Genetic variations involved in congenital and acquired long QT syndrome : identification and functional characterization

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*“ The most exciting phrase to hear in science, the one that heralds new discoveries,
is not ‘Eureka’ (I found it!) but ‘That’s funny.....’ ”*
Isaac Asimov

Contents

List of abbreviations

CHAPTER 1	Introduction	11
CHAPTER 2	A novel mutation (T65P) in the PAS domain of the potassium channel HERG results in the long QT syndrome by trafficking deficiency.	45
CHAPTER 3	Mutation analysis in congenital long QT syndrome: a case with missense mutations in KCNQ1 and SCN5A.	63
CHAPTER 4	Characterization of novel mutations E698X and P872fs877 in the C-terminus of the HERG potassium channel that cause the long QT syndrome.	73
CHAPTER 5	Analysis of the human KCNH2 (HERG) gene: identification and characterization of a novel mutation Y667X associated with long QT syndrome and a non-pathological 9 bp insertion.	91
CHAPTER 6	Two linked variations in transcriptional regulatory elements of the CYP3A5 gene constitute the major genetic determinant of polymorphic activity in humans.	99
CHAPTER 7	Genetic susceptibility to acquired long QT syndrome: the impact of congenital long QT genes and drug metabolising enzyme genes.	119
CHAPTER 8	General Discussion	137
	Summary	147
	Samenvatting	149
	Dankwoord	153
	Curriculum Vitae	155
	Publications	157
	Appendices	158

Abbreviations

AP-3	Activator protein-3
aLQTS	Acquired long QT syndrome
BAC	Bacterial artificial chromosome
bp	Base pair
BTE	Basic transcription element
cDNA	Complementary DNA
cLQTS	Congenital long QT syndrome
CLSM	Confocal laser scanning microscope
Ct	Threshold cycle
CYP450	Cytochrome P450
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FAM	6-carboxy-fluorescein
gDNA	Genomic DNA
GFP	Green fluorescent protein
HEK	Human embryonic kidney
HEPES	4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid
HERG	Human <i>ether-a-go-go</i> related gene
HRP	Horseradish peroxidase
HPLC	High-performance liquid chromatography
IgG	Immunoglobulin G
kb	Kilobase
kDa	Kilodalton
kHz	Kilo Hertz
LQTS	Long QT Syndrome
nM	Nano Molar (10^{-9} M)
MR	Metabolic ratio
MΩ	Mega Ohm
mRNA	Messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PAS	Per-Arnt-Sim
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SD	Standard deviation
SDM	Site-directed mutagenesis
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
SSCP	Single strand conformation polymorphism
TdP	Torsade de pointes
TE	Tris-EDTA
UV	Ultraviolet
Wt	Wild-type

CHAPTER 1

Introduction:

Congenital and Acquired Long QT Syndrome

PART 1: Congenital long QT syndrome

PART 2: Acquired long QT syndrome

PART 1: Congenital long QT syndrome

1.1. The history of long QT syndrome

In 1856 Meissner (1) described the story of a young student at the Leipzig School for the Deaf who collapsed and died suddenly while being admonished by the Director. As it turned out, she was the third member of the family who died suddenly, since her two deaf brothers already died at young age after periods of fright and rage. Already then, signs of genetic inheritance were apparent. One century later, in 1957, Jervell and Lange-Nielsen (2) presented the first formal description of long QT syndrome describing a Norwegian family with six children. Whilst the parents were normal, four of the six siblings were affected by deafness and a prolonged QT interval on the electrocardiogram (ECG), and three out of four affected siblings died suddenly. A few years later, in 1963, Romano (3) described a similar case of a young girl with a prolonged QT interval, experiencing syncopal attacks but without deafness. Independently, Ward (4) reported in 1964 on another family with three family members affected by syncopic attacks, prolonged QT intervals, but no deafness. Today, the Romano-Ward syndrome (RWS) is known as the autosomal dominant inherited form of the disease, whilst the Jervell Lange-Nielsen syndrome (JLNS) is defined as the autosomal recessive form. The prevalence of these disorders is estimated at approximately 1/250.000 for the Jervell and Lange-Nielsen syndrome compared to 1/10.000 for the Romano-Ward syndrome.

1.2. Clinical features and ECG characteristics

The clinical symptoms of long QT syndrome (LQTS) include the occurrence of sudden loss of consciousness (syncope), seizures, cardiac arrest or eventually even sudden death. These symptoms are in many cases preceded by “agitating” factors such as emotions (anger, rage or fright), physical exercise (swimming, football) or auditory arousal (alarm clock, telephone, thunder). Because more than twenty-five cases of LQTS were reported between 1958 and 1970, it was decided in 1979 to start a LQTS registry to study epidemiological and inherited characteristics (5). This registry led to several studies on the clinical features of LQTS in over 300 different families (6,7). The clinical features, risk factors and course of LQTS in probands (symptomatic), affected (asymptomatic) and unaffected family members were evaluated. Individuals were classified as affected on the basis of QT-interval prolongation (Figure 1) and the corrected QT-value (QT_c) was calculated with Bazett’s formula (Figure 1) (8). Major risk factors determined in these studies included QT_c value, history of cardiac event and heart rate. Compared to asymptomatic affected and non affected members, probands were more likely to have syncope at young age (50% by age 12), to be female (69%), to have a $QT_c \geq 500$ msec (52%), and to have a history of ventricular arrhythmia (47%) (6,7).

Figure 1. Reflection of a single heart beat on an ECG. The P-wave represents atrial electrical depolarisation and contraction of both atria, the QRS-complex represents left ventricular depolarisation and the end of the T-wave represents ventricular repolarization. The QT interval is defined as the distance between the beginning of the Q wave to the end of the T-wave. Bazett's formula used to calculate the QT-interval corrected for the heart rate.

Whilst prolongation of the heart rate-corrected QT-interval (QT_c) is the classical electrocardiographic feature of LQTS, several other ECG changes are in line with the spatial and temporal heterogeneity displayed by repolarisation in LQTS. These changes include T-wave (wide based, double hump) and U-wave abnormalities and relative bradycardia. The episodes of polymorphous ventricular tachycardia that may be evoked, are called Torsades de Pointes (TdP) (Figure 2). This term was first used by Dr. Dessertenne (9) in 1966 and refers to the morphologic features of the QRS complexes that are of changing amplitude and that appear to twist around an isoelectric point. This rapid ventricular arrhythmia can self terminate, but can also degenerate into ventricular fibrillation. In the latter case, a loss of synchronisation between heartbeat and pulse results in a lack of cardiac output, causes syncope and, if not defibrillated, sudden death.

Figure 2. Example of an episode of *Torsades de Pointes*.

