular tachycardia occur as the sinus rate of CPVT patients increases.

A better understanding of the mechanisms that generate the abnormal Ca^{2+} handling in CPVT patients could lead to better treatment and/or prevention of the lethal arrhythmias. Insight into possible molecular biological backgrounds of CPVT has come from the many familial CPVT cases. Using genetic tools such as genome wide screening, co-segregation analysis and sequencing it has been possible to identify underlying molecular causes of CPVT.

Molecular biological background

Autosomal dominant CPVT, the cardiac ryanodine receptor (RyR2)

Many cases of CPVT are familial, and the first extensive report with a definite linkage analysis was published by Swan *et al.*, showing linkage of CPVT with chromosome 1q42-43[34]. This was recently followed by publications which associated mutations in a gene

from the 1q42-43 region, the cardiac specific ryanodine receptor type 2 (RyR2), with CPVT[25,30,31]. Ryanodine receptor channels are intracellular Ca^{2+} release channels forming a homotetrameric membrane-spanning calcium channel on the sarcoplasmic reticulum (SR). They are characterized by a large cytosolic domain, which is probably involved in regulating the channel function, and a channel pore located at the carboxy terminal end, in the last 10% of the protein[40,41]. Three different isoforms of ryanodine receptors (RyR), each encoded by different genes, have been characterized so far. Isoform 1 (RyR1) is principally expressed in skeletal muscle, isoform 2 (RyR2) is the predominant form in cardiac muscle and isoform 3 (RyR3) is expressed at low levels in various tissues including the brain[42]. The majority of RyR2 channels in cardiomyocytes are distributed in areas of the SR membrane that lie in proximity to the T-tubule invaginations of the sarcolemmal membrane[43]. Here they are closely associated with the L-type voltage-dependent Ca^{2+} channel and this spatial association of the two channels is key to the signal amplification process that couples the excitation of myocardial cells to the actin/myosin contractile apparatus (as described above). The ryanodine receptors are not merely ion channels, they are considered macromolecular signaling complexes, as they contain binding sites for signaling proteins such as kinases, phosphatases and adaptor/anchoring proteins, which are specifically bound by several domains on the cytosolic side of the RyR channel[36]. The RyR2 gene is located on chromosome 1q42 and consists of 105 exons, which translate into 15kb of cDNA. It encodes a protein of 4967 amino acids, making it the largest known ion channel to date.

RyR2 mutations and CPVT

At present 38 mutations have been reported in 40 families[25-27,29-33] (Table 2, figure 2). All of them are missense mutations, 12 occur *de novo*, and the remaining 24 show an autosomal dominant mode of inheritance. The various mutations cluster within four regions within the RyR2 protein: the N-terminal side, around the binding site of FKBP12.6, a protein that stabilizes the RyR2 channel[44], the calcium binding site, and the channel-forming transmembrane domains (Figure 2). Interestingly, mutations in RyR1 are also clustered in roughly the same regions. RyR1 mutations are associated with rare dominantly inherited disorders of calcium signaling in striated muscle called malignant hyperthermia (MH) and central core disease (CCD)[45,46]. Thus the present data on the various mutations, suggest that CPVT shows close kinship to these diseases. Therefore, the clustering of RyR mutations in discrete areas in several Ca^{2+} related disorders indicates that these regions are important for proper protein function of all the ryanodine receptors. Recently, the topology of the transmembrane region of the RyR proteins was further defined using truncated fusion proteins[47]. The resulting model predicts that 9 of the 10 so-called transmembrane RyR2 mutations actually fall on cytoplasmatic loops connecting the various transmembrane domains, and that no RyR2 mutations fall on the

luminal side of the protein. Only 1 mutation, V4771I, is located within a transmembrane segment, and no mutations have been reported for the pore forming loop and selectivity filter M9 (figure 2). Besides the clustering into four distinct regions of the protein, it is striking that 9 RyR2 mutations occur at the same amino acid position in 18 unrelated families (figure 2). Amino acid positions 4104 and 4108 are especially interesting, as these harbor 3 *de novo* mutations and one mutation associated with a considerable number of juvenile sudden deaths[33]. Other shared mutations occur at positions 420, 2386, 2504, 4076, and 4771 in unrelated families. Finally, four unrelated probands[26] share *de novo* mutations in amino acid positions 2246 and 3946. The presence of multiple mutations occurring at the same amino acid position could suggest the presence of "mutational hotspots" within the RyR2 coding sequence. The most common source of mutations in inherited human diseases is de-amination of methylated cysteines, leading to a C->T or a G->A transition. Indeed 13 of the 38 (34%) RyR2 mutations occur at methylated cystines sites. Though, a founder effect could also underlie the various shared familial mutations. Consequently, it appears that clustering, hotspots and possibly founder effects for RyR2 mutations are relatively common in CPVT.

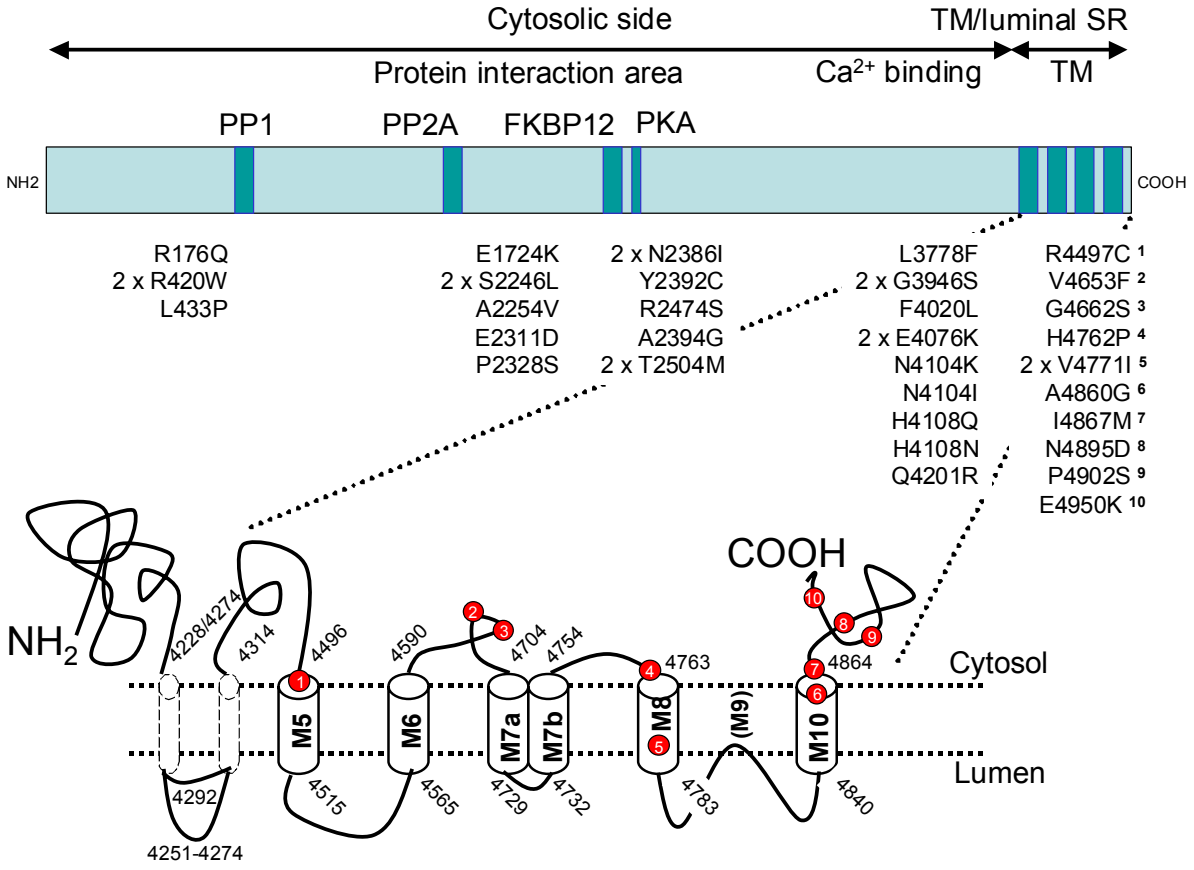

Figure 2. Overview of all published RYR2 mutations drawn on a schematic representation of the RYR2 protein. Highlighted is the transmembrane region according to the model of Du et al. (Proc Natl Acad Sci U S A 2002 Dec 24;99(26):16725-30)

RyR2 mutations, genotype-phenotype relation?

Unfortunately, there is no clear correlation among RyR2 probands between the region of mutation and the phenotype of the proband, in contrast to what is reported for RyR1 mutations[45,48]. No significant correlation has been found between the age of onset and the position of the various mutations. Moreover, there is little expression data available to compare the various mutations on a molecular level. However, using the 38 identified mutations and their corresponding phenotypes it might be possible to speculate on certain genotype-phenotype relations, since several mutations are shared between unrelated families. The amino acid positions 4104 and 4108 are most interesting since 4 mutations occur in the same small region. Three of these mutations are *de novo* and together with the high lethality of the N4104I mutation, this indicates that this region is particularly important to the RyR2 protein function. This region is located just before the C-terminal transmembrane segments and might be involved in Ca^{2+} binding[47]. Likewise, important functional parts of RyR2 protein might be located around amino acid positions 2246 and 3946 as they each share 2 independent *de novo* mutations. It's harder to compare the remaining shared mutations such as N2386I, T2504M, E4076K and V4771I. Although the penetrance for the various mutations is comparable in the affected families, the age of onset varies. In contrast to the above-mentioned mutations, which are all situated in the middle or C-terminal part of the protein (figure 2), three mutations (R176Q, R420W, L433P) occur in the N-terminal part. Interestingly, the R420W mutation produces a high number of asymptomatic individuals in two unrelated families. In addition, a high number of juvenile sudden deaths are reported for these mutations. This suggests that the clinical spectrum of RyR2 mutations can include a totally asymptomatic behaviour, and more worryingly, that the first symptoms might be sudden cardiac death. However, it is difficult to relate the N-terminal part of RyR2 with asymptomatic CPVT, as mutations close by, R176Q and L433P, both have a highly penetrant phenotype with no asymptomatic individuals. One study suggests that some mutations of the FKBP12.6 binding domain (FBD) of RyR2 would lead to arrhythmogenic right ventricular dysplasia type 2 (ARVD2) (see below), indicating that some parts of the RyR2 protein might have a genotype-phenotype relation. However, it seems likely that straight genotype-phenotype relations are complicated by the fact that RyR2 is more than an ion channel, it contains additional binding sites for kinases, phosphatases and adaptor/anchoring proteins[49]. Therefore, it is conceivable that depending on the place of the mutation various other factors get (subtly) influenced besides ion transport. Moreover, many substances such as endogenous-physiological modulators (calcium ions, lactacte, palmitoyl carnitine) and exogenous-physiological ones (caffeine, sulmazole, suramine etc) can affect the RyR2 function[50]. Consequently, although several identical mutations have overlapping phenotypes in multiple families, it is difficult to establish a firm genotype-phenotype

relationship since many modulating factors and genes affect the final phenotype of a RyR2 mutation

Mechanism(s) behind RyR2 mutations

The mechanism through which the various RyR2 mutations cause CPVT is unclear. However, the overall mechanism thought to underlie this disease is a calcium overload of the myocyte, possibly caused by leaking of Ca^{2+} from the sarcoplasmic reticulum into the cytosol (as described earlier). In contrast to RyR2, many of the mutations identified in RyR1 have been studied functionally in heterologous expression systems. The various RyR1 mutations have shown increases in caffeine-induced calcium release[51], increases in the sensitivity to calcium[52], the activation of calcium release at lower depolarizing potentials[53], and in one mutation, located in the putative pore of the channel, a defect in skeletal EC coupling rather than a "leaky" RyR1 channel[54]. All these mechanisms can contribute to a calcium overload of the cell. Recently, the first expression study with single-channel recordings of a transmembrane RyR2 CPVT mutation was reported. The mouse homologue of the human R4497C RyR2 mutation, showed an enhanced basal channel activity and the propensity for spontaneous Ca^{2+} release[55], corroborating the Ca^{2+} leak mechanism. At present little is known about the structure function relation of the RyR protein, it is therefore unclear how mutations outside the transmembrane region would cause leaking of Ca^{2+}. However, recently two studies have shed light on the role of CPVT mutations in the FKBP12.6 binding domain (FBD) of RyR2 (figure 2). One study, using the domain-peptide-probe technique, reported that the FBD binds to the N-terminal region of the RyR protein; expression of the R2474S CPVT mutation weakened this inter-domain interaction and caused hyper-activation and hyper-sensitization effects, particularly at low Ca^{2+} concentrations[56]. Interestingly, another study using the yeast two hybrid system, showed that interaction of the FBD of RyR2 with the FKBP12.6 protein was altered by RyR2 mutations[57]. Two 'ARVD2' RyR2 mutations (see below) diminished the binding of FKBP12.6 to RyR2, while the above-mentioned R2474S CPVT mutation strongly increased binding to FKBP12. Since FKBP12.6 has a strong influence on the open probability and Ca^{2+} release of the RyR channel[49], this suggests that mutations in the FBD can have profoundly different affects based on the resulting interaction with FKBP12.6. Alternatively, mutations in RyR2 could produce proteins that function as a poison peptide in the homotetrameric RyR2 channel, analogous to the effect of some long QT mutations. Since RyR channels on the SR are arranged into functional arrays, which are thought to be important in the control of Ca^{2+} release[58], the presence of ill-formed RyR channels could lead to a disruption of these arrays and possibly result in loss of appropriate excitation contraction coupling resulting in leaked Ca^{2+}. Further detailed expression studies of RyR2 mutations should shed light on the mechanisms underlying cathecholaminergic polymorphic ventricular tachycardia on the basal molecular level. In

addition, the identification of these mechanism(s) could possibly lead to more specific and effective pharmacological treatment.

RyR2 (mutations) in other diseases

Mutations in RyR2 are also reported in arrhythmogenic right ventricular dysplasia type 2 (ARVD2)[30]. However, it is uncertain whether the families and patients reported in that study do not merely represent a sub-group of CPVT, as they have all the characteristics of CPVT, including non-inducibility of polymorphic VT by programmed electrical stimulation. These findings sharply contrast with the inducibility by PES seen in the majority of ARVD patients[59]. However, these patients with 'ARVD2' RyR2 mutations have some mild abnormalities of the right ventricular wall, which normally are not found in CPVT patients. As mentioned above, a recent study from the same authors showed that the interaction between FKBP12.6 and RyR2 protein is different between 'ARVD2' and CPVT RyR2 mutations[57], implying that a different pathological mechanism might underlie the various mutations. Alternatively, the right ventricular abnormalities could be secondary to the imbalance in the intracellular calcium homeostasis, which can trigger cell death by necrosis or apoptosis[60]. However, regardless of the underlying mechanism, 'ARVD2' RyR2 patients can be identified by the same diagnostic criteria, require the same treatment, and have the same follow-up as CPVT RyR2 patients implying that they are clinically overlapping. A possible role for RyR2 involvement in chronic heart failure was recently established by experiments that showed hyperphosphorylation of RyR2 in chronic heart failure[49,61]. This hyperphosphorylation resulted in a functional change in RyR2's characteristics allowing an increased leak of Ca^{2+} out of the SR, particularly during diastole. This `diastolic Ca^{2+} leak' would, analogous to the earlier described mechanism underlying CPVT, deplete SR Ca^{2+} load and serve as a substrate for DADs. It is proposed that although phosphorylation of RyR2 occurs as a compensatory response in failing hearts, its continuation for a prolonged period will eventually become counterproductive to cardiac function. Thus far, it has not been demonstrated that RyR2 mutations in the CPVT patients lead to heart failure, however it might be possible that certain RyR2 mutations accelerate or even cause heart failure.

Autosomal recessive CPVT, the cardiac calsequestrin gene (CASQ2)

In contrast to the majority of CPVT patients with an autosomal dominant mode of inheritance, two studies have recently demonstrated a recessive form of CPVT in 4 families[29,32]. In the first study by Lahat *et al.* a large consanguineous Bedouin family was identified in which 9 children (age 7±4) from 7 related families have died suddenly. Twelve other children suffered from recurrent syncope and seizures starting at the age of

6±3. The parents of the affected individuals were asymptomatic and were all related. In contrast with the "classical" CPVT, segregation analysis suggested an autosomal recessive mode of inheritance. In concurrence with autosomal dominant CPVT variant, recessive symptomatic patients had a relative resting bradycardia, and polymorphic VT could be induced by either treadmill exercise or isoproterenol infusion. After performing a genome wide screen, a common disease locus of 16 megabases on chromosome 1p13-21 was identified, which segregated in all affected patients. They subsequently identified in all the affected patients a homozygous missense mutation in a highly conserved region of the cardiac calsequestrin gene (CASQ2), effectively defining an autosomal recessive form of CPVT (Figure 3)[29]. The CASQ2 gene encodes a protein that serves as the major Ca^{2+} reservoir within the SR lumen of cardiac myocytes. CASQ2 protein is anchored in close proximity to RyR2 and has the ability to bind extremely large numbers of Ca^{2+} cations in the SR[62]. Subsequently, we reported on the first nonsense mutations in CASQ2 in three CPVT families(figure 3)[32]. These mutations are thought to induce premature stop codons. The three probands had phenotypes very comparable with the study of Lahat (Table 1). Interestingly, the patients who experienced syncopes before the age of 7, were homozygous carriers suggesting a complete absence of calsequestrin 2. Strikingly, as in the Bedouin family, the heterozygous carriers of the mutations were devoid of any clinical symptoms or ECG anomalies. The phenotypes of the CASQ2 CPVT patients seem more severe compared to RyR2 CPVT patients. In particular the age of onset is earlier than that of the reported RyR2 mutations (Table 1). Since there is no compensation from wildtype protein, homozygous mutations probably produce a more severe phenotype and thus surface earlier. The efficacy of β-blocker therapy for CASQ2 CPVT patients matches that of RyR2 CPVT patients, as 12% (2/17) of the CASQ2 patients experienced recurrent syncope on β-blockers (Table 2). In conclusion, CASQ2 CPVT patients present with a more severe clinical phenotype including early age of onset. In addition, the CASQ2 mutations are mostly associated with consanguineous families and therefore diagnosis in CASQ2 CPVT patients is more difficult due to the absence of a positive family history. Finally, the identification of a second CPVT causative gene proves that CPVT is genetically heterogeneous, similar to other arrhythmogenic disorders.