A survey of studies between 1985 and 1991 on the diagnostic criteria of LQTS indicated that better specifications were needed (6). A fixed value of 0.44 sec as a cut-off point for the diagnosis of LQTS had limitations since women turned out to have longer averaged QT_c intervals than men (10). Furthermore, other aberrations in the

ECG profile besides the QT-interval were not yet implemented in the criteria, nor the early patterns of inheritance of LQTS. Therefore, Schwartz et al. (11) refined the diagnostic criteria for LQTS, which are indicated in Table 1. These criteria provide a scoring system based on clinical characteristics and define the probability of LQTS: a total score of ≤ 1 : low probability of LQTS, 2 to 3 points: intermediate probability of LQTS and ≥ 4 : high probability of LQTS (Table 1).

Table 1. Diagnostic criteria for LQTS according to Schwartz *et al.* (11).

ECG findings ^a	Points
QT _c ^b	
≥ 480 msec ^{1/2}	3
460-470 msec ^{1/2}	2
450 msec ^{1/2} (in males)	1
Torsade de pointes ^c	2
T-wave alternans	1
Notched T wave in three leads	1
Low heart rate for age ^d	0.5
Clinical history	
Syncope ^c	
With stress	2
Without stress	1
Congenital deafness	0.5
Family history ^e	
A) Family members with definite LQTS ^f	1
B) Unexplained sudden cardiac death below the age of 30 among immediate family members	0.5

^aIn the absence of medications or disorders known to affect these electrocardiographic features, ^bQT_c calculated by Bazett's formula, ^cMutually exclusive, ^dResting heart rate below the second percentile for age (12), ^eThe same family member cannot be counted in A and B, ^fDefinite LQTS is defined by a LQTS score ≥ 4 .

1.3. Genetic inheritance of long QT syndrome

In 1991 Keating et al. (13,14) applied genetic linkage analysis in a large 5-generation LQTS family. They established linkage chromosomal location 11p15.5 which was confirmed in six other small LQTS families (15). However, locus heterogeneity was suspected in LQTS because several groups (16-19) were unable to link other definite LQTS families to locus 11p15.5. Linkage analysis in the latter families revealed two additional loci for LQTS, respectively on chromosomes 7 and 3 (20). In order of discovery, the loci were named LQT1 (11p15.5), LQT2 (7q35-36) and LQT3 (3p21-24)

(Figure 3). Further linkage analysis, positional cloning and candidate gene approach led to the identification of the responsible genes *KCNQ1*, *KCNH2* and *SCN5A* (Figure 3). In addition, increasing numbers of families revealed three other loci LQT4 (*ANKB*), LQT5 (*KCNE1*) and LQT6 (*KCNE2*) (21-23) (Figure 3).

Figure 3. Loci and corresponding genes linked to LQTS.

1.4. Cardiac ion channel genes

1.4.1. LQT1: *KCNQ1* gene, encoding KvLQT1

In 1996 a gene with high similarity to a voltage-gated potassium channel, named *KCNQ1*, was discovered at chromosomal location 11p15.5 (24). Furthermore, sixteen distinct LQTS families were found to carry heterozygous mutations in this gene, providing further genetic evidence that mutations in *KCNQ1* cause chromosome 11 linked Romano-Ward LQTS (24). The genomic structure of the *KCNQ1* gene comprises 16 exons, encompassing approximately 400 kb (25). Several splice variants of the gene were detected (26) some of which were either non-functional (27) or expressed a dominant negative effect on other splice variants (28). The *KCNQ1* gene was found to be widely expressed in different organs or tissues including heart, lung, placenta, pancreas and inner ear, but not in skeletal muscle or brain (29). The encoded KVLQT1 protein forms the α -subunit of a voltage gated potassium channel and consists of 6 transmembrane domains with a putative voltage sensor in S4 and a potassium selective pore between S5 and S6 (Figure 4) (24). KVLQT1 α -subunits interact with a β -subunit minK, encoded by the *KCNE1* gene, to form a fully functional potassium channel (Figure 4). This channel constitutes the slow component (I_{Ks}) of the delayed-rectifier potassium (K^+) current that is responsible for the repolarisation of cardiac action potentials (30). Heterozygous mutations in the *KCNQ1* gene lead to the dominant Romano-Ward syndrome and many have been reported (31,32). Homozygous mutations in the *KCNQ1* gene may occur when the same mutation is

inherited from both the father and the mother, leading to the recessive Jervell Lange-Nielsen syndrome. As the KVLQT1 protein is also expressed in the inner ear, this syndrome also causes bilateral deafness (33). Also homozygous mutations in the β -subunit encoded by *KCNE1* lead to JLNS (34) as well as two separate heterozygous mutations in *KCNQ1* (compound heterozygosity) (35). Recently, the original genotypic descriptions of RWS and JLNS have been questioned with the discovery of homozygous (recessive) mutations in *KCNQ1* without deafness, therefore causing RW and not JLN syndrome (36,37). A complete overview of all *KCNQ1* mutations identified at present, including functional consequences where available, is presented in Appendix 1.

1.4.2. LQT2: *KCNH2* gene, encoding *HERG*

Shortly after linkage of LQTS to chromosome 7, Warmke and Ganetzky (38) identified a novel human cDNA from a hippocampal cDNA library by homology to the *Drosophila* ether-a-go-go (*eag*) gene. This human gene, initially called the human *ether-à-go-go*-related gene (*HERG*), and later assigned the gene symbol *KCNH2*, shares approximately 50% amino acid sequence homology to *eag*. The *eag* family members have, however, several common structural features such as the hydrophobic domains, a conserved domain (PAS domain) in the cytoplasmic N-terminal end (first 150 amino-acids) and a cytoplasmic C-terminal domain that is very similar to the cyclic nucleotide-binding domain (cNBD) of cyclic nucleotide-gated cation channels in vertebrate photoreceptors (38). Because the *Drosophila* *eag* gene encodes a Ca^{2+} -modulated potassium channel (39) and *KCNH2* was mapped to chromosomal location 7q35-q36, *KCNH2* became an obvious candidate for LQTS. Curran *et al.* (40) reported on additional LQTS families that were all linked to markers on chromosome 7q35-36 and delivered final proof for linkage of *KCNH2* to LQTS. They showed that *KCNH2* is strongly expressed in the heart and identified six *KCNH2* mutations associated with LQTS. The *KCNH2* gene comprises 15 exons, encompassing approximately 19 kb (41). Also *KCNH2* encodes a potassium channel α -subunit (Figure 4) and analogous to other shaker potassium channels, four of these α -subunits form a homo-tetramer (42). Studies in *Xenopus* oocytes revealed that the *HERG* potassium channel displays biophysical properties nearly identical to the rapidly activating delayed rectifier K^+ current (I_{Kr}) in cardiac myocytes (43,44). The *HERG* channel possesses very unique properties: although the *HERG* protein has the structure of the depolarisation-activated K^+ channel family with six transmembrane domains, it exhibits an inward rectification as seen in channels with only two transmembrane domains (45,46). The inward rectification in *HERG* channels is the result of very rapid voltage-dependent inactivation, reducing the conductance at more positive voltages (45). Electrophysiological studies revealed a spectrum of electrophysiological changes that lead to diminished I_{Kr} and delayed repolarisation (47): non-functionality and changed gating properties of channels. Another mechanism involves defects in biosynthetic processing of the channel proteins leading to retention of mutated proteins in the

endoplasmic reticulum (48,49). A complete overview of all *KCNH2* mutations, including functional consequences where available, is presented in Appendix 2.

Figure 4. LQTS proteins. Topology of ion channel proteins (α - and β -subunits) associated with genetic variants of LQTS. Modified from Priori *et al.* (70).

1.4.3. LQT3: *SCN5A* gene, encoding *SCN5A*

Subsequent to initial linkage of LQTS to the LQT3 locus on chromosome 3 (20), Wang *et al.* (50) showed that mutations in the cardiac sodium channel gene *SCN5A* caused LQT3. This gene was cloned and characterised earlier by Gellens *et al.* (51); it comprises 28 exons which are distributed over a genomic region of 80 kb and encodes a protein of 2016 amino-acids. The structure of the *SCN5A* protein is similar to other previously characterised sodium channels: It consists of four homologous domains (DI to DIV), each containing six membrane-spanning segments (S1 to S6) with a structure similar to the potassium channel α -subunits (Figure 4). Together, these four domains form a fully functional channel. Consequently, in contrast to *HERG* and *KVLQT1*, assembly and co-expression of multiple α -subunits is not required to produce a functional sodium channel. The *SCN5A* channel is responsible for the initial depolarising upstroke of the action potential (I_{Na}). The mutations detected by Wang *et al.* (50) were functionally studied in *Xenopus* oocytes and showed generation of a late phase of inactivation-resistant current either by dispersed reopening after the initial transient current or by both dispersed reopenings as well as long lasting bursts (52,53). In contrast to “the lack of function” in LQT1 and LQT2 mutations, LQT3 mutations mostly result in a “gain of function” mechanism, leading to a sustained, non-inactivating inward sodium current during the plateau phase, thereby explaining the prolongation of the action potential. Yet, other mechanisms are possible as An *et al.* demonstrated (54); mutation D1790G was associated with changed kinetic properties due to interactions with β -subunits and not with a persistent inward current. It was also discovered that abnormal function of *SCN5A* channels due to mutations caused other heart diseases such as the Brugada syndrome, progressive cardiac conduction defect and sudden infant death syndrome (55-58). The Brugada syndrome is a familial disease that is characterised by right bundle branch block and ST-segment elevation in leads V1-V3, but with a normal QT-interval. The first two Brugada mutations in *SCN5A*, described by Chen *et al.* (59), were located in different regions of the gene as compared to the LQTS mutations. Therefore, a distinction between the two syndromes based on position appeared logical. However, novel mutations detected in both syndromes have abolished this distinction. For instance, a single mutation (1795insD) has been detected that causes both LQTS and Brugada syndrome phenotypes (60). Another mutation has been detected in both LQTS and Brugada syndrome and is located in the same codon, changing the wild-type amino-acid to two different residues (Y1795C/Y1795H) (61). It is becoming apparent that both syndromes are very closely related in genetic respect but may differ with respect to their phenotypic manifestations. For a complete overview of all detected *SCN5A* mutations and their phenotypic representation, see Appendix 3.

1.4.4. LQT4: *ANKB* gene, encoding ankyrin-B

In 1995, linkage to a fourth LQTS locus was established in a four generation LQTS family of 65 members (21). According to the diagnostic criteria by Schwartz *et al.* (Table 1), 21 members had high probability scores for LQTS. The phenotypic characteristics of affected family members were different from those observed in other LQTS families (severe sinus node dysfunction, atrial fibrillation and uncommon T-wave morphology), which may explain the exclusion from the other known LQTS loci. Linkage analysis mapped this family to locus 4q25-27 with a maximal LOD score of 7.05. Very recently the gene responsible for LQT4 in this family has been identified (194). A loss-of-function mutation E1425G in the ankyrin-B gene (*ANKB*) was shown to cause dominantly inherited type 4 LQTS in affected family members. The *ANKB* gene is member of a family of versatile membrane adaptors and is therefore so far the only LQTS gene encoding a protein that is not an ion channel or subunit.

1.4.5. LQT5: *KCNE1* gene, encoding minK

The *KCNE1* gene was originally cloned from rat kidney and was localised to chromosomal location 21q22.1 (62). The minK protein, encoded by *KCNE1*, consists of a small 130 amino-acid protein with a single membrane-spanning domain (63,64). The human minK protein only showed functionality in *Xenopus* oocytes through interaction with endogenous KVLQT1 proteins and thereby functions itself as an ancillary β -subunit. By co-expression of α -subunits (KVLQT1) and β -subunits (minK) the channel characteristics most closely resemble the native I_{Ks} cardiac potassium current (29,30). McDonald *et al.* reported that minK may also interact with the HERG α -subunit to modulate the rapid delayed rectifier current I_{Kr} (65). MinK mammalian mRNA was detected in several tissues including heart and inner ear (66,67), which made the *KCNE1* gene an excellent candidate for both RWS and JLNS. In 1997 Splawski *et al.* reported two *KCNE1* mutations in separate RWS families both causing a reduction in I_{Ks} when coexpressed with KVLQT1 (22). Whilst first excluded as a candidate for JLNS (68), later studies indicated that mutations in *KCNE1* can also cause JLN syndrome (69). For a complete overview of all detected *KCNE1* mutations and their phenotypic representation, see Appendix 4.

1.4.6. LQT6: *KCNE2* gene, encoding MiRP1

The *KCNE2* gene was cloned and characterised in 1999 by Abbott *et al.* (23). The *KCNE2* gene was mapped to chromosome 21q22.1, close to *KCNE1*. The encoded MiRP1 protein consists of a small integral potassium channel β -subunit of 123 amino acids that contains a single transmembrane segment. Similar to co-assembly of minK with KVLQT1, the MiRP1 protein was found to co-assemble with HERG and modulate its channel function. Abbott *et al.* detected three mutations in this gene. Two of these were detected in congenital LQTS families, one other was detected in a patient with drug-induced arrhythmia. For an overview of detected mutations in *KCNE2* and their functional significance, see Appendix 5.