Mechanism(s) behind CASQ2 mutation(s)

How mutations in CASQ2 can induce the polymorphic VT still remains to be elucidated as no expression studies have been reported thus far. However, it is known that CASQ2 and RyR2 function together in close proximity, as they are bound by junctin- and triadin proteins (see below) in a stable protein complex required for the normal operation of Ca^{2+} release[63,64]. Moreover, the clinical characteristics of RyR2 CPVT mutations and CASQ2 mutation seem to overlap (Table 1). Thus it would seem logical that a mechanism similar to that proposed for RyR2 mutations, i.e. Ca^{2+} overload, is also responsible for the effects seen in CASQ2 patients. A possible loss or disruption of the RyR2-CASQ2 protein complex by mutations in CASQ2 could influence the open probability and Ca^{2+} release properties of the RyR2 ion channel, resulting in Ca^{2+} leaking. Obviously, detailed studies of the various CASQ2 mutations would shed light on the underlying mechanism(s).

Genetic heterogeneity for CPVT

With the discovery of genetic heterogeneity for CPVT the question arises whether there are additional genes involved in the disease. Evidence for this comes from the fact that in at least 29 CPVT families no causative mutation has been found (Table 2), although a familial history of juvenile sudden death was present in 52% (15/29) of them, implying an underlying hereditary cause. It is likely that some mutations in RyR2 or CASQ2 might have been missed due to incomplete screening of the genes, or the lack of sensitivity of the screening techniques used. On the other hand, given the recent discovery of the CASQ2 mutations, other proteins could be involved in CPVT, implicating further genetic heterogeneity, in analogy with other arrhythmogenic disorders such as the long QT syndrome[65]. As many CPVT cases are familial, it is "unfortunate" that in all the large

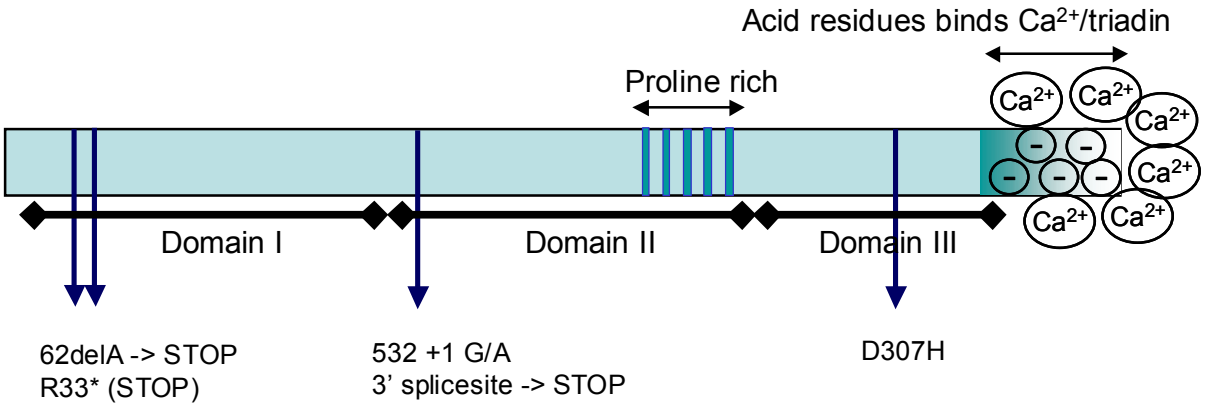

Figure 3. Schematic representation of the CASQ2 protein, with all the published CASQ2 mutations. The protein consists of three similar domains, and a Ca^{2+} binding domain.

CPVT families underlying mutations have been found. As described above, the causative mutations in CPVT patients have so far been found in RyR2 and CASQ2, both proteins that are involved in Ca^{2+} release from the SR. Given that Ca^{2+} overload seems to be the trigger that is specific in CPVT patients, other genes involved in the Ca^{2+} release and re-uptake pathway are attractive candidates. It is known that both calsequestrin and the ryanodine receptor have interactions with two other proteins named junctin and triadin[63,64]. Junctin is a 26-kD transmembrane calsequestrin-binding protein detected in junctional sarcoplasmic reticulum (SR) from cardiac and striated muscle tissue and co-localizes with the ryanodine receptor and triadin. Triadin, a protein found in triadic junctions, is intrinsic to the terminal cisternae and is closely associated with the ryanodine receptor. Both these proteins are capable of binding calsequestrin and the ryanodine receptor on their luminal site[66]. By this fashion they anchor calsequestrin to the SR membrane in close proximity to RyR. Cooperation of these four proteins, the ryanodine receptor, calsequestrin, junctin and triadin is required for proper Ca^{2+} release from the SR. Therefore, since junctin and triadin are both expressed in the heart, they would seem likely candidate genes for CPVT. Alternatively, other Ca^{2+} binding proteins located in the cardiac SR, like histidine rich binding protein (HRC) and sarcalumenin might be targets[67,68]. The other part of the pathway, the re-uptake of Ca^{2+} into the SR is mediated largely by SERCA2 and phospholamban[69]. SERCA2 is an ATP-driven Ca^{2+} pump located in the SR. It specifically maintains low cytosolic Ca^{2+} concentrations by actively transporting Ca^{2+} from the cytosol into SR lumen. It is regulated by phopholamban an inhibitor of SERCA2 in the unphosphorylated state. Phospholamban is therefore, through modulation of SERCA2 activity, a key regulator of cytosolic Ca^{2+} levels[70]. Thus mutations affecting either SERCA2 or phopholamban could lead to excess Ca^{2+} in the cytosol be preventing or slowing re-uptake of Ca^{2+} into the SR lumen. Finally, other possible candidate genes for CPVT are the kinases, phosphates and other modulators that bind to RyR2 and might regulate and influence the function of the ryanodine receptor. Though, since no skeletal involvement has been shown in CPVT patients it is most likely that additional candidate genes must be cardiac specific, probably limiting their numbers. Nevertheless, it seems likely that other yet unidentified genes might underlie further cases of CPVT.

Synopsis

Catecholaminergic polymorphic ventricular tachycardia is a distinct arrhythmogenic disease. The hallmark of CPVT is the reproducible occurrence of polymorphic ventricular tachycardia during exercise or stress at heart rates above 110 beats per minute (with or without syncope). During the polymorphic VT the ECG shows a typical bi-

directional pattern of QRS complexes. Furthermore, CPVT is characterised by a positive family history for syncope and SCD in relation to specific triggers (emotion, stress, exercise); the patient's history is characterized by syncope and SCD in response to similar triggers. In addition there are no clinical structural heart abnormalities, and the resting ECG including the QTc interval is normal. Sinus bradycardia may be a common finding, especially in young children. Exercise testing and Holter monitoring are effective tools to confirm a diagnosis of CPVT. The mean age of onset of the symptoms is 10 years, and both genders are susceptible. CPVT has a high mortality rate of up to 50% by the age of 30 years. β-blocker therapy appears effective in the majority of the patients, and the maximal well-tolerated dosages should be prescribed and Holter recordings and exercise tests should be repeated periodically to assure that the degree of sinus tachycardia that precedes onset of arrhythmias is not reached. Moreover, once the diagnosis is established it is crucial to make the patients aware of the necessity of faultless compliance to the β-blocker therapy given the high lethality of the disease and to avoid exercise and related triggers. In a small group of patients β-blocking treatment alone is not effective, however in combination with an ICD these relapsing patients should be adequately protected. Molecular analysis has shown that there is a small group of CPVT patients with an apparently normal phenotype, even after exercise testing. Worryingly, some of these phenotypically normal CPVT patients with normal exercise tests, do experience syncope and sudden cardiac death, implying that an asymptomatic phenotype doesn't guarantee protection from CPVT. Therefore, we also recommend the use of β-blocker therapy in asymptomatic mutation carriers. Finally, given the risk of juvenile sudden death (50%) and the efficacy of β-blocker/ICD therapy for CPVT, the identification of large numbers of mutations calls for genetic screening, early diagnosis and subsequent preventive strategies. In addition, the evidence that mutations in the RyR2 and CASQ2 genes underlie CPVT could lead to a more specific and effective pharmacological treatment.

Abbreviations

ARVD	arrhythmogenic right ventricular dysplasia
CASQ2	cardiac calsequestrin
CPVT	catecholaminergic polymorphic ventricular tachycardia
DAD	delayed after depolarization
PES	programmed eletrical stimulation
ICD	implantable cardiac defibrillator
VT	ventricular tachycardia
RyR1/3	ryanodine receptor type 1/3
SERCA2	sarcoplasmic/endoplasmic reticulum Ca(2+)-ATPase type 2
SCD	sudden cardiac death
SR	sarcoplasmic reticulum
TM	trans-membrane

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Chapter **5**

Absence of Calsequestrin 2 Causes Severe Forms of Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by syncopal events and sudden cardiac death (SCD) at young age during physical stress or emotion, in the absence of structural heart disease. We report the first nonsense mutations in the cardiac calsequestrin gene, *CASQ2*, in three catecholaminergic polymorphic ventricular tachycardia (CPVT) families. The three mutations, a nonsense R33X, a splicing 532+1 G>A, and a 1-bp deletion, 62delA, are thought to induce premature stop codons. Two patients, who experienced syncopes before the age of 7, were homozygous carriers suggesting a complete absence of calsequestrin 2. One patient was heterozygous for the stop codon and experienced syncopes from the age of 11 on. Despite the different mutations, there is little phenotypic variation of CPVT for the CASQ2 mutations. Of the 16 heterozygous carriers of these various mutations, 14 were devoid of clinical symptoms or ECG anomalies, while 2 of them had ventricular arrhythmias at ECG on exercise tests. In line with this, the diagnosis of the probands was difficult due to the absence of a positive family history. In conclusion, these additional three CASQ2 CPVT families suggest that *CASQ2* mutations are more common than previously thought and produce a severe form of CPVT.

Keywords

calsequestrin, tachycardia, syncope, arrhythmia, genetics

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an uncommon arrhythmogenic disorder occurring in children and adolescents with a structural normal heart. Patients suffering from CPVT are exposed to syncopal events and sudden cardiac death (SCD) at young age, due to stress-induced ventricular tachyarrhythmia[1]. The resting electrocardiogram, including the QTc interval, is generally normal but sinus bradycardia may be observed. CPVT has a highly malignant course when untreated, with estimates of mortality ranging from 30 to 50 percent by the age of 20 to 30 years[2]. Furthermore, there is a clear correlation between the age of the first syncope and the severity of the disease, with a worse prognosis in case of early occurrence. Beta-blockers without sympathomimetic activity are clinically effective in reducing syncope, but sometimes an implantable defibrillator may be needed[3].

The genetic basis of this inherited arrhythmogenic disorder has been established by the identification of autosomal dominant mutations in the cardiac ryanodine receptor gene (*RYR2*)[4,5]. More recently, Lahat *et al.* reported on one consanguineous CPVT family and their associated homozygous missense mutation in calsequestrin 2 (*CASQ2*), effectively describing a recessive form of CPVT[6]. Both genes play a crucial role in the excitation-contraction coupling; they are involved in the release of calcium from the sarcoplasmatic reticulum (SR) prior to contraction of the myocyte[7].

Genetic and clinical evaluation of our CPVT population allowed us to identify three new mutations in *CASQ2*. In this article we report on two CPVT families with phenotypes fitting with a recessive mode of inheritance, one family with a possible autosomal dominant mode of inheritance, and their associated nonsense mutations.

Methods

CPVT Families

The probands of the three families were referred to Paris or Amsterdam for syncope (n=2) and rescued cardiac arrest (n=1). CPVT diagnosis was established by documenting bi-directional VT, which was reproducibly induced by exercise stress testing and/or isoproterenol infusion in the absence of structural heart abnormalities as assessed by clinical history, blood chemistry, electrocardiography, and echocardiography. All probands had a normal QTc interval at rest. A familial history of syncope or sudden death was absent in families I and II. Family members were evaluated by 12-lead resting ECG,

exercise test and Holter recording. All individuals gave informed consent to the clinical and genetic study, which was approved by the internal ethics committee.

Genotyping of candidate genes

Mutation screening was performed on genomic DNA samples that were extracted from peripheral blood lymphocytes using standard methods. The genomic sequence of the *CASQ2* gene (accession no. NM_001232) was used to design intronic primers for all 11 exons and the 5' putative promoter region. PCR amplified fragments were analysed by uni-directional sequencing on an ABI 377 sequencer (Applied Biosystems) with BigDye terminator (Perkin Elmer). Aberrant sequences were re-amplified and re-sequenced to exclude polymerase errors. A mixed control group from France and The Netherlands of 200 alleles from healthy and unrelated subjects was used to exclude the possibility of a polymorphism. Additionally to the *CASQ2* screening, the entire coding regions of *SCN5A* (accession no. NM_000335), histidine rich binding protein (HRC accession no. NM_002152), phospholamban (PLN accession no. NM_002667), and 50% of *RYR2* (areas with known mutation/function, accession no. NM_001035) were screened using intronic primers. Moreover, in two of the three probands the coding regions of *KCNQ1* (accession no. NM_000218), *KCNH2* (accession no. NM_000238), *KCNE1* (accession no. NM_000219), and *KCNK1* (accession no. NM_002245) were also screened.

Results

Exclusion of *HRC*, *PLN* and *RYR2* coding regions

Direct DNA sequencing of all protein-coding areas of the candidate genes, histidine rich calcium binding protein (*HRC*), phospholamban (*PLN*), and sequencing of 50% of the ryanodine receptor type 2 (*RyR2*) gene failed to reveal any mutations in the 3 probands reported here (data not shown). In contrast, direct sequencing of all the coding regions of the *CASQ2* gene revealed 3 new mutations, each private to a distinct family.

CASQ2 mutations in CPVT families

Family I

A 6.5-year-old girl (II-2, figure 2) was initially referred after syncope occurred subsequent to a facial trauma while playing in school. A neurological evaluation was performed without detecting any anomaly at MRI. A second syncopal event occurred one month later while running. The resting ECG was normal regarding heart rate and QTc interval (Table 1). During hospitalisation, a Holter recording demonstrated salvos of PVT at a

triggering rate of 100 bpm, preceded by isolated polymorphic ventricular bigeminy. An exercise test triggered ventricular bigeminy and polymorphic salvos (see figure 1). Nadolol was started at 80 mg daily, but a new stress-induced syncopal event occurred 6 months later and the nadolol dosage was increased to 160 mg daily. The child remained asymptomatic for 4 years. The last Holter recording still demonstrated isolated polymorphic VPB. A new stress-induced syncopal event occurred recently under 160mg of nadolol and an ICD was proposed to the patient. The family pedigree suggests a recessive pattern of transmission, however the family is not aware of consanguinity. The family history was negative for sudden death or syncope, and exercise tests did not reveal any stress induced ventricular arrhythmia in the remaining family.

The probands' coding regions of *KCNQ1, KCNE1, KCNH2, KCNK1* and *SCN5A* did not reveal any abnormal conformers by PCR-SSCP screening. In contrast, screening of *CASQ2* revealed a homozygous 1-basepair deletion in the proband at nucleotide 62 of exon 1 (62delA figure 2). This deletion generates a frameshift in the reading frame, which leads to a stop codon 14 aminoacids downstream from the deletion. Both parents and four of the probands brothers are silent heterozygous carriers without any symptoms or ECG abnormalities at exercise testing (Table 1). One brother inherited two wildtype alleles, and his phenotype is indistinguishable from that of his parents or brothers (Table 1). Since the proband is homozygous for this deletion inducing stop codon, we assume that there is a total absence of functional CASQ2 protein in the proband.