1.5. Involvement of LQTS genes in the cardiac action potential

The electrical potential of myocardial cells is based on a series of ionic events. The delicate balance between these ionic currents form the cardiac action potential. For several of these currents the responsible genes have been identified (Figure 5). The proteins (and genes) that were described in the previous sections because of their involvement in LQTS, all fulfil important contributions to the cardiac action potential (Figure 6). The *SCN5A* protein is largely responsible for the depolarising upstroke (phase 0, I_{Na}), while the potassium channel proteins *KVLQT1*, *HERG*, *minK* and *MiRP1* are mainly involved in the rapid and slow component of cardiac repolarisation (phase 2 and 3, I_{Kr} and I_{Ks}). Mutations in the genes encoding these proteins cause a prolongation of the ventricular action potential by a reduction in outward currents (I_{Kr} , I_{Ks}) or an enhancement of inward current (I_{Na}). The functional consequences of a mutation can be studied by expression of mutated channels in heterologous systems such as *Xenopus* oocytes or mammalian cell lines. Mutations have shown to lead to a broad scala of dysfunctional channels among which non-functionality, reduced or modified gating at the plasma membrane, or non-expression at the cell membrane by deficient transport from the endoplasmic reticulum. As a consequence of tetrameric channel formation by α -subunits (*KVLQT1* and *HERG*), heterozygous mutations, which are detected in the majority of RWS patients, may cause additional negative effects on channel function (Figure 7).

Figure 5. Ionic currents and responsible genes. Separated ionic currents contributing to the regulation of the cardiac action potential. The time dependency of activation of the currents is shown in relation to the phases of the action potential and the direction of the flow: inward currents are represented under the line, outward currents are represented above the line. From Snyders *et al.* (74).

Incorporation of mutant subunits can disrupt the function of the wild-type subunits (47,71), which is called a "dominant negative" effect. A strong dominant negative effect is expected if one abnormal subunit is sufficient to completely inactivate

channel function. In this case, only 6.25% of the channels will be functional (Figure 7). Mutations in the LQT3 gene *SCN5A* usually cause extra sodium channel activity during the plateau phase of the action potential ("gain of function"), leading to additional inward current and thereby prolonging action potential duration (53,72).

Figure 6. LQTS genes involved in the cardiac action potential. Many proteins together form the cardiac action potential (Figure 5), of which so far 5 are implicated in the LQTS. The time course of the action potential can be divided into five phases: upstroke of rapid depolarisation (phase 0), which is mostly the result of rapid inflow of sodium ions (*SCN5A*); early repolarisation after the peak (phase 1) is primarily the result of K^+ current I_{Kr} ; the plateau (phase 2) where there is a balance of the inward currents caused by calcium and sodium and outward currents caused by chloride and potassium (I_{Kr} , *HERG*, *MiRP1*); repolarisation after the plateau (phase 3), predominantly caused by outward potassium current (I_{Ks} , *KVLQT1*, *minK*); and the period between the maximum negativity (maximum diastolic potential) and the upstroke of the next action potential (phase 4) caused by the balance between slow inward sodium current and outward potassium current. From Towbin *et al.* (73).

Figure 7. Tetrameric channel formation. Functional potassium channels are formed by coassembly of four *KLTQ1* or *HERG* α -subunits. In the dominant RW syndrome, patients inherit one normal allele and one mutant allele from their parents. With the availability of 50% wild-type subunits (light grey) and 50% mutant subunits (dark grey), the statistical distribution of possible co-assembled tetramers is presented above.

1.6. Genotype-phenotype correlations in LQTS

The availability of genetic screening and the continuously growing number of LQTS families have initiated correlation studies between genotype and phenotype. On one hand, correlations were made on the basis of clinical symptoms (age at onset, recurrences and lethality). For example, Zareba *et al.* (75) indicated that the risk of

experiencing cardiac events was higher in LQT1 and LQT2 mutation carriers, although the likelihood of dying at an event was significantly higher among LQT3 carriers. On the other hand, correlations were made on the basis of ECG characteristics. Moss *et al.* (76) showed that ECG T-wave patterns were different among LQT1, LQT2 and LQT3 forms; specific details per genotype include the unusual large distance between the start of the Q-wave and the beginning of the T-wave in LQT3 mutation carriers, the extremely low T-wave amplitude in LQT2 mutation carriers and the long duration time of the T-wave in LQT1 mutation carriers. Although a typical pattern cannot always be found, this method can eventually be useful to select the first gene to be screened for mutations (77). An additional characteristic that may point to the gene affected, is the trigger for cardiac events. Exercise related events seem to dominate the clinical picture in LQT1 (78), with a particularly high incidence caused by swimming (79-81). Triggers initiating cardiac events in LQT2 are dominated by auditory stimuli such as alarm clocks, thunder, telephone ring, etc. (82,83). The majority of cardiac events in LQT3 patients is triggered by rest or sleep (83).

1.7. Therapy in congenital Long QT Syndrome

1.7.1. Acute treatment

Intravenous administration of magnesium is usually very effective to rapidly end non self-terminating TdP and prevent recurrences. Magnesium is therefore the first choice of treatment, although the exact mechanism of action is unknown (84). If magnesium therapy fails, cardiac pacing may be effective. Cardiac pacing prevents pauses and shortens the QT_c-interval by enhancing repolarising potassium currents (85).

1.7.2. Chronic treatment

1.7.2.1. β -Adrenergic blocking drugs

β -blockers are the first choice of treatment for symptomatic LQTS patients. The effectiveness of β -blockers lies in the suppression of adrenergic stimulation, which is often the trigger for arrhythmias (86,87). Advantages of this treatment include high effectiveness and no need for direct surgery. Long-term use of β -blockers has shown to significantly reduce cardiac events in LQTS patients (88). However, β -blocker therapy is more effective in LQT1 and LQT2 patients than LQT3 patients. The latter group may benefit more from a sodium channel blocker such as mexiletine (89). Patients who remain symptomatic despite β -blocker therapy require additional treatment (87). With more genetic information becoming available, effectiveness of therapy may be optimised in the future when it can be adjusted to genotype.

1.7.2.2. Cardiac pacemakers

Additional cardiac pacing is required in patients who remain symptomatic despite maximal doses of β -blockers (90,91). Especially patients with pause-dependent or bradycardiac arrhythmias may benefit from this therapeutic option. These include

many LQT3 mutation carriers of whom the majority experience symptoms at rest during slow heart rates (92). Pacemaker therapy should always be complemented with β -blockers to suppress possible arrhythmias triggered by adrenergic stimulation (93).

1.7.2.3. *Left Cardiac Sympathetic Denervation*

Initially, an unequal contribution of left sympathetic cardiac innervation compared with right sympathetic innervation was considered responsible for QT prolongation. Therefore, in case of failing results with β -adrenoceptor blockade, left sympathetic denervation was introduced in the late 1960s (94). The discovery that cardiac ion channel malfunctions are responsible for LQTS has called this “sympathetic imbalance” theory into question. Long-term studies have shown however that left cardiac sympathetic denervation reduces arrhythmic risk when β -blockers fail, possibly also by blocking adrenergic pathways (95). Like pacemakers, this therapeutic option should also be complemented with β -blockers.

1.7.2.4. *Implantable Cardioverter Defibrillator*

Implantable cardioverter defibrillators (ICD's) were introduced in the 1980s (96). This device does not prevent torsade de pointes, but rather prevents sudden death when torsades de pointes is prolonged or degenerates into ventricular fibrillation (VF). The ICD will give shocks to bring the heart back to sinus rhythm, and therefore works as a “fail-save” mechanism. This therapy, in combination with β -blockers, has shown to reduce mortality rates in patients with spontaneous life-threatening arrhythmias (97). When considering ICD implantation, one should keep in mind economical issues such as costs, limited lifetime expectancy and long-term complications (98,99). In addition, personal issues such as the psychological effect of inappropriate shocks and restrictions on sports or driving should not be overlooked (100,101).

1.7.2.5. *Gene specific therapy*

The identification of the molecular basis of LQTS opened the door towards gene-specific and possibly mutation-specific therapy. Realising that LQT genotypes differ in clinical and electrocardiographic characteristics has led to the assumption that blocking the Na^+ inward current may be a more effective treatment in LQT3 patients while enhancement of repolarising K^+ currents may be more beneficial in LQT1 and LQT2 patients. Schwartz *et al.* (89) tested the efficacy of mexiletine, a known Na^+ channel blocker, in LQT2 and LQT3 patients and showed that mexiletine significantly shortens the QT interval among LQT3 patients but not in LQT2 patients. In addition, this study showed that LQT3 patients shorten their QT interval much more than LQT2 patients in response to an increased heart rate. Therefore, restriction of exercise may not be needed in LQT3 patients. Two other sodium channel blockers, lidocaine and tocainide, have also proven to shorten abnormal QT prolongation in LQT3 mutation carriers (102). Beta-blockers remain the first line of therapy to suppress adrenergic activated triggers in LQT1 and LQT2 patients, although other options are also being tested. Since these patients have inadequate potassium outflow currents, augmentation of the outward currents and shortening of QT-intervals can be achieved

by raising potassium serum levels (103,104). Even therapies for patients with a specific mutation are now being developed. For example, long-term normalisation of the QT interval was accomplished with flecainide but not with lidocaine (105) for the D1790G mutation in *SCN5A*. Also trafficking deficient mutations in the *KCNH2* gene and the *SCN5A* gene are the topic of mutation-specific therapy. For example, the trafficking deficient mutation N470D in *KCNH2* could be rescued with fexofenadine without blocking the channels (106) and mutation M1766L in *SCN5A* could be rescued with mexiletine, although the persistent late sodium current displayed by the rescued protein still needs to be resolved (107). The development of gene-specific and mutation-specific therapy is promising and may significantly improve treatment for LQTS patients. As mutation-specific therapy will usually only be useful in a single family, the financial support to develop such therapies will probably be the greatest challenge.

PART 2: Acquired long QT syndrome

QT prolongation and increased QT dispersion are risk factors in a number of cardiovascular diseases. Many different classes of drugs have QT-prolonging potential, including anti-arrhythmics, antihistamines, antibiotics and antipsychotics (Table 2). The largest group form the class III anti-arrhythmics, which very effectively stop reentrant tachycardias by selectively blocking K^+ channels. By doing so, however, they also prolong the cardiac repolarization and may therefore induce TdP. Between 1% and 8% of the patients receiving these QT-prolonging antiarrhythmic drugs develop TdP (108). Because the clinically observed symptoms clearly resemble the symptoms in congenital long QT syndrome, this disorder is being referred to as acquired long QT syndrome.

In addition to the trigger drug(s), other risk factors are usually involved in acquired LQTS, such as female sex, electrolyte disturbances, other heart disease, concomitant medication and baseline QT prolongation. In the following sections the most important acquired LQTS causing drugs (section 1.8) and risk factors (section 1.9) are discussed.

Table 2. Drugs that prolong the QT-interval and/or induce TdP.Source: www.Torsades.org.

Type of drugs	Examples
Anti-arrhythmic drugs	amiodarone, disopyramide, dofetilide, flecainide, ibutilide, procainide, quinidine, sotalol
Anti-biotics	clarithromycin, erythromycin, gatifloxacin, levofloxacin, moxifloxacin, sparfloxacin
Anti-depressants	fluoxetine, paroxetine, sertraline, venlafaxine
Anti-histamines Anti-psychotics	astemizole, terfenadine chlorpromazine, droperidol, haloperidol, mesoridazine, pimozide, quetiapine, risperidone, thioridazine, ziprasidone
Miscellaneous	arsenic trioxide, bepridil, cisapride, dolasetron, felbamate, foscarnet, fosphenytoin, halofantrine, indapamide, isradipine, levomethadyl, moexipril, nicardipine, naratriptan, octreotide, pentamidine, salmeterol, sumatriptan, tacrolimus, tamoxifen, tizanidine, zolmitriptan

1.8. Drugs causing QT-prolongation and torsades de pointes

1.8.1. Antiarrhythmic drugs

Class IA drugs

Examples of Class IA drugs causing TdP are quinidine, disopyramide and procainamide. These agents decrease ventricular automaticity and thereby reduce ectopic beats that may trigger reentrant tachycardias. The effect of these drugs in slowing conduction velocity in fast channel tissue is the basis for their use in the control of the rate of existing reentrant tachycardias. Between 1% and 3% of patients receiving quinidine develop TdP, the majority in the presence of hypokalemia, slow heart rates or concomitant medication (109). Case reports on TdP during disopyramide and procainamide treatment have also been reported (110), although with less frequency than quinidine.