Family II

The proband of family II (III-1 figure 2) is a 10-year-old male patient initially referred to the paediatric neurologist at the age 3 for breath holding spells. Spells increased both in

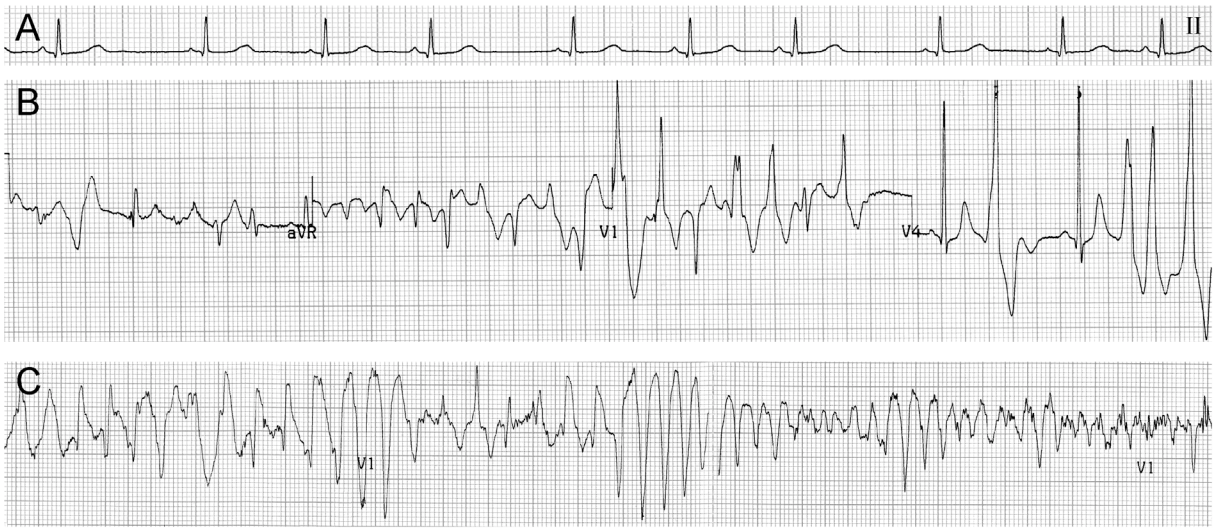

Figure 1. ECG recording in lead II of the proband of family I showing a normal QTc interval (428ms) and a slow heart rate (70 bpm) at admission (A). Examples of polymorphic ventricular premature beats at the beginning of the stress test (B) followed by self-terminating polymorphic salvos as the heart rate increases above 100 bpm (C). Recorded at 25mm/s and 10mm/mV.

frequency and severity over time with symptoms including paleness, cyanosis, and tachypnea. An epileptic disorder was suggested and carbamazepine therapy was initialised. The patient was resuscitated at age 7 after he experienced syncope during exercise. The ECG obtained immediately after the resuscitation efforts showed sinus bradycardia (HR 65 bpm) and a prolonged QTc interval (550 msec). Propranolol therapy was initiated at 2mg/kg, and the QTc interval normalized on subsequent ECGs. Despite this, the patient developed PVTs at heart rates greater than 125 bpm, which led to an increase in propranolol dosage to 4 mg/kg. Since then the patient has remained free of tachycardia and symptoms with a follow-up of 3 years (table 1). The proband is the oldest child of consanguineous parents (first cousins) and has two younger sibs (aged 8 and 6 years) who are asymptomatic. The remaining family members are also asymptomatic and no sudden death has been reported. Subsequent exercise tests and Holter recordings of the family revealed that the proband's brother (III-3) had doublets and runs of PVT, for which he was treated by beta-blockers at the age of 5. Exercise tests were normal for the rest of the family members.

After exclusion of the above mentioned candidate genes we screened *CASQ2* and found a homozygous intronic basepair change (G to A), 1bp downstream of exon 4 (532 +1 G/A figure 2). The intronic G/A basepair change in Family II occurs at the fully conserved 532 +1 position in the 5' splice site[9]. The effect of the mutation would either be the abolishment of the authentic donor splice site, and the subsequent utilization of a completely conserved cryptic donor splice site 55 basepairs downstream. Or alternatively, exon skipping could produce a *CASQ2* transcript lacking exon 4. Unfortunately, we cannot determine *in vitro* the exact splicing effect, as there is no patient heart tissue available, and attempts to amplify *CASQ2* mRNA from the probands native lymphocytes failed. However, regardless of the mechanism, a frameshift occurs in the reading frame and a premature stop codon for *CASQ2* is introduced by this +1 G/A mutation. Genotyping of the family revealed that the youngest brother (III-3) of the proband, with a CPVT phenotype, also carries the homozygous splicing defect. The parents, their other

TABLE 1. Clinical Data of CASQ2 CPVT families

	Mutation	Age 1st syncope	Age diagnose	Current age	HR diag. bpm	Median HR age group[10]	QTc ms	PVT threshold, bpm	Symptoms	Treatment
Proband										
Family I (n=1)	62delA	6.5	6.5	14	70	88	428	100	Exercise induced syncope	nadolol 80mg/D
Family II (n=1)	532 +1 G/A	5	7	10	63	88	403	120	Syncope/Cardiac Arrest	propanolol 4x30mg/D
Family III (n=1)	R33*	11	18	24	46	74	440	95	Exercise induced syncope	nadolol 80mg/D
	mean±SD	8±3	11±7	15±8	60±12		424±19	105±13		
Heterozygous Carriers										
Family I (n=6)	62delA	-	-	26±7	79±9		422±16	-	-	-
Family II (n=4)	532 +1 G/A	-	-	25±14	73±11		413±6	-	-	-
Family III (n=5)	R33*	-	-	48±19	70±9		387±21	-	-	-
Wildtype										
Family II (n=1)	-	-	-	12	82		425	-	-	-
Family III (n=10)	-	-	-	39±22	78±7		391±26	-	-	-

son (II-2), and 2 other family members are heterozygous for the splicing defect, fitting with the recessive mode of transmission (figure 2). As the proband is homozygous for the 532 +1 G/A splicing mutation, we assume that there is a total absence of functional CASQ2 protein in this patient as observed in Family I.

Family III

The proband of Family III (III-3, figure 2) presented with syncopal events since the age of 11 years. These syncopal episodes were of short duration and occurred at the end of exercise. The initial diagnosis was vagal syncope, and CPVT was not diagnosed until the 18th year. At that time, a Holter recording demonstrated numerous polymorphic ventricular premature beats (VPB) with couplets during sinus tachycardia. Resting ECG showed sinus bradycardia and a borderline QTc interval (table 1). A subsequent stress test confirmed CPVT by showing polymorphic ventricular salvos above 135 bpm. Nadolol (80mg/day) was started leading to the disappeareance of syncopal events. She has been completely asymptomatic under nadolol with a 6-year follow-up. The family history was

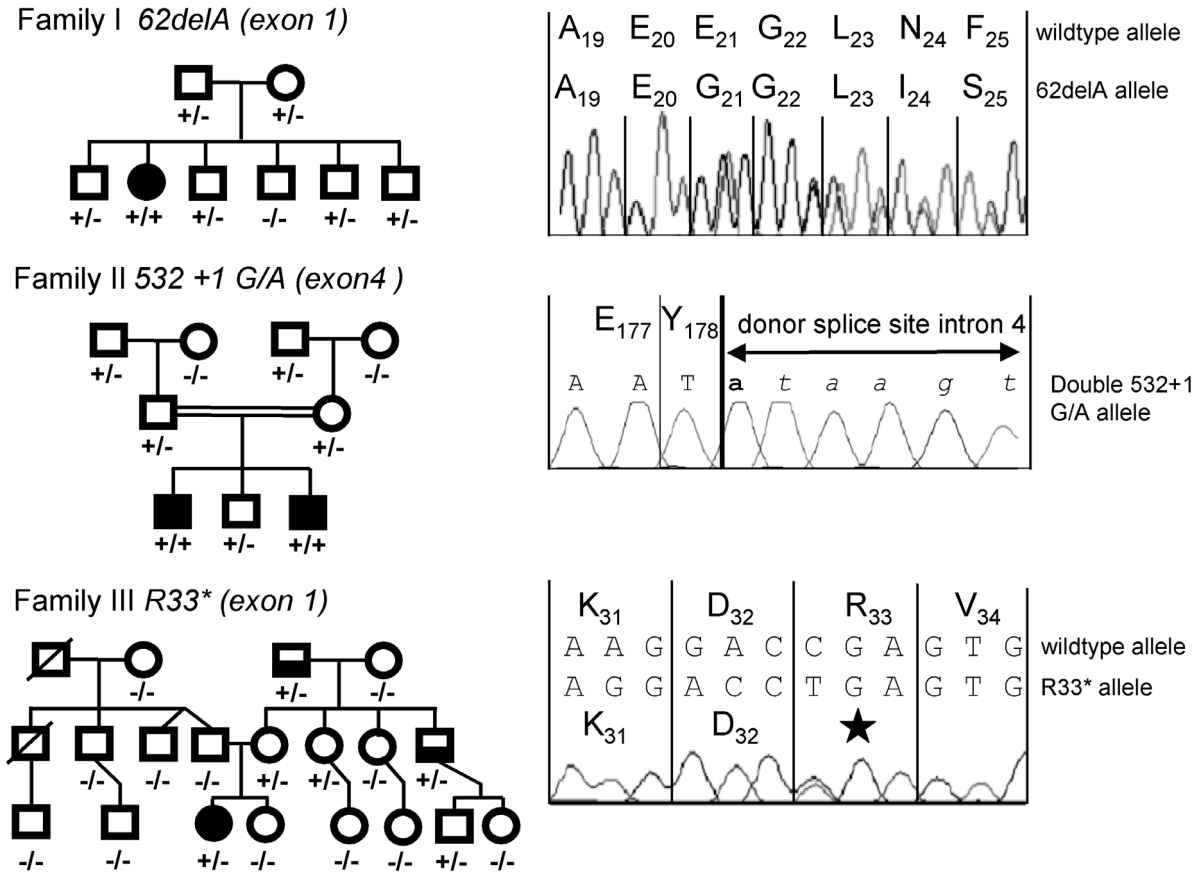

Figure 2. Pedigrees of the three CPVT CASQ2 families and their associated mutations, the solid symbols indicate affected individuals with a bi-directional VT, syncope, and a CPVT diagnosis. Half filled symbols are individuals with VPB and bigeminy on the ECG during exercise tests, unfilled symbols are asymptomatic individuals after exercise testing. "-" indicates presence of the mutation, mutations are displayed according to their aminoacid number or nucleotide number where applicable. Sequence given of individual I-1 in family I, III-1 in family II, and III-3 in family III.

negative for syncope or sudden death; nonetheless exercise tests of the family demonstrated episodes of bigemini and premature ventricular beats in both the maternal grand-father (I-3) and uncle (II-8).

The coding regions of *KCNQ1*, *KCNE1*, *KCNH2*, *KCNK1* and *SCN5A* did not reveal any abnormal conformers by PCR-SSCP screening. Subsequent sequencing of *CASQ2* revealed a heterozygous mutation in the first exon of *CASQ2*, which changes an arginine into a stop codon at position 33 (R33*). Segregation analysis of the family suggests that this nonsense mutation was inherited from the mothers side with five additional carriers (figure 2). The proband's mother, her sister and a nephew carry the heterozygous R33X nonsense mutation but are phenotypically normal and they had no ECG abnormalities during exercise tests. The grandfather and uncle do have mild CPVT symptoms detectable during exercise tests as described above, but never had syncope.

Discussion

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by syncopal events and sudden cardiac death (SCD) at young age during physical stress or emotion, in the absence of structural heart disease. Diagnosis of CPVT is difficult since rest ECG's are usually normal. Holter recordings and especially exercise tests are useful in uncovering CPVT. Mutations in calcium handling proteins located in the sarcoplasmic reticulum (SR), such as RYR2 and CASQ2, are associated with CPVT. RYR2 CPVT mutations have an autosomal dominant mode of transmission, while the one published CASQ2 CPVT mutation has an autosomal recessive transmission. We report here the first nonsense mutations in the cardiac calsequestrin gene, *CASQ2*, in three additional CPVT families.

In contrast to the array of different mutations leading to CASQ2 CPVT (see below), the phenotypes of the three probands share many similarities: the probands reported syncope were always preceded by exercise or emotion, and exercise testing in the probands showed an average threshold of 110bpm before the appearance of PVT, which was reproducibly induced in the same proband. Their QTc interval was within normal parameters[10], however the probands have a relative resting bradycardia (60 bpm), which differs from healthy children in their age groups (88 and 73 bpm) (table 1)[10]. Interestingly, all probands had runs of PVT and doublets on their Holter recordings, without any reported symptoms, thus Holter recordings might be useful in detecting (CASQ2) CPVT. Treatment with β-blockers has a favorable overall outcome, 2 of the 3 probands are symptom free with a mean follow-up period of 4 years, for the other proband an ICD is being considered. The above mentioned phenotype: exercise induced syncope, PVT

thresholds, normal QTc intervals and bradycardia, are in concordance with the homozygous carriers of the consanguineous family described by Lahat, who have an average heart rate of 64bpm, a PVT threshold of 110bpm and a QTc interval of 420msec[6]. Thus, regardless of the precise nature of the mutation or its effect (see below), it appears that CASQ2 mutations generate a homogeneous phenotype. Nevertheless, the homozygous carriers of the family described by Lahat and our reported CASQ2 probands may represent a specific early age of onset subgroup among CASQ2 CPVT probands, which doesn't exclude the possibility of CASQ2 CPVT probands with a different phenotype including later age of onset. The age of onset of CPVT symptoms appears to differ somewhat between our three CASQ2 CPVT families. The two homozygous CASQ2 probands, of families I and II, have an earlier age of onset than the heterozygous proband of familiy III (6 vs. 10.5 years). This might be explained by the autosomal recessive transmission, as there is no compensation from wildtype protein, homozygous mutations produce a more severe phenotype and thus surface earlier. Comparison between CASQ2 CPVT probands and published RYR2 CPVT probands shows a similarity in CPVT symptoms, threshold of PVT and QTc interval. The effectiveness of the therapy also seems similar between those groups. A recent publication, with a comparable follow-up time, shows that in 7 of 19 genotyped RYR2 probands, episodes of VT/VF occurred while on β-blockers of equivalent dosage[3]. In line with those RYR2 probands, the CASQ2 proband of family I experienced a stress related syncope while on β-blockers, the other two CASQ2 probands remain symptom free. The comparison of the relative bradycardia seen in the genotyped CASQ2 probands with genotyped RYR2 probands is difficult to make, as RYR2 genotyped probands heartrates are either pooled within a single family or not reported[4,5]. In an overview of non-genotyped CPVT patients, which did not include our CASQ2 probands, Leenhardt describes bradycardia similar to our CASQ2 probands[1]. However, since that patient group most likely contained both CASQ2 and RYR2 mutation carriers, it is difficult to separate the two. Interestingly, sino-atrial node cells, which serve as the primary pacemaker of the heart, have RYR channels and functional SR[12]. Moreover, substances that interfere with SR function have a negative chronotropic effect on the SA node[13]. Thus, the bradycardia seen in the CASQ2 probands could be a direct effect of the impaired Ca^{2+} handling of their SA nodal cells. Overall, it seems that there is a large phenotypic overlap amongst CASQ2 probands, and a smaller overlap between CASQ2 probands and published RYR2 probands.

It is striking that (induced) nonsense CASQ2 carriers in the three families are asymptomatic or mildly affected, despite the fact that they probably have half of the normal amount of CASQ2 protein. However, it has been shown that the predominant

consequence of nonsense mutations is not the synthesis of truncated proteins. Rather the majority of nonsense transcripts are recognized and efficiently degraded by the cell via a pathway known as Nonsense Mediated mRNA Decay (NMD)[14]. Moreover, in diseases such as β-thalassemia and Marfan syndrome, NMD is a potent modulator of the phenotype; nonsense mutations in the first part of a gene usually result in very mild or asymptomatic phenotypes. In contrast, nonsense mutations in later exons are not degraded and result in truncated protein, possible interaction with the wildtype protein, and a severe phenotype[15]. Since our reported nonsense mutations occur early in the gene, we believe that NMD might explain why nonsense CASQ2 carriers are asymptomatic or mildy affected. Nonetheless, although our nonsense heterozygous CASQ2 mutation carriers are not inducible by exercise testing, they most likely have compromised Ca^{2+} binding ability in the SR. Alarmingly, phenotypically normal RYR2 mutation carriers with normal exercise tests, do have syncope and sudden cardiac death, implying that an asymptomatic phenotype doesn't guarantee protection from CPVT[16]. In this respect, a long-term follow-up study in asymptomatic heterozygous CASQ2 mutation carriers might reveal if they are (also) at risk for developing CPVT.

Conversely, nonsense homozygotes, arguably putative null alleles, as seen in families I and II, are severely affected. In accordance with NMD, homozygosity for these nonsense mutations would result in complete absence of functional CASQ2 protein. As described earlier RYR2 homozygote knockout mice die during embryonic life[11], thus it seems that properly regulated calcium release is crucial. Similarly, a SR lacking buffered calcium, due to absence of CASQ2, would seem incompatible with life, since regulated calcium release would be compromised. Since both homozygous probands survived the first years, a mechanism must exist which provides calcium buffering in the SR in the absence of functional CASQ2 protein. One possibility is the up regulation of other Ca^{2+} binding proteins such as histidine rich binding protein (HRC) or sarcalumenin, which both have been identified in the SR of cardiac muscle and shown to bind Ca^{2+} reminiscent to calsequestrin[17,18]. Alternatively an increase or up regulation of phospholamban and SERCA activity could produce an adequate level of calcium in the absence of the buffering power of CASQ2. Unfortunately, we are unable to verify this since there is no heart tissue available. Attempts to amplify or detect CASQ2 in native lymphocytes failed, probably due to its heart specificity. Regardless of the mechanism, it is clear that homozygosity for nonsense mutations in CASQ2 co-segregates with the CPVT and produces a severe phenotype. In this respect it is important to analyze pedigrees of CPVT probands carefully as consanguinity might infer CASQ2 involvement. Moreover, sporadic CPVT cases without familial involvement may be explained by recessive transmission of CASQ2 mutations.

In family III we found a heterozygous nonsense mutation that is not only present in the proband, but also in five additional family members. However, as opposed to the heterozygous nonsense carriers in families I and II, it seems that some nonsense carriers in family III have at least a mild phenotype upon exercise. The R33X nonsense mutation produces episodes of bigiminy and VPB upon exercise testing in two out of the five carriers, besides the proband, although they have no reported syncope. The remaining three nonsense carriers of family III do not exhibit any symptoms of CPVT. The mild phenotype in the two carriers may represent a phenocopy caused by underlying ischemic heart disease or another common cardiac disease. Alternatively, a reason for the mild or absent phenotypes in the heterozygous carriers, and the strongly affected proband of family III could be reduced penetrance, analogous to a recent study on CPVT which reports that 17% of RYR2 mutation carriers have no phenotype[3]. This would make the R33X mutation the first autosomal dominant mutation for CASQ2. Alternatively, based on the unaffected parents and the similarity in phenotype between the homozygous CASQ2 probands and the heterozygous CASQ2 proband, a second mutation in the calcium handling pathway, besides a CASQ2 mutation, might be present in the heterozygous CASQ2 proband leading to CPVT. In this way, the clearly affected proband and the mildly affected family members are explained by a multifactorial form of CPVT. With regard to this we excluded several calcium handling and ion channel genes by sequencing, though we cannot eliminate the possibility of mutations in other genes acting as a second disease allele in the heterozygous proband of family III. However, a (influence of) second gene altering calcium handling will be difficult to find in the asymptomatic parents, as it is not producing a phenotype, in spite of exercise testing. Concluding, the heterozygous nonsense mutation in family III shows a different phenotype from nonsense mutations in families I and II, either the family's distinct phenotype is altered by incomplete penetrance, or the Ca^{2+} handling in the proband is further compromised by an additional mutation suggesting a multifactorial form of CPVT.