Class III drugs

Examples of Class III drugs causing TdP are sotalol, amiodarone, ibutilide and dofetilide. Class III drugs increase action potential duration (APD) without effecting conduction by selectively blocking K^+ channels in fast channel tissue. Reentry is made more difficult when the refractory period of myocardial fibers in the reentrant circuit is prolonged to such an extent that the propagating reentrant impulse no longer finds

excitable myocardium. However, these drugs can also be pro-arrhythmic because prolongation of the APD can lead to “early after depolarisations” and cause TdP. Sotalol has been associated with TdP in numerous case studies, especially in the presence of hypokalemia (111) or overdose (112). In a large sotalol study, between 2% and 5% of the treated patients developed TdP, with threefold greater odds for women to develop TdP than men (113). Case reports on TdP evoked by amiodarone are numerous, although the overall incidence of TdP under amiodarone therapy is relatively small and below 1% (114). Ibutilide and dofetilide are new generation Class III anti-arrhythmic drugs. Unlike sotalol and amiodarone, these new drugs mediate their effect selectively by prolonging APD through augmentation or blockade of specific ionic currents. Approximately 3% to 4% of patients receiving intravenous dofetilide and 0.8% to 1.5% of patients on oral dofetilide therapy experience TdP (115). Development of TdP is a well-recognised side effect of all the above-described drugs, although this does not withhold them from still being frequently used. The reasons are probably because these drugs are used to prevent death in the presence of acute life-threatening arrhythmias and because competitive drugs devoid of TdP-inducing side effects are currently lacking.

1.8.2. Second generation antihistamines

Terfenadine and Astemizole

Antihistamines are therapeutic agents used for the treatment of allergic diseases, e.g. seasonal and perennial rhinitis (cold), urticaria (nettle rash) and pruritus (itches). Because of their excellent safety record in the past half century, most of them can be obtained without prescription. Cases of TdP development caused by terfenadine and/or astemizole treatment have been reported since the 1980's, initially only due to overdoses, but later also due to normal doses or concomitant medication (116-118). After several “black box” warnings, terfenadine was removed from the US market in 1997 followed by astemizole one year later. Several *in vivo* studies in laboratory animal models have indicated that both terfenadine and astemizole can prolong the QT interval (119,120). Additional *in vitro* studies indicated that both drugs significantly block I_{Kr} (HERG) current in a dose-dependent manner (121).

1.8.3. Antibiotics

Erythromycin and Clarithromycin

Erythromycin and clarithromycin are antibiotics used to treat a wide variety of bacterial infections, such as infections of the respiratory tract, the middle ear, and skin. Drici *et al.* (122) reported 346 cases of erythromycin related to cardiac arrhythmia in the FDA SRS database between 1970 and 1996. Forty-nine of these cases were life threatening ventricular arrhythmias or death directly related to intravenous erythromycin lactobionate (123). Development of TdP with the sole administration of clarithromycin has been reported but is rare (124). Whilst both drugs can be the sole cause of TdP development, the majority of reported cases involved concomitant

administration of another QT-prolonging drug (See also section 1.9.4). For instance, in case of the prokinetic drug cisapride 65% of the published reports involved concomitant administration of either erythromycin or clarithromycin (125). In vivo and in vitro studies have investigated the cardiotoxic effect of erythromycin. Daleau *et al.* (126) demonstrated that erythromycin inhibits I_{Kr} in guinea pig myocytes while Rubart *et al.* (127) demonstrated that prolongation of the QT interval in canine Purkinje fibers was also due to I_{Kr} blockade. Antzelevitch *et al.* showed that erythromycin preferentially prolonged APD in canine M-cells but not in endo- or epicardial cells. The resulting marked dispersion of repolarization across the ventricular wall creates “early after depolarisations” and was shown to set the stage for TdP development (128). Recently, Volberg *et al.* showed that both clarithromycin and erythromycin block stably expressed HERG channels in HEK293 cells in a concentration dependent manner (129).

1.8.4. Cholinergic antagonists

Cisapride

Cisapride is a gastrointestinal prokinetic agent that has been widely prescribed for the treatment of a variety of gastrointestinal motility disorders, including gastroparesis and gastroesophageal reflux disease (130). Until 1999, cisapride was associated with 341 reports of heart rhythm disturbances including QT prolongation, syncope, serious ventricular arrhythmia (TdP) and sudden death (131). After 3 “black box” warnings, cisapride was withdrawn from the market in the USA in 2000. Several electrophysiological experiments and animal studies have determined a reasonable explanation for cisapride’s QT-prolonging and TdP-inducing abilities; cisapride has proven to be a very potent blocker of HERG potassium channels (I_{Kr}) (132-134) and has demonstrated to cause “early after depolarisations” in anaesthetized rabbits (135).

1.8.5. Antipsychotics

Thioridazine, Haloperidol and Droperidol

Thioridazine is a phenothiazine used to treat schizophrenia and other psychotic disturbances such as depression and anxiety. It is also used for the treatment of autistic children and of epileptic children with behavioural disorders. Haloperidol is an antipsychotic drug mainly used to treat schizophrenia, but is also being prescribed for Gilles de la Tourette syndrome and manias. Both thioridazine and haloperidol have sedative effects that make them very suitable for violent and agitated patients. Droperidol is usually applied as a sedative or a treatment for nausea and vomiting. The first reports of sudden arrhythmic death with thioridazine appeared in 1963 (136) and reports until today are numerous. Newer drugs haloperidol (137,138) and droperidol have also been associated with TdP (139,140). In vivo experiments in perfused guinea pig hearts and in vitro experiments in tsA201 cells have demonstrated that thioridazine blocks the delayed rectifier potassium current I_{Kr} (HERG) (141). Experiments in *Xenopus* oocytes indicated that haloperidol blocks the HERG potassium

channel by binding to the channels in the inactivated state at haloperidol concentrations similar to plasma levels found in patients with haloperidol-induced TdP (142). Finally, experiments in HEK293 cells showed very potent HERG block of droperidol at therapeutic concentrations (143).

1.9. Risk factors for acquired LQTS

1.9.1. Age and gender

During childhood the length of QT_c intervals are similar for girls and boys. However, during puberty men shorten their QT_c interval, whilst the QT_c intervals for women remain unchanged (144). These age- and sex- differences are also apparent in individuals affected by congenital long QT. Locati *et al.* showed that, consistent with QT_c -interval development, the risk of cardiac events in LQTS mutation carriers is higher in males until puberty and higher in females during adulthood (145). Analogous to the gender differences observed in the clinical manifestations of congenital LQTS (146), there is also a higher risk for adult women to develop acquired LQTS. This higher risk for women to develop acquired arrhythmia is illustrated by the large number of feminine case reports (122,147) (Table 3 and 5). The mechanisms for the age and sex differences remain currently unclear, although preliminary animal studies have indicated that sex hormones may contribute to QT interval shortening in males or the lack of shortening in females (148,149).

1.9.2. Electrolyte disturbances

Electrolyte disturbances such as hypokalemia are reported as a contributing factor in many individual TdP case reports. This additional risk factor is explained by the modulation of I_{Kr} by external potassium (150). Unlike other K^+ currents, the magnitude of outward HERG current amplitude is paradoxically reduced upon removal of extracellular K^+ . The reduced HERG current amplitude thus explains the prolonged ventricular repolarization in hypokalemic patients. Because the majority of the drugs causing acquired LQTS act through blockade of the HERG channel, hypokalemia can exaggerate this effect (151). As a consequence, administration of potassium supplements can sometimes be useful in these patients.