In summary, we report the first nonsense mutations in the cardiac calsequestrin gene, *CASQ2*, in three CPVT families. Sequencing of *CASQ2* revealed three different nonsense (inducing) mutations. However, despite the different mutations, there is little inter-phenotypic variation of CPVT for the *CASQ2* mutations, besides the age of onset in homozygous probands. Family pedigrees suggest that two *CASQ2* mutations are homozygous (consanguineous) and the other one is either autosomal dominant with reduced penetrance, or a component of a multifactorial form of CPVT. The unforeseeable risk of juvenile sudden death (50%) and the proven efficacy of beta-

blocker/ICD therapy for CPVT, emphasize the role of genetic screening for early diagnosis and preventive strategies. In addition, the evidence that mutations in the *RYR2* and *CASQ2* gene are involved in CPVT could lead to a more specific and effective pharmacological treatment. Finally, it is noteworthy that complete absence of CASQ2 in our two homozygous probands, as predicted from the mutations, is compatible with life.

Limitations

We identified two frameshifting mutations, present in the probands of families I and II, and we argue that these frameshifts lead to null alleles. Since we did not have myocytes of the probands, and are for obvious reasons not able to get them, we cannot show *in vitro* that there is an absence of CASQ2 protein in the probands and prove the null allele hypothesis.

Acknowledgements

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Chapter **6**

Catecholaminergic polymorphic Ventricular Tachycardia: RYR2 Mutations, Bradycardia, and Follow-up of the Patients

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Submitted to JACC

Abstract

Objectives

We sought to assess the underlying genetic cause, the clinical features, and the response to therapy in 24 catecholaminergic polymorphic ventricular tachycardia (CPVT) probands.

Background

CPVT is a rare arrhythmogenic disorder characterized by recurrent syncopal events and sudden cardiac death (SCD) at young age triggered by physical stress or emotion, in the absence of structural heart disease.

Methods

We collected twenty-four CPVT probands with documented polymorphic ventricular arrhythmias occurring during physical or emotional stress with a normal heart. Genetic and phenotypic characterization of the probands and their family members, allowed us to assess the clinical features, response to therapy and genotype-phenotype correlation.

Results

13 missense mutations were identified in the cardiac ryanodine receptor (*RYR2*), 12 are novel and change evolutionary highly conserved residues. Little inter-phenotypic variation was found among the CPVT patients with (12) or without RYR2 mutations (12), but a significant resting sinus bradycardia was observed in both groups. With a mean follow-up period of 86±18 months, 97% of the identified CPVT patients remain symptom free on β-blocker treatment.

Conclusions

RYR2 mutations cause CPVT in half of our patients and are associated with a significant bradycardia, more important in males than in females. Bradycardia is also observed in "genetically undetermined" CPVT patients, which could direct the search for molecular diagnosis in (young) patients without structural heart disease presenting with syncopal events and a normal resting ECG. "Individually adapted" β-blocking treatment has a favorable overall outcome in our group of CPVT patients. Given the risk of sudden death and the efficacy of β-blocker therapy, the identification of large numbers of RYR2 mutations calls for genetic screening, early diagnosis and subsequent preventive strategies.

Keywords

ryanodine receptor, tachycardia, syncope, arrhythmia, genetics

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by adrenergic-induced bidirectional and polymorphic ventricular tachycardia[1]. It occurs in children and adolescents and causes syncope and sudden cardiac death at a young age, in absence of structural heart disease. The resting electrocardiogram (ECG) including the QTc interval, is thought to be normal. The mortality of CPVT is extremely high reaching 30-50% by the age of 30 years when untreated[2]. Furthermore, there is a clear correlation between the age of the first syncope and the severity of the disease, with a worse prognosis in case of early occurrence. Beta-blockers without sympathomimetic activity are clinically effective in reducing syncope, and the implantation of an intra cardiac defibrillator is rarely needed in these patients[3].

The genetic basis of CPVT had been initially elucidated by the establishment of linkage between the disease and chromosomal region 1q42[2]. Subsequently, several groups independently discovered autosomal dominant missense mutations in the ryanodine type 2 receptor (*RYR2*) associated with CPVT[4,5]. The *RYR2* gene, located on 1q42, encodes the cardiac ryanodine receptor, which is the major calcium release channel on the sarcoplasmic reticulum (SR) in cardiomyocytes[6]. More recently, two studies reported consanguineous CPVT families associated with homozygous missense and nonsense mutations in calsequestrin 2 (*CASQ2*), a Ca^{2+} binding protein located in the SR, thus describing a recessive form of CPVT[7,8]. Both RYR2 and CASQ2 play a crucial role in the excitation-contraction coupling, by their involvement in the storage and release of Ca^{2+} from the SR, which subsequently activates cardiomyocyte contraction.

We report here the genetic and clinical characterization of 24 probands and their family members, which allowed us to assess the clinical features, the response to therapy and the genotype-phenotype correlation of this disease in a large group of patients. We identified *RYR2* missense mutations in 12 CPVT families. We provide evidence that RYR2 mutation carriers and all phenotypically affected CPVT patients have a significant resting sinus bradycardia and report the high efficacy of the β-blocking treatment, when well-adapted and followed in our group patients.

Methods

CPVT Families

The probands of the 24 families were referred to Paris or Amsterdam, CPVT diagnosis was established by documenting the occurrence of the characteristic ECG pattern of the

disease (monomorphic ventricular premature beats followed by bi-directional ventricular tachycardia and salvos of polymorphic ventricular tachycardia) in the absence of structural heart abnormalities as assessed by clinical examination, blood chemistry, electrocardiography, and echocardiography. The QTc interval was calculated with the Bazett formula; normal resting heart rates were established with reference to published criteria[9]. When a CPVT diagnosis was established in the proband, 12-lead resting ECG, exercise test and Holter recording were proposed to family members and accepted in 74% of the cases. Apart from the 24 probands mentioned above, we collected 3 additional CPVT probands, which are not included in the present study. In these probands we have previously identified CASQ2 mutations[8]. All individuals gave informed consent to the clinical and genetic study, which was approved by the internal ethics committee.

Genotyping of candidate genes

Mutation screening was performed on genomic DNA samples extracted from peripheral blood lymphocytes using standard methods. The genomic sequence of the *RYR2* gene (accession no. NM_001035) was used to design intronic primers for 45 exons, covering areas with known function or mutations, resulting in the amplification of 53 fragments (6365 bp cDNA). PCR amplified fragments were analyzed by uni-directional sequencing with ABI PRISM BigDye Terminator cycle sequencing ready reactions kit and run on a 3700 Genetic Analyzer (Applied Biosystems). Aberrant sequences were re-amplified and re-sequenced to exclude polymerase errors. A mixed control group of 100 healthy and unrelated subjects from France and The Netherlands was used. Additionally to the *RYR2* screening, the entire coding regions of cardiac calsequestrin (*CASQ2* accession no. NM_001232), histidine rich binding protein (*HRC* accession no. NM_002152), and phospholamban (*PLN* accession no. NM_002667), were screened using intronic primers.

Genotyping of parents

Genotypes of parents of *de novo* probands were determined using the Powerplex 1.2 system (Promega) according to the instructions provided.

Statistical analysis

Data are presented as mean±SEM. RR intervals of resting ECGs were measured and converted to beats per minute, and subsequently compared to age and gender matched control heart rates[9]. The deviations from these control heart rates were evaluated among different groups. The effect of the genotype, phenotype and gender on heart rates, as well

as the interaction between genotype and gender or phenotype and gender were tested with the Mixed Model Analysis of Variance (SPSS, version 11.0) in which family membership was used as a repeated factor. Statistical significance was accepted at $p < 0.05$.

Results

Clinical Evaluation Probands

The 24 probands were referred because of syncopal events (n=18) or rescued cardiac arrest (n=6) related to life-threatening arrhythmias (polymorphic ventricular tachycardia or ventricular fibrillation) occurring during physical or emotional stress (11 males, table 1). Syncope occurred in 23 of 24 probands, as the first manifestation of the disease. Nine of these probands presented with seizures during the course of their syncopal events. The mean age of onset of the symptoms was 13±2 years among the probands (range 3 to 51 years of age, table 1). Two probands experienced sudden cardiac death; one prior to diagnosis, which was retrospectively established based on DNA and clinical records; the other died after CPVT diagnosis was established due to non-compliance to β-blocker treatment. Diagnosis of CPVT was confirmed in the probands with the use of exercise tests (23/24), which reproducibly showed the occurrence of the characteristic ECG pattern of the disease (monomorphic ventricular premature beats followed by bi-directional ventricular tachycardia and salvos of polymorphic ventricular tachycardia) in all living probands. The resting ECG was normal, including the QTc interval. However,

TABLE 1. Clinical Data of CPVT probands

RYR2 mutation	Gender	Age of onset, y	Penetrance %	Rest ECG pre med, bpm	Median HR age group[9]	Deviation bpm	QTc, ms	Symptoms	Followup, y	Origin
Fam.4 F4020L	M	4	n/a	70	98	28	410	x syncope, seizure	SD at 20	French
Fam.8 H4108N	F	4	*de novo*	50	73	23	374	x syncope, seizure, CA	2	French
Fam.9 H4108Q	F	6,5	*de novo*	70	89	19	400	x syncope	12	Dutch
Fam.7 N4104I	M	7	100	60	88	28	400	x syncope, seizure	2	French
Fam.2 A2254V	M	8	100	66	84	18	410	CA x VT	22	Dutch
Fam.1 E1724K	F	9	100	43	78	35	380	x syncope	15	Dutch
Fam.3 A2394G	M	9	100	60	78	18	440	x syncope, seizure, CA	6	French
Fam.6 E4076K	F	10	66	58	76	18	405	x syncope	3	Dutch
Fam.11 V4771I	F	12	n/a	…	…	…	…	x syncope, seizure	7	French
Fam.12 P4902S	F	13	65	60	76	16	414	x syncope	6	French Polynesia
Fam.10 H4762P G4662S	F	13	compound	…	…	…	410	x syncope	28	French
Fam.5 E4076K	M	51	64	73	adult	-3	351	x syncope, seizure	4	French
		12±4				**20±2**	**400±7**		**8±2**	
Non-genotyped										
…	F	3	…	…	…	…	400	x syncope	7	French
…	M	8	…	…	…	…	400	x syncope	5	French
…	M	11	…	63	77	14	400	x syncope	5	Dutch
…	F	11	…	60	79	19	400	x syncope, seizure	2,5	French
…	M	12	…	…	…	…	420	CA x VT	10	French
…	F	12	…	60	78	18	420	x syncope, seizure, CA	0,5	French
…	M	12	…	40	76	36	391	x syncope	18	Dutch
…	F	13	…	88	77	-11	360	x syncope, seizure, CA	14	French
…	F	14	…	…	…	…	337	x syncope	2	French
…	F	16	…	…	…	…	421	x syncope	1,5	French
…	M	27	…	75	70	-5	380	x syncope	14	Dutch
…	M	33	…	68	70	2	410	x syncope	SD at 33	Dutch
		14±2				**10±6**	**395±7**		**7±2**	
Combined		**13±2**				**14±3**	**397±5**		**8±1**	

x syncope= exercise induced syncope, CA= cardiac arrest, VT= ventricular tachycardia, SD= sudden death, n/a= not available, Mean±SEM

the mean resting heart rate was lower than that of age-matched groups, see below (Table 1). We did not observe any significant differences in clinical parameters between probands with a RYR2 mutation (see below) and without an identified mutation (Table 1).

Clinical Evaluation of Family Members

In our 24 families, we identified 41 additional clinically affected family members aging from 11 to 62 (Table 2). In all of them arrhythmic events were induced during exercise testing and had a pattern similar to that observed in the corresponding proband. Previous syncopal events were reported in 28 of these 41 of clinically affected family members, leading to a high percentage (66%) of syncopal events in the entire population. Moreover, a history of sudden cardiac death was present in 16 of 24 families (66%), with a total of 37 lethal events (15 in male subjects) with a mean age 24±11 years.

Treatement and follow-up of CPVT patients

The 65 clinically affected patients and 10 silent RYR2 mutation carriers (see below) were treated with β-blockers (1 to 2 mg/kg per day nadolol, 3 to 4 mg/kg per day propranolol, 50 to 100mg per day metoprolol), which were individually titrated until the maximal heart rate was <110bpm at exercise tests or Holter recordings, in order to prevent polymorphic ventricular salvos. The mean dosage of nadolol was 1.6±0.15 mg/kg. β-blocker treatment had a favourable overall outcome, as 73/75 (97%) patients were symptom free with a mean follow-up period of 86±18 months, though some continued to have PVBs at exercise tests. One patient died on β-blockers due to cardiac arrest and failure to

TABLE 2. Clinical Data of CPVT Patients According to Genotype

	RYR2 CPVT	non-RYR2 CPVT	*P*
Number of probands	12	12	NS
Families with ≥ 2 affected	7/12	8/12	NS
Families with juvenile SD	7/12	9/12	NS
Clinically affected	37	28	NS
Phenotype undetermined	4	NA	NS
Silent gene carrier	10	NA	NS
Gender	21M/30F	14M/14F	NS
Exercise-related syncope	32/51	20/28	NS
Patients on β-blockers	47/51	28/28	NS
Follow-up, months	96±24	64±20	NS
VT/VF on β-blockers	1/47	1/28	NS

Mean±SEM

resuscitate, and another experienced syncope. An ICD was implanted in two RYR2 positive patients; the first was implanted at age 51 because the etiology of the syncope in the context of a familial sudden death was unknown; the other at age 18 because of poor compliance in the symptomatic sister of the proband of Family 3. Over a follow-up of 1 year, the second patient received two appropriate shocks to terminate ventricular fibrillation; both patients remained on β-blockers. In four RYR2 mutation carriers we couldn't establish a definite clinical phenotype, as they were too young (<5 years) to perform exercise testing (figure 1). However, as they were phenotypically normal, these children were not treated thus far.

Exclusion of candidate gene coding regions

Direct DNA sequencing of all protein-coding areas of the candidate genes *CASQ2*, *HRC*, and *PLN* failed to reveal any mutation in the 24 probands reported in the present study (data not shown). Moreover, in families 3,4,9, and 11 the coding regions of

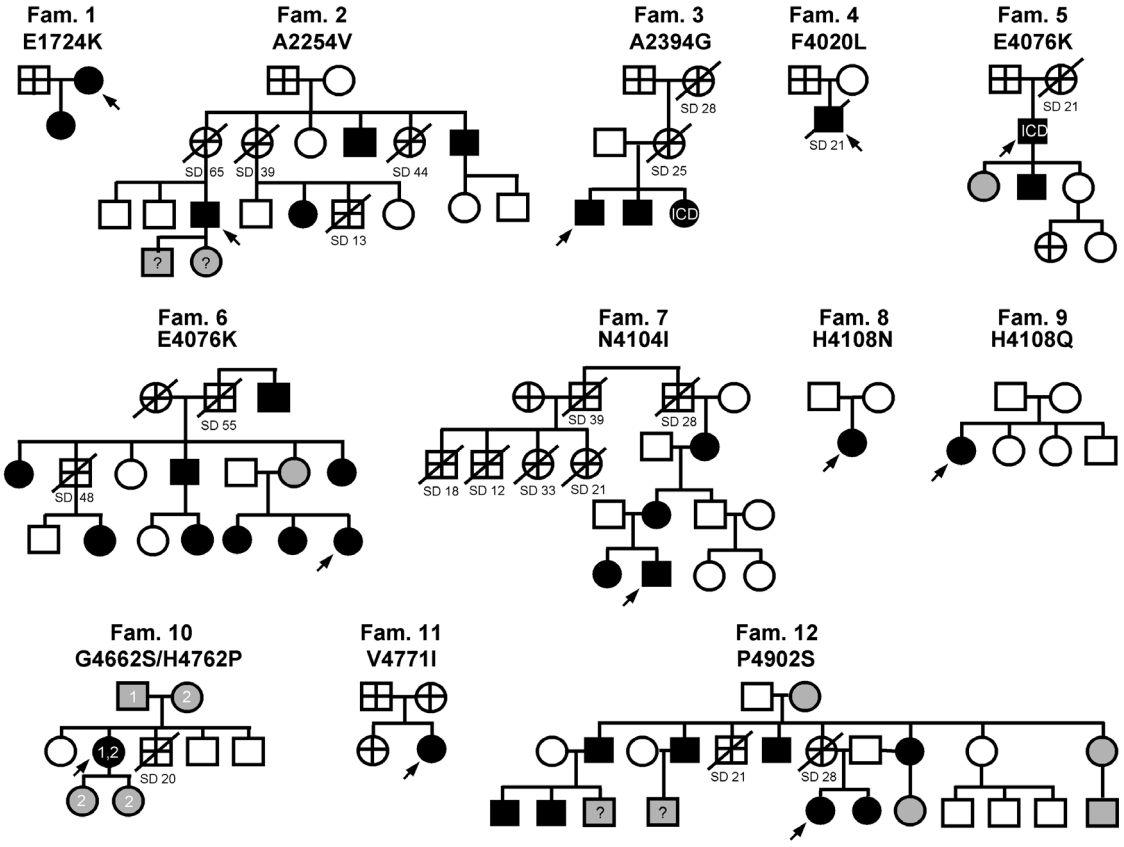

Figure 1. Overview of the 12 CPVT families with identified RYR2 mutations and their clinical phenotypes. Black symbols indicate a CPVT phenotype with presence of a RYR2 mutation, open symbols represent clinically unaffected persons without a RYR2 mutation, grey symbols indicate persons with a RYR2 mutation, who are clinically unaffected, and non-genotyped individuals are indicated by a cross. Question marks indicate persons with a RYR2 mutation, but undetermined phenotype. Probands of each family are indicated by an arrow, deaths are indicated by a transverse line. CPVT related sudden cardiac deaths are indicated by SD and the age of the death in years. In Family 10 number 1 corresponds to the presence of the G4662S mutation and number 2 that of the H4762P mutation.

KCNQ1, *KCNH2*, *SCN5A* and *KCNE1* did not show any aberrations.

Genetic Evaluation of CPVT families

In contrast, direct sequencing of the coding region of *RYR2* revealed 13 mutations in 12 of the 24 CPVT probands (50%), of which 12 were novel (Table 1, figures 1,2). In 8 out of 12 RYR2 mutation carrier probands (66%) the genetic defect was inherited in an autosomal dominant manner. Two mutations, H4108N and H4108Q, occurred *de novo* in Families 8 and 9, in which the parental transmission was confirmed. For two mutations, F4020L and V4771I, it was impossible to establish an inheritance due to unavailability of parental DNA (Families 4 and 11). Four of the eight autosomal dominant families display complete penetrance based on stress tests (families 1,2,3,7 figure 1). In contrast, the other four families (5, 6, 10, and 12) show incomplete penetrance (Figure 1, table 1), with 10 mutation carriers being clinically unaffected. Interestingly, eight of these clinically unaffected mutation carriers were females (31±8 years). As mentioned above, we couldn't establish a genotype-phenotype relation in four RYR2 mutation carriers (3 male), as they were too young (<5 years) to perform exercise testing (Figure 1).

The mutation E4076K was identified in two families. We excluded a familial relationship

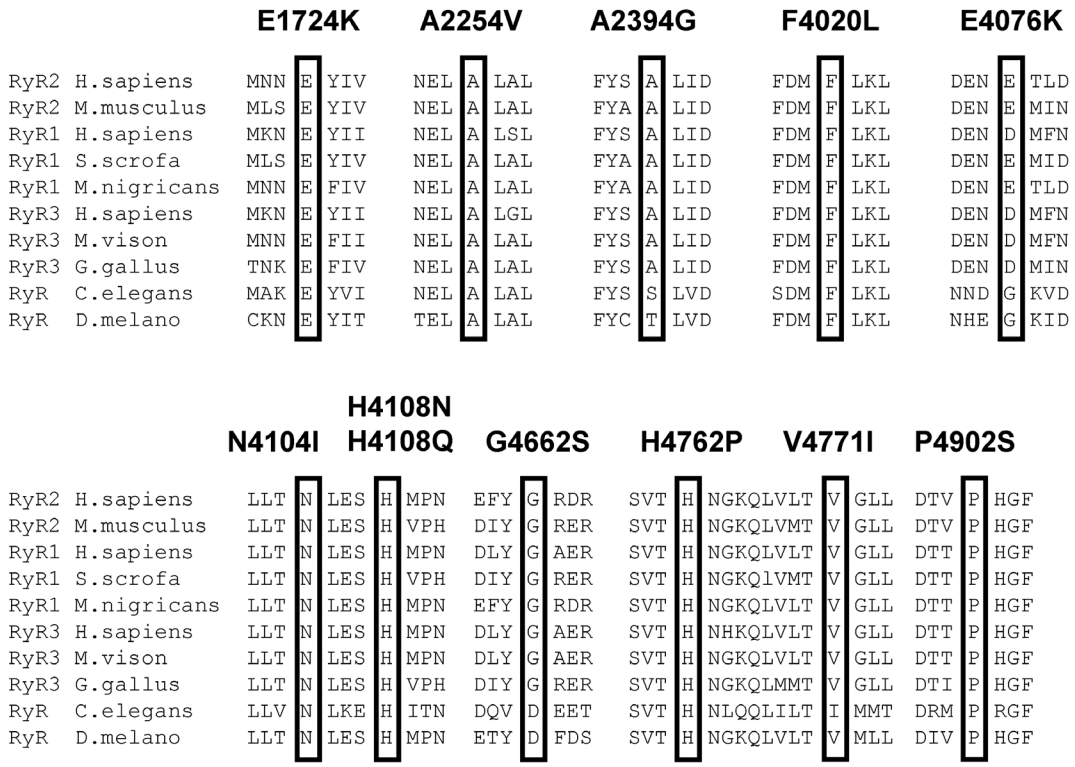

Figure 2. Alignment of RYR protein sequences of multiple species. Amino acid positions at which the various RYR2 mutations occur are indicated by the boxed areas.

between these families by the use of a rare polymorphism located in exon 37 (-136 G/A), which co-segregates with the mutation in Family 6, but was absent in Family 5.

In families 8 and 9, two independent *de novo* mutations change the same histidine 4108 resulting in its substitution into aspargine and glutamine respectively (Figure 1). Another mutation, N4104I, was identified in Family 7 only 4 amino acids upstream. The clinical features of the three probands are quite similar regarding symptoms, low heart rate and early age of onset (Table 1).

In Family 10 the proband carries two separate mutations (G4662S and H4762P). Genetic analysis shows that her parents each carry a single mutation. The two children of the proband both inherited the H4762P mutation (Figure 1). Her parents and her two children are clinically unaffected.

All the 12 mutations identified occurred in evolutionary highly conserved positions in the RYR2 protein (Figure 3). The mutations clustered into three distinct regions, the binding site of FKBP12.6 and other modulatory proteins (3 mutations), the Ca^{2+} binding site (6 mutations), and the pore forming transmembrane domains (4 mutations) (Figure 3).

Comparison of the clinical characteristics in genotyped and non-genotyped CPVT patients

The 51 RYR2 mutation carriers (including the 12 RYR2 positive probands) were

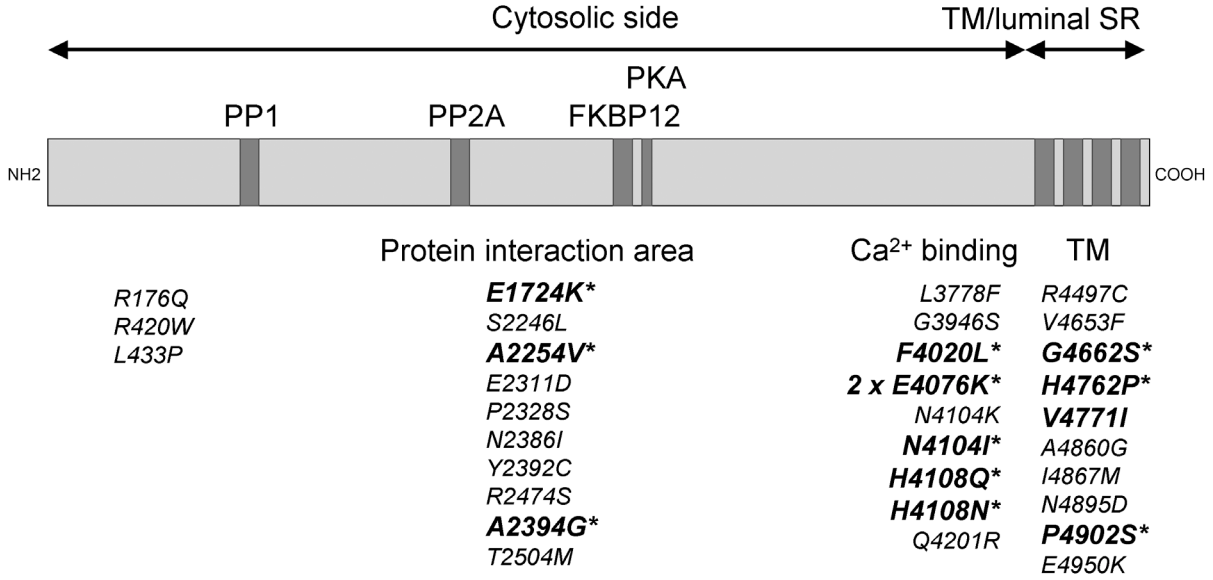

Figure 3. Schematic representation of the RYR2 protein, with an overview of predicted function and topology, and the location of all the known RYR2 mutations. Indicated in bold are RYR2 mutations identified in this study with novel mutations marked with an *, the other mutations have been previously reported[3-5,12,19].

compared with the 28 RYR2 negative CPVT patients (including the 12 RYR2 negative probands). No statistical differences were observed between the two groups regarding clinical parameters such as exercise-related syncope, number of juvenile deaths, or response to antiadrenergic therapy (Table 2).

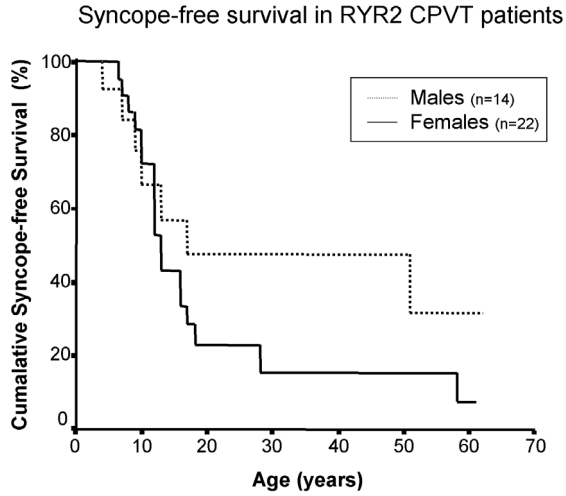

Figure 4. Kaplan-Meier analysis of cumulative syncope-free survival in 36 untreated genotyped RYR2 CPVT patients by gender.

Absence of gender difference in syncope free survival

From the 51 RYR2 mutation carriers, 25 had a reliable indication of the year of their first (CPVT related) syncope, whereas 11 RYR2 carriers never experienced syncope (of whom 8 came from Family 12); in the remaining 15 individuals this information was not available. Gender specific Kaplan-Meier curves of syncope free survival were constructed with this information (Figure 4). The apparent difference in the age of occurrence of the first syncope between males and females is not statistically significant, and is due to the 8 members in Family 12 without a reported syncope. Exclusion of this specific family shows male and female syncope-free survival curves to be overlapping (data not shown).

Heart rate in RYR2 mutation carriers is significantly lower

Initial resting ECGs before treatment were reviewed in 67 genotyped individuals (40 RYR2 mutation carriers, 27 family members without a RYR2 mutation). The resting heart rate of the 12 RYR2 CPVT probands is on average 20 beats per minute (bpm) lower than that of age and gender matched controls[9] (Table 1). Likewise, the only statistically significant difference in ECG parameters between RYR2 mutation carriers and individuals without a RYR2 mutation was the resting heart rate. RYR2 carriers had in general a lower heart rate than their family members without a mutation, irrespective of mutation or family, as they deviated significantly more from age and gender matched control heart rates (-12 bpm vs 2 bpm, p=0.002 Figure 5). A further analysis of the genotyped population according to gender revealed that male RYR2 carriers deviated more from control heart rates than female RYR2 carriers (-19 bpm vs. -7 bpm Figure 5). There was however no gender difference of deviation from control rates among family members without a mutation. Although there was a trend towards a gender effect on heart rate and

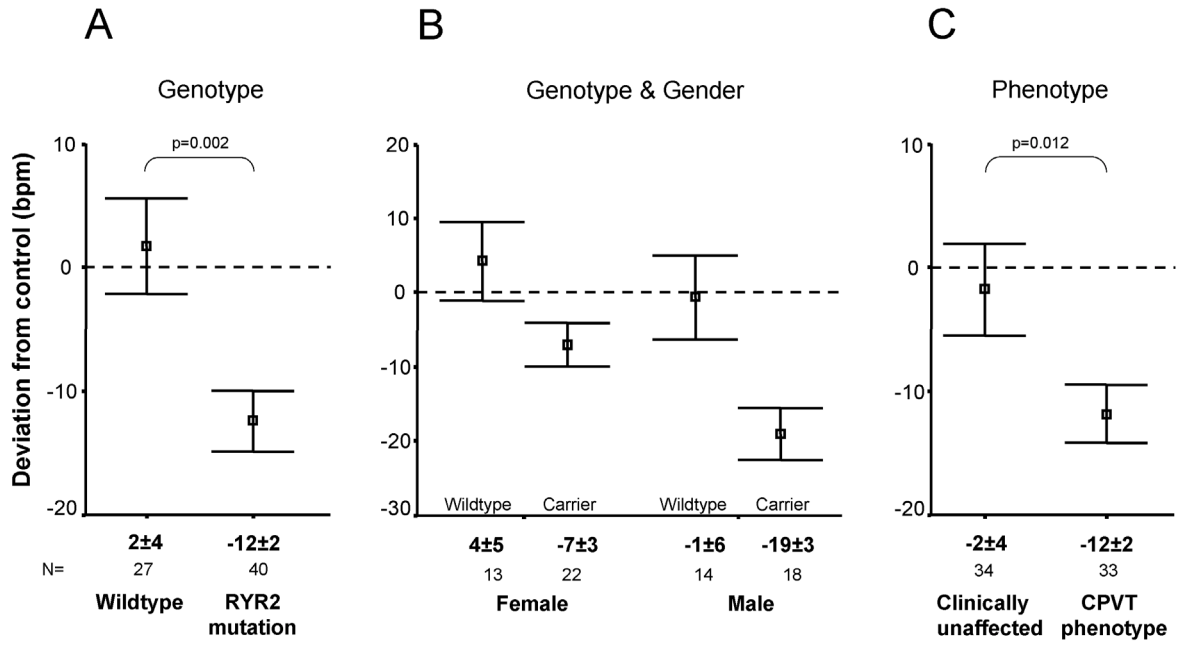

Figure 5. Comparisons between resting heart rates of 67 genotyped individuals (40 family members with a RYR2 mutation, 27 family members without a RYR2 mutation). (A) wildtype family members versus RYR2 mutation carriers, (B) the effect of gender and genotype on the resting heart rate in wildtype family members and RYR2 mutation carriers, and (C) clinically unaffected individuals (27 wildtype family members and 7 silent RYR2 mutation carriers) versus phenotypically affected CPVT patients (33 mutation carriers). Individual resting heart rates were corrected for age and gender and compared to control rates[9] set at 0.

genotype, the difference was not significant (p=0.067). Interestingly, the heart rate of phenotypically affected CPVT patients, was also significantly lower than that of their clinically unaffected family members (including 7 silent RYR2 mutation carriers) in comparison with age and gender matched controls (-12 vs –2 bpm, p=0.012).

Discussion

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by syncopal events and sudden cardiac death (SCD) occurring in young subjects during physical stress or emotion, in the absence of structural heart disease. Diagnosis of CPVT might be difficult since the resting ECG is usually normal. The diagnosis of CPVT is therefore most frequently established by means of Holter recordings and especially exercise testing. Mutations in calcium handling proteins located in the SR, such as RYR2 and CASQ2, have been associated with CPVT. We report here on the genetic and clinical evaluation of a relatively large group of CPVT families and their associated RYR2 mutations.

The phenotypes of the CPVT probands share many similarities: syncopal episodes were usually triggered by exercise or emotion. During exercise testing there was a threshold in

the heart rate before the appearance of ventricular arrhythmias, which was individually highly reproducible. The initial resting ECG demonstrated a normal QTc interval in all probands, however, most of them had, compared to control values[9], sinus bradycardia (see below, Figure 5). The age of onset of CPVT symptoms in our RYR2 CPVT probands (12±4 years), and the overall age of onset among all our CPVT probands (13±2 years) are comparable to previously reported (RYR2) probands[3,10]. However, some CPVT probands may present a much later age of onset, as 2 of our probands were asymptomatic until their late 20s, and one until 51 years of age. In 38% (9/24) of our CPVT probands exercise induced syncopal episodes were accompanied by seizures. Based on these seizures, they were initially referred to a neurologist who recommended anti-epileptic treatment. However, this did not lead to syncope resolution, and in most cases the diagnosis of CPVT was established only after an exercise ECG or a subsequent syncopal event. Treatment with β-blockers had a favorable overall outcome in our group, as 22 of the 24 genotyped probands are symptom free with a mean follow-up period of 8±1 years. The remaining 2 probands unfortunately experienced sudden cardiac death; prior to diagnosis in one patient (in whom the diagnosis was established retrospectively) and due to non-compliance after appropriate diagnosis and treatment in the second one. Moreover, of the 75 patients of the 24 families, 97% are symptom free with a mean follow-up period of 86±18 months, though some continued to have PVBs at exercise tests. The β-blockers doses required to keep the patients symptom free are higher than those used in the long-QT syndrome[11]. Importantly, we did not observe any difference in exercise-related syncope, age of onset of symptoms, or gender between RYR2 CPVT patients and patients without an identified mutation. In contrast to our group of patients, a recent publication with comparable follow-up time shows that 7 of 19 genotyped RYR2 probands had episodes of VT/VF while on β-blockers[3]. The discrepancy in efficacy of β-blocker treatment between the two studies could reflect a difference in β-blocker dosage, a difference in underlying RYR2 mutations or a bias in the selection of (severely affected) CPVT families in the latter study. Obviously, larger groups of genotyped CPVT probands are needed to address the issue of β-blocker efficacy in CPVT.

Analogous to other inherited arrhythmogenic diseases, RYR2 CPVT has a variable expressivity. Ten out of the 51 RYR2 mutation carriers (20%) presented no ventricular arrhythmia during exercise test. Interestingly, eight of these silent gene carriers are female, hinting a possible gender bias, which is in line with a recent study that points male gender out as a risk factor for syncope in genotyped RYR2 patients[3]. However, we did not observe a significant gender based difference in syncope free survival in our genotyped RYR2 patients, as indicated in that study. This could reflect the fact that more genotyped RYR2 patients were included in our analysis, or it could be due to the presence of

different mutations in our CPVT families as compared to the other study. Phenotypically normal RYR2 mutation carriers with normal exercise tests do have syncope and sudden cardiac death, implying that an asymptomatic phenotype doesn't guarantee protection from CPVT[12].