1.9.3. Other heart disease

Not surprisingly, patients with underlying structural heart diseases are more vulnerable to drug-induced TdP. QT prolongation in patients recovering from myocardial infarction has been well documented (152). It was shown that 9 out of 13 patients suffering from TdP had an underlying structural heart disease such as ischaemic, valvular or hypertensive heart disease (153). As also seen in congenital LQTS type 3, bradycardia can also play an important role in the trigger for TdP (154).

1.9.4. Underlying congenital LQTS

In 1982, Moss *et al.* (155) hypothesized that some drug-induced arrhythmias may depend on genetic predisposition and represent “forme fruste” cases of the congenital LQTS. This hypothesis was intensified later when it became apparent that some *KCNH2* mutation carriers had normal QT intervals (156). It was hypothesized that the latter group of individuals would be particularly susceptible to drug-induced LQTS. Indeed, sporadic forme fruste cases of congenital LQTS have been reported (Table 3). In addition, common polymorphisms in LQTS genes might modify arrhythmia susceptibility in potential gene carriers. Sesti *et al.* (157) demonstrated that a common polymorphism in *KCNE2* (T8A) contributed to acquired LQTS because of altered sensitivity of HERG/MiRP1 channels to an antibiotic (Table 3). However, studies in larger groups of acquired LQTS patients (158,159) showed that only a minority of the cases could be explained by underlying mutations in currently known LQTS genes. The majority of acquired LQTS patients often carry several risk factors, but still the development of life-threatening arrhythmias remains unpredictable.

1.9.5. Concomitant medication and metabolism

Drug interactions occur when the effectiveness or toxicity of a drug is altered by the administration of another drug. Often this is caused by an inhibition of the metabolism of a drug due to co-administration of another drug. Such drug metabolism inhibition results in elevated plasma drug concentrations with an increased potential for adverse effects (164).

The family of cytochrome P450 (CYP450) enzymes accounts for the majority of oxidative biotransformation of xenobiotics and endogenous chemicals. In the human genome more than 50 different CYP450 genes are present, that are classified into different subfamilies on the basis of sequence homology. One of these subfamilies is the CYP3A family, consisting of CYP3A4, CYP3A5 and CYP3A7 respectively. The CYP3A members are the most abundant CYP450 enzymes in the human liver and small intestine (165) and catalyse the oxidative, peroxidative and reductive metabolism of structurally diverse endobiotics, drugs and procarcinogenic molecules (166). The total CYP3A activity is responsible for the biotransformation of approximately 60% of all oxidised drugs (167). The contribution of each of these CYP3A genes to the total CYP3A activity is still largely under debate. It has been shown that CYP3A7 is predominantly expressed in foetal life and that its expression seems to be silenced shortly after birth. However, some people express CYP3A7 mRNA into adulthood and contribution of CYP3A7 in these individuals to metabolism is unclear (168).

The CYP3A4 and CYP3A5 enzymes have wide overlapping substrate specificity and it is therefore very difficult to separate the metabolism of these two enzymes. Many of the drugs described in the previous sections are, at least among others, substrates or inhibitors of the CYP3A4/5 enzymes (Table 4). These substrates however also block the

HERG potassium rectifier current. Inhibition leads to elevated drug plasma levels and this can result in a higher potency for HERG block, QT-prolongation and an increased risk of developing TdP. Drug-induced TdP as a result of concomitant drug administration is illustrated by the increasing number of individual case reports (Table 5).

For both CYP3A4 and CYP3A5 activity there is evidence for a wide variation in expression. Several studies have shown, for instance, that in only 10% to 30% of adult human livers CYP3A5 expression can be detected (191). The genetic basis for this variability in expression, if any, is currently under research (192,193). The contribution of CYP3A5 expression may however play an important role as a study by Gibbs *et al.* (194) demonstrated: it was shown that liver and intestinal microsomes that contained an equal or greater amount of CYP3A5 in addition to CYP3A4, were less susceptible to inhibition by ketoconazole and fluconazole. It was therefore concluded that variable CYP3A5/CYP3A4 enzyme expression in gut and liver may contribute significantly to the inter-individual variability in adverse drug-drug interactions, such as TdP, associated with concomitant ketoconazole- and fluconazole administration.

Table 3. Specific mutations reported in acquired long QT syndrome case studies. Individual case report studies in which drugs in combination with an underlying mutation in one of the known congenital LQTS genes led to serious ventricular arrhythmia.

Gene	Mutation	Amino-acid change	Drugs	Risk factors	Symptoms	Ref.
KCNQ1	944A>G	Y315C	Cisapride	hypokalemia, elderly woman (77 yrs)	Sudden loss of consciousness, cardiac arrest	(160)
	1663 C>T	R555C	terfenadine or dysoperamide or mefloquine	congenital LQTS	sudden death, syncope, TdP	(31)
	1747 C>T	R583C	Dofetilide		TdP	(158)
KCNH2	1039 C>T	P347S	cisapride + clarithromycin + sulfamethoxazole	elderly woman (77 yrs), congestive heart failure, ventricular pacemaker	TdP, QTc>600 ms (normal 440 ms)	(162), (176)
	1048 C>T	R328C	Not specified		TdP	(159)
KCNE2	2350 C>T	R784W	Amiodarone		TdP	(158)
	22 A>G	T8A	sulfamethoxazole		QTc>600 ms	(157)
	25 C>G	Q9E	clarithromycin	elderly woman (76 yrs), diabetic, history of stroke, hypokalemia	TdP, VF requiring defibrillation	(23)
	161 T>C	M54T	procainamide		TdP	(157)
	170 T>C	I57T	oxatamide		TdP	(157)
SCN5A	347 C>T	A116V	quinidine + mexiletine	Woman (55 yrs), history of cardiac arrest	Syncope with TdP	(157)
	1844 G>A	G615E	quinidine		TdP	(158)
	1852 C>T	L618F	quinidine		TdP	(158)
	3748 T>C	F1250L	sotalol		TdP	(158)
	4999 G>A	V1667I	halofantrine	QTc(baseline)>440 ms, congenital LQTS	Syncope, TdP, heart block, QTc>600 ms	(161)
	5474 T>C	L1825P	cisapride	elderly woman (70 yrs)	TdP	(163)

Table 4. Drugs metabolised by cytochrome P450 3A4/5 and its clinically most relevant inhibitors.