In this study we report that RYR2 mutation carriers have a significant lower heart rate than their genetically unaffected family members on the resting ECGs, irrespective of mutation position or family. Moreover, there is also a significant difference in the deviation from control heart rates between genders; male carriers have a lower heart rate than female carriers. This is in concordance with the fact that females have on average a slightly higher heart rate[13]. Interestingly we also observed a significant relation between phenotype and heart rate, hinting at a possible diagnostic marker. Unfortunately, on the whole, these findings cannot be compared to the previous RYR2 CPVT studies, as the heart rates in these studies were either pooled within a single family or not reported[3,5]. Recently though, a clinical study of 29 non-genotyped Japanese CPVT patients, with a similar age of onset, also demonstrated sinus bradycardia corroborating our results[14]. A possible link between ryanodine receptors and heart rate is the presence of RYR channels and functional SR in sino-atrial (SA) node cells, which serve as the primary pacemaker of the heart[15]. Moreover, substances that interfere with SR function, such as ryanodine and cyclopiazonic acid, have a negative chronotropic effect[16]. Thus, the bradycardia seen in the RYR2 mutation carriers could be a direct effect of the impaired Ca^{2+} handling of their SA nodal cells. Alternatively, it could represent a feedback mechanism by the vagal system; a low average heart rate reduces the likelihood to reach the deleterious threshold at which CPVT is induced.

We identified 13 *RYR2* missense mutations, of which 12 are novel, in 24 probands diagnosed with CPVT. Since we did not screen the entire coding region of RYR2 in the remaining unidentified probands, it is possible that we missed some *RYR2* mutations. Alternatively, other genes that alter Ca^{2+} handling of the SR could be involved. Overall, the 12 *RYR2* mutations presented in this study cluster within three functionally important regions of RYR2: the binding site for the FKBP12.6 protein that stabilizes the RYR2 channel, the calcium binding site, and the channel-forming transmembrane domains (Figure 3). Other studies on RYR2 CPVT also showed a similar mutational clustering into these three distinct regions[3,5]. Moreover, diseases such as malignant hyperthermia (MH) and central core disease (CCD), which are associated with mutations in RYR1, also show mutational clustering in the same areas[17,18]. Unfortunately, there is no clear correlation among RYR2 probands between the region of mutation and the phenotype of the proband, in contrast to what was reported for RYR1 probands[17]. However, the clustering

itself can be used to preferentially screen exons in non-genotyped probands, as the majority of the identified mutations fall within 20 exons.

Mutations in *RYR2* do not only cluster into the three protein areas described above, but also within discrete mutational hotspots. We found three mutations in close proximity to each other, two at amino acid position 4108 (H4108N, H4108Q) and one at position 4104 (N4104I). Moreover, a recent study also reported a CPVT associated mutation at position 4104 (N4104K)[3]. Interestingly, both the 4108 mutations and the reported N4104K occurred *de novo*. The occurrence of three independent *de novo* mutations in this area, together with the high lethality of our N4104I mutation, seems to indicate that this area is particularly important to the RYR2 protein function. Other potential hotspots for *RYR2* mutations are amino acid 4076, where we identified an identical mutation (E4076K) in two unrelated families (6&7), leading to a similar penetrance (70%). Consequently, it appears that clustering and hotspots of RYR2 mutations are relatively common in CPVT. *In vitro* expression studies of these mutations could provide insight into the exact function of these specific regions.

In summary, genetic and phenotypic characterization of our CPVT population, allowed us to assess the clinical features, response to therapy and the genotype-phenotype correlation. We identified 13 mutations in evolutionary highly conserved residues in the cardiac ryanodine receptor in 12 CPVT families. In addition, we found that RYR2 mutation carriers and phenotypically affected CPVT patients have a significant resting sinus bradycardia. Consequently, observations of sinus bradycardia and normal QTc intervals in (young) patients with stress induced syncopal events and seizures are significant; it suggests CPVT, especially when there is a family history of sudden death. Finally, given the risk of juvenile sudden death (50%) and the efficacy of β-blocker/ICD therapy for CPVT, the identification of large numbers of *RYR2* mutations calls for genetic screening, early diagnosis and subsequent preventive strategies.

Acknowledgments

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Chapter **7**

Involvement of the Kir2 Gene Family in Catecholaminergic Polymorphic Ventricular Tachycardia; Analysis for Mutations and Identification of Numerous Pseudogenes

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Submitted to Human Genetics

Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by recurrent syncopal events and sudden cardiac death (SCD) at young age triggered by physical stress or emotion, in the absence of structural heart disease. We previously collected twenty-seven CPVT probands with documented polymorphic ventricular arrhythmias occurring during physical or emotional stress with a structurally normal heart. In 12 of these probands we were unable to find an underlying mutation in either the RYR2 or the CASQ2 gene, implying further genetic heterogeneity for CPVT. Mutations in the KCNJ2 (Kir2.1) gene underlie Andersen syndrome (AS), a disease characterized by a wide range of phenotypic abnormalities, including periodic paralysis, dysmorphic features and polymorphic venctricular tachycardia. Therefore, we sought to find out whether mutations of the Kir2 gene family might underlie some of the genetically unclassified CPVT cases. Systematic screening of all the four members of the Kir2 gene family led to the identification of an autosomal dominant missense mutation in the KCNJ2 gene, R67W in one individual in whom it presents solely with CPVT without any features of Andersen syndrome. We assessed the clinical and genetic characteristics and the response to therapy of this KCNJ2 CPVT mutation. In addition, we identified a large number of KCNJ12-like pseudogenes, polymorphisms and associated mutations, which has consequences for mutational screening. Finally, we report on the exclusion of the candidate genes KCNJ12, KCNJ4 and KCNJ14 in our CPVT population of 27 probands.

Keywords

inwardly rectifying K^+ channel, Kir2 family, tachycardia, syncope, arrhythmia

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by adrenergic-induced bidirectional and polymorphic ventricular tachycardia[1]. It occurs in children and adolescents and causes syncope and sudden cardiac death at a young age, in absence of structural heart disease. Mortality in CPVT is extremely high, reaching 30-50% by the age of 30 years when untreated[2]. Furthermore, there is a clear correlation between the age of the first syncope and the severity of the disease, with a worse prognosis in case of early occurrence. Beta-blockers without sympathomimetic activity are clinically effective in reducing syncope, and the implantation of an intra cardiac defibrillator is rarely needed in these patients[3].

The genetic basis of CPVT had been elucidated by the establishment of linkage between the disease and chromosomal region 1q42[2]. Afterward, several groups independently discovered autosomal dominant missense mutations in the ryanodine type 2 receptor gene (RYR2) located at this chromosomal locus[4,5]. More recently, two studies reported on consanguineous CPVT families associated with homozygous missense and nonsense mutations in the calsequestrin 2 gene (CASQ2)[6,7]. The gene products of RYR2 and CASQ2 play a crucial role in the excitation-contraction coupling, by their involvement in the storage and release of Ca^{2+} from the SR, which subsequently activates cardiomyocyte contraction. Evidence of further genetic heterogeneity comes from the fact that a causal mutation was not found in 40% of the familial cases of CPVT[3,8].

Andersen syndrome (AS) is a condition mainly characterized by potassium-sensitive periodic paralysis, dysmorphic features, and polymorphic ventricular tachycardia. AS is associated with mutations in the KCNJ2 (Kir2.1) gene[9,10], and can result in a pleiotropy of phenotypic variations including all, some or just a single characteristic of the AS. Recently, a large AS family was reported wherein a specific KCNJ2 mutation (R67W) co-segregated with a cardiac phenotype in females and with a skeletal muscle phenotype in males[11]. The proband of this family exhibited, besides mild dysmorphic features, patent CPVT features including syncope, exercise induced polymorphic ventricular tachycardia, in the presence of a normal QTc interval. Since KCNJ2 mutations can result in a wide range of abnormalities, and as the Kir2 gene family is expressed in the heart[12,13], we sought to find out whether mutations of the Kir2 gene family might underlie some of our genetically unclassified CPVT cases.

We report here on the identification of a KCNJ2 R67W mutation leading solely to CPVT in a proband lacking any AS features, adding to further genetic heterogeneity for CPVT. We assessed the clinical phenotype and the response to therapy of the R67W mutation in

the proband. In addition, we identified a large number of KCNJ12-like genes, likely pseudogenes, which has consequences for mutational screening. Furthermore, we report on the exclusion of the candidate genes KCNJ12, KCNJ4 and KCNJ14 in our CPVT population of 27 probands. Finally, mutations in KCNJ2 can result in a multitude of phenotypes including CPVT; this should prompt investigators to screen KCNJ2 in CPVT patients for whom mutations in RYR2 and CASQ2 have been excluded.

Methods:

CPVT Families

The probands of the 27 families were referred to the Paris or Amsterdam clinic, CPVT diagnosis was established by documenting the occurrence of the characteristic ECG pattern of the disease (monomorphic ventricular premature beats followed by bi-directional ventricular tachycardia and salvos of polymorphic ventricular tachycardia) in the absence of structural heart abnormalities as assessed by clinical examination, blood chemistry, electrocardiography, and echocardiography. In 15 of these probands we have previously identified CASQ2 mutations (n=3), and RYR2 mutations (n=12, 43% of the RYR2 gene screened)[7,8]. All individuals gave informed consent for clinical and genetic study, which was approved by the internal ethics committee of each centre.

Genotyping of candidate genes

Mutation screening was performed on genomic DNA samples extracted from peripheral blood lymphocytes using standard methods. The genomic sequence of the four members of the Kir2 gene family, KCNJ2 (Kir2.1, accession no. NM_000891), KCNJ12 (Kir2.2, accession no. NM_021012), KCNJ4 (Kir2.3, accession no. NM_004981), and KCNJ14 (Kir2.4, accession no. NM_013348) were used to design intronic primers. PCR-amplified fragments were analysed by uni-directional sequencing with the ABI PRISM BigDye Terminator kit and run on a 3700 Genetic Analyzer (Applied Biosystems). Aberrant sequences were re-amplified and re-sequenced to exclude polymerase errors. A control group of 200 unrelated alleles from France and The Netherlands was used. In addition to the Kir2 family screening, the entire coding regions of cardiac calsequestrin (CASQ2 accession no. NM_001232), histidine rich binding protein (HRC accession no. NM_002152), phospholamban (PLN accession no. NM_002667), and 45 exons of the RYR2 gene, containing all the alleged crucial areas and published RYR2 mutations, (accession no. NM_001035) covering 43% of the coding sequence were screened using intronic primers.

Cloning of allele-specific amplicons

To amplify the KCNJ12 gene, we synthesized forward and reverse primers based on the published sequence (NCBI accession NM_021012). Both primers contained restriction sites for subsequent cloning: *Bam*HI/*Xho*I/KCNJ12/Forw: 5'-aca-gga-tcc-ctc-gag-gcc-tgg-agc-tag-cct-ggg-3', and Reverse/KCNJ12/*Eco*RI: 5'-tgt-gaa-ttc-tgg-atg-ctg-cat-gtc-ggc-ca-3'. PCR amplified genomic DNA was digested by *Bam*HI/*Eco*RI, and cloned into the pSKII vector and transformed into *E. Coli* XL1 and selected in a LB-Ampicilllin plate with X-Gal and IPTG for blue-white screening.

RT-PCR/mRNA

The reverse transcription was performed using an oligo-dT primer linked to an adaptor to exclude any genomic contamination, 5'-polyT_{17}-gac-tcg-aga-cat-gtg-atg-tgt-gaa-c-3'. The amplification was subsequently carried out with a KCNJ12 forward primer 5'-cag-cca-tga-cca-ccc-agg-3' and a reverse primer specific to the adaptor sequence of the oligo-dT primer (5'-gac-tcg-aga-cat-gtg-atg-tgt-gaa-c-3'). The reverse transcription itself was carried out with Superscript reverse transcriptase II (Invitrogen, USA) at 42ºC for 1 hour, according to the manufacturer's instructions on $PolyA^+$ human heart RNA of a 38-year old female (Stratagene, USA), and on total RNA from right atrial appendages obtained from patients who underwent open-heart coronary bypass grafts. In the latter case, total RNA was extracted using the RNAeasy mini-kit according to the instructions provided (Qiagen, USA).

Results

Exclusion of candidate genes in CPVT probands

Direct DNA sequencing of all the protein-coding areas of the candidate genes KCNJ4 (Kir2.3) and KCNJ14 (Kir2.4), HRC, and phospholamban did not reveal any additional mutation in the 27 probands, besides the previously reported ones[7,8].

Identification of R67W KCNJ2 mutation

Screening of the KCNJ2 (Kir2.1) gene of all the probands revealed one mutation in a proband in whom no RYR2 or CASQ2 mutation was found. The mutation leads to a substitution of an evolutionary highly conserved arginine for a tryptophan at amino acid

position 67 (Figure 1). The R67W mutation was absent from the mother and brother of the proband, and from 220 control alleles.

Clinical Evaluation of the Proband and Family Members

The proband was an 11-year-old boy (II-1, figure 1) who was initially rushed to the emergency room after syncope occurred following exercise. Polymorphic ventricular tachycardia was recorded at the hospital and normal sinus rhythm was restored by lidocaine. He was released from the hospital but experienced eight additional syncopal attacks in the subsequent eight years. Following this, the patient was treated with β-blockers (propranolol 1mg/kg/day) and has since remained symptom free with a follow-up of 6 years. The resting ECG of the proband (Figure 2) before medication displays numerous ectopic beats and a normal QTc interval. The family history was negative for sudden death or syncope, and exercise tests did not reveal any stress induced ventricular arrhythmia in the remaining family members. The typical dysmorphic features of the Andersen syndrome, namely small mandible, hypertelorism, clinodactyly, or scoliosis did not register in any of the family members including the proband nor did they exhibit signs of periodic paralysis.

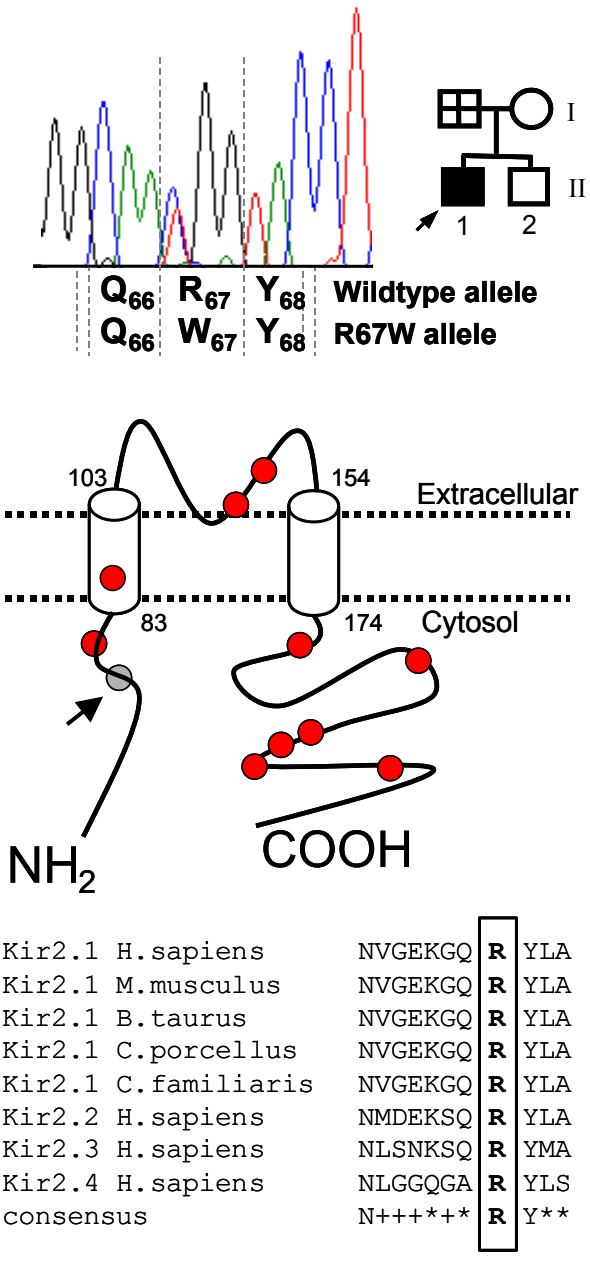

Figure 1. Pedigree of the proband is depicted. Solid indicates presence of the mutation and positive phenotype, cross indicates no DNA available, open indicates absence of mutation and phenotype. Middle panel, overview of published KCNJ12 mutations and the presence of the R67W mutation (arrow) in the KCNJ2 protein. Bottom panel, alignment of the Kir2.x proteins of varies species, note that the R67 is complete conserved.

KCNJ12-like pseudogenes

Sequencing of the KCNJ12 gene

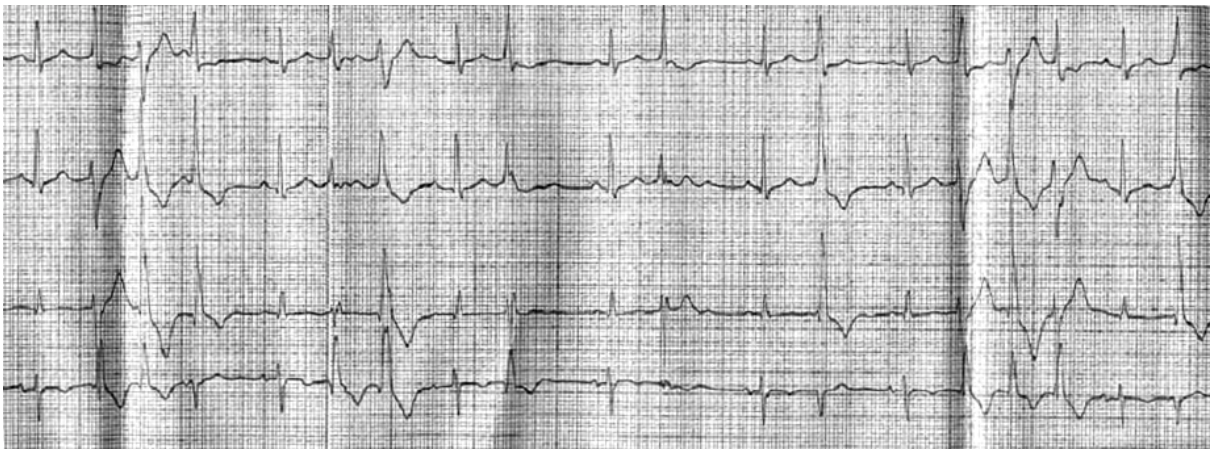

Figure 2. Rest ECG of the proband, note the numerous ectopic beats and the normal QT interval.

Initial DNA sequencing of the KCNJ12 gene (Kir2.2) with two sets of primers (F1-R1, F2-R2, Table 1), revealed over a dozen possible polymorphic sites. Interestingly, for 13 of these sites, all the 27 CPVT probands appeared to be heterozygous. Moreover, further screening indicated heterozygosity for these 13 polymorphic sites in 220 unrelated control alleles (Table 2).

Table 1. Primers used to amplify Kir2.2-(pseudogenes)

Primers 5'-3'	
Kir2.2F1	ccg ccc tgc ctg gag cta g
Kir2.2R1	agc gac cgc ggg gcc tct c
Kir2.2F2	cat gtg gcg tgt ggg taa c
Kir2.2R2	gat gta ctc gcc ctc ctc

In 3 CPVT probands we found unique sequence aberrations not shared by the other probands; a c to g nucleotide substitution, leading to a change of the conserved leucine to phenylalanine (F281L), an insertion of a thymidine at nucleotide position 758 (758insT) in codon 253 leading to a stop codon 20 amino acids downstream, and a change from t to c resulting in the introduction of a stop-codon at position 407 (Q407X). Subsequent screening revealed the presence of these putative mutations in unrelated controls alleles (F281L 2 out of 220, 758insT 1 out of 220, Q407X 1 out of 220 alleles).

Identification of numerous KCNJ12-like pseudogenes

The fact that 127 unrelated people were heterozygous for 13 "polymorphic sites", and the fact that stop-codon (inducing) mutations were found in unrelated control alleles, suggested that during the PCR-amplification of KCNJ12, other closely related KCNJ12-like sequences were being amplified. To prove this hypothesis we cloned allele-specific amplicons from the three CPVT probands carrying the Q407X "mutation", the 758insT "mutation" and the F281L "mutation". Interestingly, after evaluation of over 50 clones from each proband, we were able to establish that each proband carried at least 18 different KCNJ12-like alleles (Table 3). This implies that at least 9 different KCNJ12-like genes exist in the human genome (Figure 4). Two sets of the identified alleles matched

Table 2. Sequence variations in Kir2.2-(pseudogenes)

Nucleotide[3]	Amino	Accession #	Nucleotide[3]	Amino	Accession #	Nucleotide[3]	Amino	Accession #	Nucleotide[3]	Amino	Accession #
9 gca/gcg	A3A	rs3752032	346 gag/aag	**E113K**		745 atc/gtc	**I249V**	rs4985866	1025 tcg/ttg	**S342L**	
44 tcg/ttg[1]	**S15L**	rs1657738	353 cgc/cac[1]	**R118H**	rs1657740	750 gtg/gtt[2]	**750insT**		1056 aca/acg	T352T	
81 ggc/ggt	G27G		354 cgc/cgg[1]	**R118H**	rs1657741	753 att/atc	I251I		1068 agt/agc	S356S	
87 aac/aat	N29N		415 aag/gag	**L139E**		782 cgc/cac	**R261H**		1086 gtg/gta	V362V	
106 gtg/ttg	**V36L**		425 acc/aac	**T142N**		785 atc/agc	**I262S**		1110 agc/agt[1]	S370S	rs1612176
116 cgg/cag	**R39Q**	rs3752033	433 ggc/agc	**G145N**		798 tcg/tca	S266S		1110 agc/agg	**S370R**	
119 cgc/cac	R40H	rs3752034	456 acg/aca	T152T		811 ttg/ctg	L271L		1128 tat/tac[1]	Y376Y	rs1657745
128 cgc/cac	**R43H**		477 ccg/ctg[1]	**P156L**	rs1714864	816 cat/cac	H272H	hCV26029658	1129 gag/aag	**E377K**	
167 gag/gcg	**E56A**	rs1714865	517 aac/gac	**D173N**		819 gag/gaa[1]	E273E	rs1657744	1188 gac/gat	D396D	hCV26029659
213 atg/ata	**M71I**		554 gta/gca	**A185V**		834 agc/agt	S278S		1203 gac/gat[1]	D401D	
243 cgg/cgc[1]	R81R	rs1657739	576 cac/cag[1]	**Q192H**	rs1657742	843 ttc/ttg[2]	**F281L**		1203 gac/gaa	**D401E**	
258 atc/ata	I86I	hCV27390157	597 aat/aac	N199N		863 ctg/cag	**L288Q**		1211 agc/atc	**S404I**	
264 tcg/tca	S88S		618 gat/gac	D206D		865 gag/cag	**E289Q**		1219 cag/tag[2]	**Q407X**	
294 ttc/ttt	F98F		631 ctc/ttc	**L211F**		869 acg/atg	**T290M**		1251 ggc/ggt	G417G	
297 ggc/ggt	G99G		647 ggc/ggt[1]	G216G	rs1657743	873 gac/gat	D291D		1286 gag/ggg[1]	**E429G**	
298 atc/gtc[1]	**I100V**		657 cgt/cgc	R219R		889 gtc/atc	**V297I**		1298 tga/taa	*433*	
315 atc/att	I105I		705 ccg/cca	P235P		906 atg/att	**M302I**				
327 cac/cat	H109H		738 ctg/cta	L246L		997-9 del gag	**+333-**				

[1]heterozygous in 127 unrelated individuals [2]"mutations" in Kir2.2-like pseudo-genes; amino acid changes in bold
[3]nucleotide position 1 refers to the adenosine of the initiation codon of KCNJ12 as reported in NM_021012 or of putative initiation codon of the respective pseudogene

with the known genes KCNJ12 (Kir2.2) and KCNJN1 (Kir2.2v)[14]. These alleles differed at 67 different sites, (Table 2). Based on the differences in the sequence of the 18 alleles we constructed a phylogenetic tree (Figure 4). This suggested that for five genes we had found both parental alleles, two of which were KCNJ12 (Kir2.2) and KCNJN1 (Kir2.2v). The remaining alleles differed substantially from each other; they could thus also represent single alleles of different genes, suggesting that there might even be as much as 11 KCNJ12 pseudo-genes present in the human genome. All the "mutations" identified in the control population (F281L, 758insT and Q407X) were present on pseudogene alleles.

Table3. 18 alleles of Kir2.2-like genes sorted according to 45 sequence variations[1]

	9	44	87	106	116	119	128	167	213	243	264	294	297	298	705	738	745	753	782	785	798	811	816	819	834	863	865	869	873	889	906	997/9	1025	1056	1068	1086	1110	1128	1129	1188	1203	1211	1251	1286	1298
KCNJ12ψ1a1	g	t	c	g	g	g	g	c	g	c	g	c	c	g	g	g	a	c	g	t	g	t	t	a	c	t	g	c	c	g	g	+	c	g	t	a	t	t	g	c	t	g	c	g	g
KCNJ12ψ1a2	g	c	c	g	g	g	g	a	g	g	g	c	c	a	g	g	a	c	g	t	g	t	t	a	c	t	g	c	c	g	g	+	c	g	t	a	t	t	g	c	t	g	c	g	g
KCNJ12ψ2	g	t	c	g	a	a	g	a	g	c	g	c	c	g	g	g	g	c	g	t	g	t	t	a	c	t	g	c	c	g	g	+	c	g	t	a	t	t	g	c	t	g	c	g	g
KCNJ12ψ3a1	g	c	c	g	g	g	a	a	a	g	a	t	t	g	g	g	a	c	g	t	g	t	t	a	c	t	g	c	c	g	g	+	c	g	t	a	t	t	g	c	t	g	c	g	g
KCNJ12ψ3a2	a	c	c	g	g	g	a	a	a	g	a	t	t	g	g	g	a	c	g	t	g	t	t	a	c	t	g	c	c	g	g	+	c	g	t	a	t	t	g	c	t	g	c	g	g
KCNJ12ψ1a4	a	c	t	t	g	g	a	a	g	g	g	c	c	a	g	g	a	c	g	t	g	t	t	g	c	t	g	c	c	g	g	+	c	g	t	a	c	c	g	c	c	g	c	a	g
KCNJ12_a1	g	c	c	g	g	g	g	a	g	g	g	c	c	a	g	g	a	c	g	t	g	t	t	g	c	t	g	c	c	g	g	+	c	g	t	a	c	c	g	c	c	g	c	a	g
KCNJ12_a2	g	c	c	g	g	g	g	a	g	g	g	c	c	a	g	g	a	c	g	t	g	t	t	g	c	a	g	c	c	g	g	+	c	g	t	a	c	c	g	c	c	g	t	a	a
KCNJ12ψ5	g	c	c	g	g	g	g	a	g	c	g	c	c	g	g	g	a	c	g	t	g	t	t	a	c	t	g	c	c	g	g	-	t	a	c	g	c	c	g	c	c	g	c	a	g
KCNJ12ψ6	g	t	c	g	g	g	g	c	g	c	g	c	c	g	g	g	a	c	g	t	g	t	t	a	c	t	c	t	t	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a
KCNJ12ψ7	a	c	c	g	g	g	a	a	a	g	a	t	t	g	g	a	a	t	a	g	a	c	c	g	t	t	c	t	t	a	t	-	t	a	c	g	t	t	g	c	t	g	c	g	g
KCNJ12ψ8a1	a	c	t	t	g	g	a	a	a	g	a	t	t	g	a	a	a	c	a	g	a	c	c	g	t	t	c	t	c	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a
KCNJ12ψ8a2	a	c	t	t	g	g	a	a	a	g	a	t	t	g	a	a	a	c	a	g	a	c	c	g	t	t	c	t	c	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	g
KCNJ12ψ9	a	c	t	t	g	g	a	a	a	g	a	t	t	g	a	a	a	c	a	g	a	c	c	g	t	t	c	t	c	a	t	-	t	a	c	g	g	c	a	c	c	g	c	a	g
KCNJ12ψ10	g	c	c	g	g	g	g	a	g	g	g	c	c	a	g	a	a	t	a	g	a	c	c	g	t	t	c	t	t	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a
KCNJ12ψ11	g	c	c	g	g	g	g	a	a	g	a	t	t	g	a	a	a	c	a	g	a	c	c	g	t	t	c	t	c	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a
KCNJN1_a1	g	c	c	g	g	g	a	a	a	g	a	t	t	g	g	a	a	t	a	g	a	c	c	g	t	t	c	t	t	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a
KCNJN1_a2	a	c	c	g	g	g	a	a	a	g	a	t	t	g	g	a	a	t	a	g	a	c	c	g	t	t	c	t	t	a	t	-	t	a	c	g	g	c	a	t	a	t	t	a	a

[1] Nucleotide position 1 refers to the adenosine of the initiation codon of KCNJ12 as reported in NM_021012

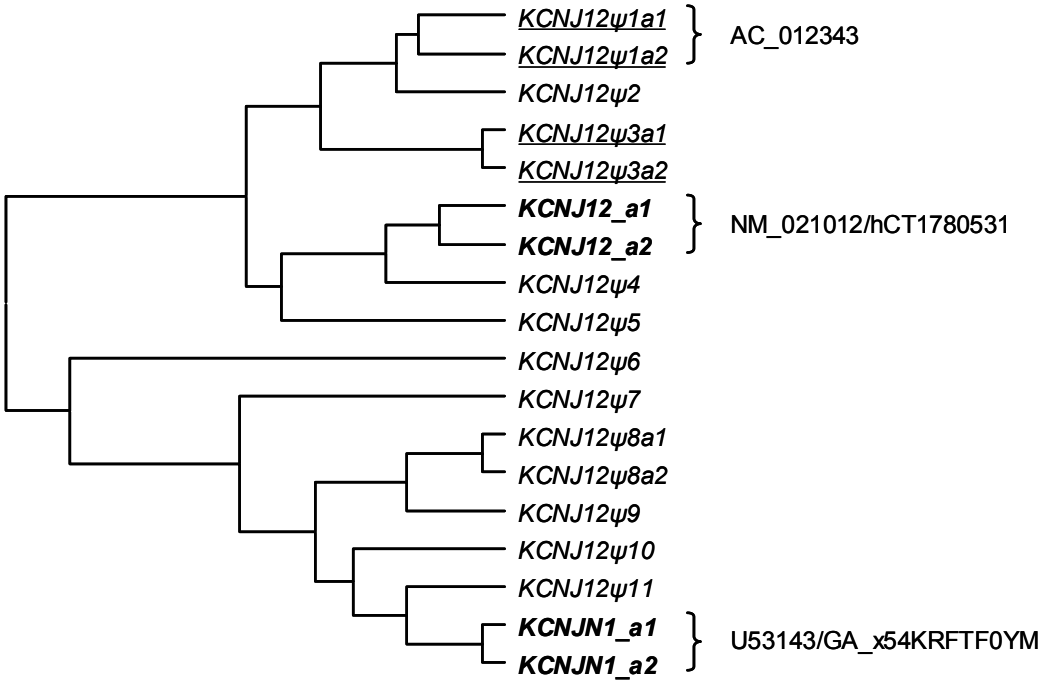

Figure 4. Phylogenetic tree based on the differences between the various alleles of KCNJ12-like sequences of one individual carrying the Q407X "mutation". Numbers to the right are accession numbers for NCBI or Celera, which are a complete match with that allele. Bold alleles are previously known genes, and the underlined alleles contain the Q407X "mutation".

Further evidence for KCNJ12-like pseudo-genes in the human genome

Evidence for the presence of multiple copies of KCNJ12-like genes also comes from both the NCBI and Celera databases. Through a BLAST search using KCNJ12 specific sequences, we found a HTGS sequence (accession no. AC_012343) containing a perfect matching copy to pseudogene *KCNJ12ψ1* (Figure 4). Furthermore, we identified four genomic sequences that lacked the first 826 basepairs of KCNJ12 (accession no. hCT1828275, hCT1828660, hCT1764796, and NT_010799), implying that they are not functional as they lack a proper promoter and initiation site, hinting at the presence of yet more pseudogenes. The known genes KCNJ12 and KCNJN1, and all of the above-mentioned sequences map to the same area of chromosome 17, between 22 and 27 megabases. However, this region contains a 3 megabases contig gap, so the remaining unidentified Kir2.2-like genes might be present there.

Only KCNJ12 alleles are expressed in human heart

Using an adaptor-polyT system, to circumvent genomic contamination, and gene specific primers that do not lie over polymorphic sites, we amplified KCNJ12 fragments from both human atrial appendage RNA and human whole heart RNA. Cloning and sequencing of these fragments revealed that all clones had an absolute match to the KCNJ12 gene and none of the pseudo-gene alleles (data not shown).

Discussion

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a rare arrhythmogenic disorder characterized by syncopal events and sudden cardiac death (SCD) occurring in young subjects during physical stress or emotion, in the absence of structural heart disease. Diagnosis of CPVT is difficult since the resting ECG is usually normal; the diagnosis of CPVT is therefore most frequently established by means of Holter recordings and especially exercise testing. Mutations in calcium handling proteins located in the SR, namely RYR2 and CASQ2, have been associated with CPVT. We report here on the identification of a mutation in KCNJ2 in a boy presenting with CPVT. In addition, we identified a large number of KCNJ12-like pseudogenes and report on the exclusion of the candidate genes KCNJ12, KCNJ4 and KCNJ14 in our CPVT population of 27 probands.

The phenotypes of CPVT patients share many similarities: the syncopal episodes are usually triggered by exercise or emotion, during exercise testing there is a threshold in the heart rate before the appearance of ventricular arrhythmias, and the initial resting ECG including the QTc interval is normal. The proband reported in this study shares many of these CPVT characteristics, including a normal QTc interval. However, his resting ECG is characterized by numerous ectopic beats, a feature commonly reported in Andersen syndrome patients[15]. As has previously been noted, Andersen syndrome has a pleiotropic phenotypic expression and the dysmorphic features of Andersen syndrome are often mild and nondysfiguring. Bearing this in mind however, we could not register any patent Andersen features such as periodic paralysis or dysmorphic features in the proband. Regardless of the exact phenotype, the proband is at risk for lethal arrhythmias and is successfully treated with β-blockers resulting in a symptom-free follow-up of 6 years, analogous to CPVT patients[8]. It seems that the R67W mutation produces in our proband a phenotype more resembling CPVT than Andersen syndrome, suggesting that these two diseases are more related than previously appreciated.

We believe that the different phenotypes of mutations in KCNJ2 (e.g. Andersen and CPVT) might be explained by the specific effects of the mutation on protein expression. A recent study demonstrated that the surface expression of the KCNJ2 (Kir2.1) protein is regulated by its cytoplasmatic domains at distinct steps of intracellular protein transport[16]. Interestingly, several highly conserved positive charges in the N-terminus of the KCNJ2 protein, including R67, are necessary for post-Golgi trafficking to the plasma membrane. Substitution of R67 for a neutral amino acid led to accumulation of the mutant channel within the Golgi-complex, and an absence of the mutant channel on the cell surface[16]. As the R67W mutation described herein also changes a positively charged arginine for a

neutral tryptophan, it seems likely that retention in the Golgi-complex underlies the phenotype seen in our proband with the R67W mutation. Moreover, the co-expression of the R67W KCNJ2 mutation with the wildtype protein in human tsA201 cells demonstrated a complete absence of inward current[16], consistent with an absolute loss of channel function and retention in the Golgi-complex. In contrast, the majority of the KCNJ2 mutations associated with Andersen syndrome show mild to strong dominant-negative effects on wildtype KCNJ2 channels, but none of them completely abolish the KCNJ2 current[9,10]. This implies that these mutations do produce a current in combination with the wild-type protein and therefore exert their effect at the plasma membrane level rather than in the Golgi-complex.

The presumed functional effect of the R67W mutation, an absolute loss of channel function, is in line with the numerous ectopic beats on the probands resting ECG. An absence of functional KCNJ2 protein on the myocyte membrane would significantly increase the propensity towards spontaneous depolarisations of the myocyte during diastole, which is normally kept in check by the presence of the strong inwardly rectifiying currents of KCNJ2. These spontaneous depolarisations can lead to delayed or early afterdepolarisations, which in turn can initiate (lethal) cardiac arrhythmias such as polymorphic ventricular tachycardia, leading to syncope as seen in the proband.

As stated, KCNJ2 mutations give a pleiotropic phenotypic expression, however even further variability is suggested by a recent report of an Andersen family also carrying the R67W mutation[11]. Similar to our proband, the R67W mutation carriers of this family have a normal QTc interval and exercise induced syncope, but in addition exhibit gender-specific cardiac (female) and skeletal muscle (male) phenotypes. Moreover, mutation carriers of this family of both genders have dysmorphic features typical for the Andersen syndrome. Thus, it seems that the same mutation causes different phenotypes. Indeed, variability in phenotypic expression is suggested by studies that show that Kir2.x channels can form heteromeric channels[17,18], and that these heteromeric channels have properties different from those of homomeric channels. This assembly of heteromeric channels is a potential source of physiological variability and might thus account for the phenotypic differences between the two studies. Alternatively, the discrepancy in phenotype between R67W mutation carriers could also be due to differences in the genetic background such as the presence of polymorphisms or mutations in functionally related proteins, influencing the final phenotype. However, overall it is hard to make a solid comparison between the two phenotypes as we only have one affected patient.

We report the presence of at least 9 KCNJ12-like genes in unrelated individuals, of which only KCNJ12 and KCNJN1 have been previously described[14,19], and we propose that 8 of them are pseudo-genes. This is substantiated by the presence of numerous stop codon (inducing) sequence variations in their sequences, the occurrence of these variations in unrelated control alleles, the frequent amino acid changes in functional domains of the protein, and the fact that we could only find expression of KCNJ12 in human heart RNA. Finally, most of the variations listed in the KCNJ12 genbank entry NM_021012 can be accounted for by nucleotide differences between KCNJ12 and its pseudo-genes and are therefore not polymorphisms within KCNJ12. It is not surprising that we could not find expression of the previously reported KCNJN1 gene, as the original cloning of the KCNJN1 gene was done from human genomic DNA rather than from RNA[14]. Taken together, we believe that the human genome contains at least 8 non-expressed KCNJ12-like pseudogenes besides the functional KCNJ12, and we report the absence of the expression of the KCNJN1 gene in the human heart.

In summary, systematic screening of all the four members of the Kir2 gene family led to the identification of an autosomal dominant missense mutation in the KCNJ2 gene, R67W, presenting solely with CPVT in a male proband without any features of AS, adding to further genetic heterogeneity for CPVT. In addition, we identified a large number of KCNJ12-like pseudogenes, which has consequences for mutational screening in both CPVT and AS, and we report on the exclusion of the candidate genes KCNJ12, KCNJ4 and KCNJ14 in our CPVT population of 27 probands. Finally, mutations in KCNJ2 result in a multitude of phenotypes including CPVT possibly depending on the site of the mutation; this should prompt investigators to screen KCNJ2 in CPVT patients for whom mutations in RYR2 and CASQ2 have been excluded.

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Summary / Samenvatting

Summary

Sudden cardiac death is a major health problem associated with significant mortality and morbidity. It is associated with the occurrence of potentially lethal disturbances of the cardiac rhythm, so called ventricular tachyarrhythmias. Most of these sudden cardiac deaths occur in the setting of structural heart diseases such as coronary artery disease, congestive heart failure, and ischemia. In contrast, sudden cardiac death solely due to primary ventricular fibrillation, i.e., without apparent evidence of structural heart disease, occurs in ±5% of victims of sudden cardiac death. Recently, many of these so-called idiopathic sudden cardiac deaths were shown to have an underlying genetic cause. These heritable cardiac arrhythmia diseases have since been named 'ion channelopathies'. The morbidity and mortality of these ion channelopathies are significant and research has started to define the problems at a clinical and molecular level. Options for treatment include pharmacotherapy and implanted devices (such as pacemakers and defibrillators). Although antiarrhythmic medications can greatly reduce the arrhythmic events in the ion channelopathies, their systemic effects are sometimes poorly tolerated. Moreover, implanted devices, albeit very effective, are associated with a lifetime commitment to repeated procedures, significant expense and possible complications. Therefore, given the high risk of sudden cardiac death, the imperfect therapeutic options at hand, and the many familial occurrences of ion channelopathies, it is of utmost importance to uncover the various molecular and genetic backgrounds of these lethal inheritable cardiac arrhythmias; with detailed genetic information, it might be possible to understand the underlying molecular mechanisms, develop new therapeutic options, carry out preventive strategies, establish early diagnoses, and perform pre-symptomatic genetic screenings. The scope of the thesis is therefore to uncover the various genetic and clinical aspects of some inheritable cardiac arrhythmias.

In *chapter 2*, we describe the screening of *SCN5A* in a large 8-generation kindred characterized by a high incidence of nocturnal sudden death, and QT-interval prolongation and the "Brugada ECG" occurring in the same subjects. We identified a single mutation resulting in an insertion of aspartic acid in the C-terminal domain of the protein. Expression of wild-type and mutant Na^+ channels in *Xenopus* oocytes revealed that the mutation gives rise to significant changes in the kinetics of the SCN5A channel, which are thought to be responsible for both phenotypes. LQT_3 and Brugada syndrome are allelic disorders that can share a common genotype.

In *chapter 3*, we followed a candidate gene approach for the involvement of the *KCND2* and *KCND3* genes (*Kv4.2* and *Kv4.3*) in the pathogenesis of the long QT syndrome

(LQTS) and Brugada syndrome. We therefore determined the genomic organisation and the chromosomal localisation of *KCND2* and *KCND3*. Screening of both *KCND2* and *KCND3* in LQTS and Brugada patients in whom no causal mutations in other candidate ion channels had been found was negative, implying that additional causative genes underlie the LQTS and Brugada syndrome.

In *chapter 4* we have aimed to (1) summarize the clinical data of the various recent publications on catecholaminergic polymorphic ventricular tachycardia, (2) describe the underlying molecular causes and pathophysiological mechanism(s) and (3) propose diagnostic guidelines for CPVT. We established that sinus bradycardia is a common finding in CPVT patients, especially in young children. Moreover exercise testing and Holter monitoring are effective tools to confirm a diagnosis of CPVT. The mean age of onset of the CPVT symptoms is 10 years, and both genders are susceptible. β-blocker therapy appears effective in the majority of the patients, although a small group of patients exists in whom β-blocking treatment alone is not effective. Furthermore, molecular analysis has shown that there is a small group of asymptomatic CPVT patients, even after exercise testing, who do carry a mutation.

In *chapter 5*, we studied the autosomal recessive form of catecholaminergic polymorphic ventricular tachycardia (CPVT). We reported the first nonsense mutations in the cardiac calsequestrin gene, *CASQ*2. The three mutations are all thought to induce premature stop codons. Two patients were homozygous carriers, suggesting a complete absence of calsequestrin 2. We show that heterozygous carriers were devoid of clinical symptoms or ECG anomalies. In line with this, the diagnosis of the recessive probands was difficult because of the absence of a positive family history.

In *chapter 6*, we sought to assess the underlying genetic cause, the clinical features, and the response to therapy in 24 catecholaminergic polymorphic ventricular tachycardia (CPVT) probands. Genetic and phenotypic characterization of the probands and their family members, allowed us to assess the clinical features, response to therapy and genotype-phenotype correlation. 13 missense mutations were identified in the cardiac ryanodine receptor (*RYR2*). Little inter-phenotypic variation was found among the CPVT patients with (12) or without *RYR2* mutations (12), but a significant resting sinus bradycardia was observed in both groups. With a mean follow-up period of 86±18 months, 97% of the identified CPVT patients remain symptom free on β-blocker treatment.

In *chapter 7* we describe the screening of a group of CPVT patients, in whom we were unable to find an underlying mutation in either the *RYR2* or the *CASQ2* gene, implying

further genetic heterogeneity for CPVT. Systematic screening of all the four members of the *Kir2* gene family led to the identification of an autosomal dominant missense mutation in the *Kir2.1* gene, R67W, which associates solely with CPVT without any features of the Andersen syndrome. We assessed the clinical and genetic characteristics and the response to therapy of this Kir2.1 CPVT mutation. In addition, we identified a large number of Kir2.2-like pseudo-genes, polymorphic sites and associated mutations, and report on the exclusion of the candidate genes *Kir2.2*, *Kir2.3* and *Kir2.4* in our CPVT population.

Samenvatting

Plotse hartdood is een belangrijk gezondheidsprobleem dat geassocieerd is met een hoge mortaliteit en morbiditeit. Het wordt gekenmerkt door het optreden van potentieel letale verstoringen van het hartritme, een zogenaamde ventriculaire tachy-aritmie. Het merendeel van de gevallen van plotse hartdood treedt op in het licht van onderliggende structurele hartziekten, zoals coronair lijden, hartfalen, en ischemie. In een klein gedeelte van de gevallen van plotse hartdood, ongeveer 5%, treden de ventriculaire tachy-aritmieën op *zonder* onderliggende structurele afwijking(en). Recentelijk zijn voor een aantal van deze gevallen van zogenaamde idiopathische plotse hartdood de onderliggende genetische oorzaken gevonden. Deze groep van overerfbare cardiale aritmieën staat sindsdien bekent als 'ionkanaal ziekten'. Aangezien de mortaliteit en morbiditeit van deze ionkanaal ziekten erg hoog zijn, is begonnen met onderzoek naar deze ziekten op een klinisch en moleculair niveau. De behandeling voor deze ionkanaal ziekten bestaat momenteel uit medicatie en/of het implanteren van pacemakers of defibrillatoren. Ofschoon de anti-aritmica het aantal aritmische gebeurtenissen fors weten te reduceren, hebben deze medicijnen soms ernstige bijwerkingen. En, alhoewel pacemakers en defibrillatoren erg effectief zijn, gaan ze niettemin gepaard met een levenslange blootstelling aan ingrepen en mogelijke complicaties en zijn de kosten erg hoog. Vanwege het grote risico op plotse hartdood, de niet bevredigende therapeutische oplossingen, en het voorkomen in familiair verband, is het zaak de verschillende moleculaire en genetische achtergronden van deze soms letale overerfbare hartritmestoornissen te onderzoeken. De aldus opgedane kennis zou het mogelijk maken de onderliggende moleculaire achtergronden beter te begrijpen, nieuwe therapeutische oplossingen te creëren, de preventie te verbeteren, en de ziektes vroegtijdig, zelfs presymptomatisch, te kunnen opsporen. Het doel van dit proefschrift is derhalve de verschillende genetische en klinische aspecten van een aantal overerfbare hartritmestoornissen te achterhalen.

In *hoofdstuk 2* beschrijven we de karakterisatie van een familie van acht generaties die gekenmerkt wordt door een hoge incidentie van plotse hartdood gedurende de nacht, een verlenging van het QT-interval en een typisch "Brugada ECG", in één en dezelfde patiënt. In alle patiënten vonden we na onderzoek van het *SCN5A* gen, één mutatie die resulteert in een insertie van aspartaat-zuur in het C-terminale domein van het SCN5A eiwit. Expressie analyse van zowel de wildtype als de mutant kanalen in *Xenopus* eieren laat significante afwijkingen aan de kinetiek van het mutante SCN5A kanaal zien, die in verband kunnen worden gebracht met beide fenotypes. Dus het lijkt erop dat LQT3 en Brugada allelische ziekten zijn die *zelfs* veroorzaakt kunnen worden door één en dezelfde mutatie.

In *hoofdstuk 3* hebben we onderzocht of de *KCND2* en *KCND3* genen (*Kv4.2* en *Kv4.3*) betrokken zijn bij de pathogenese van het Lange QT- en het Brugada syndroom. Hiervoor hebben we de genomische organisatie van de genen en hun plaats op de chromosomen bepaald. Vervolgens hebben we in de *KCND2* en *KCND3* genen gezocht naar mutaties in een gemengde Lange QT- en Brugada syndroom patiënten-groep, waarvan bekend was dat er geen mutaties gevonden waren in andere ion kanalen. In deze groep vonden we echter ook geen afwijkingen in zowel *KCND2* als *KCND3* wat aangeeft dat deze ziekten genetisch heterogeen zijn.

In *hoofdstuk 4* geven we een overzicht van alle recente literatuur op het gebied van Catecholaminerge Polymorfe Ventriculaire Tachycardie, met een nadruk op (1) de klinische data, (2) de onderliggende moleculaire oorzaken en pathologische mechanismen, en (3) stellen we richtlijnen op voor de behandeling van CPVT. Het blijkt dat sinusbradycardie vaak voorkomt in CPVT patiënten, met name in jonge kinderen. Inspanningstesten en Holter opnames zijn beide effectieve middelen om CPVT op te sporen en de diagnose te bevestigen. De gemiddelde leeftijd waarop CPVT zich openbaart ligt rond de 10 jaar, en beide geslachten zijn aangedaan. β-blockers vormen een effectieve therapie in de meerderheid van de patiënten, maar er is een kleine groep waarin β-blockers alleen niet genoeg zijn. Bovendien laat de moleculaire analyse van de verschillende patiëntengroepen zien dat er asymptomatische CPVT patiënten zijn, ook na inspanning, die wel drager zijn van een mutatie. Het is evenwel onduidelijk of zij ook risico lopen op plotse hartdood.

In *hoofdstuk 5*, hebben we gekeken naar de autosomaal recessieve vorm van Catecholaminerge Polymorfe Ventriculaire Tachycardieën (CPVT). We rapporteren de eerste stop-codon mutaties in het cardiale calsequestrine gen, *CASQ2*. Van alle gevonden mutaties wordt verwacht dat ze een prematuur stop-codon creëren. Twee patiënten zijn zelfs homozygoot voor hun stop-codon mutatie, wat suggereert dat ze totaal geen calsequestrine 2 aanmaken. Bovendien laten we zien dat heterozygote dragers van *CASQ2* mutaties geen klinische symptomen of ECG afwijkingen hebben. Daardoor is de diagnose van recessieve CPVT patiënten moeilijk omdat een positieve familiare geschiedenis vaak ontbreekt.

In *hoofdstuk 6*, hebben we de onderliggende genetische oorzaken, de klinische aspecten, en de respons op therapie in 24 patiënten met Catecholaminerge Polymorfe Ventriculaire Tachycardie (CPVT) onderzocht. Gedetailleerde genetische en fenotypische karakterisering van de patiënten en hun familie leden gaf ons inzicht in de globale

klinische aspecten en de correlatie tussen het genotype en het fenotype. We vonden in totaal 13 missense mutaties in deze patiëntengroep in het cardiale ryanodine receptor gen (*RYR2*). Er bleek nauwelijks sprake te zijn van variatie in het fenotype tussen de patiënten *met* (12) en *zonder* (12) gevonden RYR2 mutatie. Bij beide patiëntengroepen was er sprake van een significante sinusbradycardie. Het grote merendeel van de patiënten (97% procent) is symptoomvrij gebleven op een β-blocker behandeling met een gemiddelde follow-up van 86±18 maanden.

In *hoofdstuk 7* beschrijven wij het zoeken naar mutaties in een patiëntengroep waarin we voorheen mutaties in *RYR2* en *CASQ2* hebben uitgesloten. Dit impliceerde dat CPVT genetisch heterogeen is. Het systematisch zoeken in de vier leden van de *Kir2* gen familie leidde tot de identificatie van een autosomaal dominant overervende missense mutatie in Kir2.1, R67W. Deze mutatie is hier slechts geassocieerd met CPVT, zonder enig klinisch kenmerk van het Andersen syndroom. We hebben de klinische en genetische aspecten en de respons op therapie van deze Kir2.1 CPVT mutatie bepaald. Bovendien vonden we een groot aantal Kir2.2-achtige pseudo-genen, polymorfe plaatsen en geassocieerde mutaties, en konden we een oorzakelijk verband voor de kandidaat genen *Kir2.2*, *Kir2.3* en *Kir2.4* uitsluiten in onze CPVT patiënten.

Dankwoord

Dankwoord

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List of Publications

List of Publications

Bezzina C, Veldkamp MW, van Den Berg MP, Postma AV, Rook MB, Viersma JW, van Langen IM, Tan-Sindhunata G, Bink-Boelkens MT, van Der Hout AH, Mannens MMAM, Wilde AAM. A single Na(+) channel mutation causing both long-QT and Brugada syndromes. *Circ Res. 1999 Dec 3-17;85(12):1206-13.*

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Postma AV, Bhuiyan ZA, Shkolnikova M, Denjoy I, Beekman L, Koopmann TT, Mannens MMAM, Wilde AAM, Guicheney P, Bezzina CR Involvement of the Kir2 Gene Family in Catecholaminergic Polymorphic Ventricular Tachycardia; Analysis for Mutations and Identification of Numerous Pseudogenes. *Submitted to Human Genetics*

Postma AV, Denjoy I, Guicheney P, Wilde AAM Catecholaminergic Polymorphic Ventricular Tachycardia: Clinical Data, Therapy and Molecular Biological Backgrounds. *To be submitted*

Awards:

Keystone Symposium Scholarship Winner February 2002 "Molecular Biology of the Heart"

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