CYP3A4/5 substrates		CYP3A4/5 inhibitors
Amiodarone	Midazolam	Cimetidine
Astemizole	Nifedipine	Clarithromycin
Carbamazepine	Pimozide	Cyclosporin
Cisapride	Propafenone	Diltiazem
Cyclosporin	Quinidine	Erythromycin
Dapsone	Tacrolimus	Fluconazole
Diltiazem	Tamoxifen	Fluoxetine
Disopyramide	Terfenadine	Fluvoxamine
Erythromycin	Testosterone	Grapefruit juice
Felodipine	Valproic acid	Itraconazole
Lidocaine	Verapamil	Ketoconazole
Indinavir		Miconazole
Lovastatin		Ritonavir

Adapted from Dresser *et al.*, 2000 (164).

Table 5. Reported cases of acquired LQTS by concomitant medication.

Drug 1	Drug 2	Sex	Age (yrs)	Symptoms	Reference
Astemizole	Ketoconazole	Female	63	TdP	(169)
Astemizole	Erythromycin	Female	30	TdP	(170)
Astemizole	Cimetidine	Female	77	TdP	(171)
Terfenadine	Itraconazole	Female	26	TdP	(172)
Terfenadine	Ketoconazole	Female	39	TdP	(118)
Terfenadine	Itraconazole	Female	36	TdP	(189)
Cisapride	Ketoconazole	Female	57	TdP, VF	(175)
Cisapride	Erythromycin	Female	18	TdP	(175)
Cisapride	Clarithromycin	Female	77	TdP	(176)
Cisapride	Clarithromycin	Female	53	TdP	(177)
Cisapride	Erythromycin	Male	64	TdP	(178)
Cisapride	Erythromycin	Male	30	TdP	(179)
Cisapride	Diltiazem	Female	45	Syncope, QT prolonged	(187)
Cisapride	Erythromycin + Fluconazole	Female	8	VT	(180)
Cisapride	Clarithromycin	Female	52	TdP	(181)
Cisapride	Clarithromycin	Male	83	TdP	(181)
Quinidine	Propafenone	Female	77	TdP	(182)
Quinidine	Erythromycin	Male	95	TdP, cardiac arrest	(173)
Quinidine	Verapamil	Female	65	TdP	(174)
Disopyramide	Clarithromycin	Female	76	TdP	(184)
Pimozide	Clarithromycin	Male	27	SD	(185)
Mosapride	Flecainide	Male	68	TdP, PM	(186)
Disopyramide	Clarithromycin	Female	76	TdP	(188)
Droperidol	Cyclopropazaprine + Fluoxetine	Female	59	TdP, VF	(183)

TdP= Torsades de Pointes, VF= ventricular fibrillation, VT= ventricular tachycardia, SD= sudden death.

Aims of this thesis

The first aim of this thesis was to study congenital LQTS. The identification and subsequent characterization of (novel) LQTS mutations fulfils several purposes for both the affected family and the clinicians who counsel and treat them. The first aim was therefore to apply genetic screening for the identification of (novel) causal mutations in the probands of phenotypically diagnosed LQTS families (Chapters 2, 3 and 4). The novel identified mutations can then be used to identify other “silent” mutation carriers within the family and provide proper counselling about the individual risks and treatment options. Therapy may be improved if the molecular and biochemical mechanisms behind the pathogenic nature of mutations are understood. To serve this aim a heterologous expression system (human embryonic kidney cells) was used to functionally characterize the identified mutations (Chapters 2 and 4).

The second part of this thesis aims to study the input of genetic variations on the development of acquired LQTS. In Chapter 5, we investigated the possibility of underlying genetic susceptibility of a volunteer who participated in a clinical trial and clearly showed QT prolongation after drug intake. Because this study indicated that genetic susceptibility in acquired LQTS might be underestimated, we subsequently evaluated the impact of underlying congenital LQTS mutations in a group of 34 acquired LQTS patients (Chapter 7). Concomitant medication is a reported major risk factor in acquired LQTS and often results in an inhibition of hepatic enzymes responsible for the metabolic pathway of the drugs. Among these enzymes, the CYP3A5 enzyme has a wide variation in expression, although possible underlying genetic determinants are unknown. In Chapter 6 we attempted to explain this variable CYP3A5 expression. Finally, we evaluated the importance and relevance of these findings in a larger group of acquired LQTS patients (Chapter 7).

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CHAPTER 2

A novel mutation (T65P) in the PAS domain of the human potassium channel (HERG) results in the long QT syndrome by trafficking deficiency

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Abstract

The congenital long QT syndrome is a cardiac disease characterized by an increased susceptibility to ventricular arrhythmias. The clinical hallmark is a prolongation of the QT interval that reflects a delay in repolarization caused by mutations in cardiac ion channel genes. Mutations in the human *ether-à-go-go* related gene *KCNH2* (HERG) can cause a reduction in I_{Kr} , one of the currents responsible for cardiac repolarization. We describe the identification and characterization of a novel missense mutation T65P in the Per-Arnt-Sim (PAS) domain of HERG, resulting in defective trafficking of the protein to the cell membrane. Defective folding of the mutant protein could be restored by decreased cell incubation temperature and pharmacologically by cisapride and E-4031. When trafficking was restored by growing cells at 27°C, the kinetics of the mutated channel resembled that of wild type channels although the rate of activation, deactivation and recovery from inactivation were accelerated. No positive evidence for the formation of hetero-tetramers was obtained by co-expression of WT with mutant subunits at 37°C. As a consequence the clinical symptoms may be explained rather by haplo-insufficiency than by dominant negative effects. This study is the first to relate a PAS-domain mutation in HERG to a trafficking deficiency at body temperature, apart from effects on channel deactivation.

Introduction

Congenital long QT Syndrome (cLQTS) is a disorder of the electrical system of the heart. The disorder manifests itself by a prolonged QT-interval on the electrocardiogram (ECG) and by a propensity for tachyarrhythmias causing syncope's and sudden death (1). The human *ether-à-go-go*-related gene *KCNH2*, encoding the α -subunits of the voltage-gated potassium channel HERG, is one of five genes linked to this disease. The HERG potassium channel has been shown to encode the α -subunit of the cardiac rapidly activating, delayed rectifying current I_{Kr} (2) that plays an important role in the repolarization of the cardiac action potential (3,4). Although the role of other factors contributing to I_{Kr} current, such as *KCNH2* splice variants (5,6,7) and interacting β -subunits (8) are still under investigation, it is clear that mutations in the *KCNH2* gene are one of the most common causes of congenital LQTS. In most cases these mutations reduce the outward potassium current during repolarization, and thus lengthen the action potential duration, which increases the risk of ventricular arrhythmia (9). More than one hundred different mutations in the *KCNH2* gene have been described for cLQTS families including frame shifts, deletions, insertions, missense and nonsense mutations (10,11). There is a great deal of genetic evidence that the mutations identified are the cause of the disease in the different families however, in many cases the molecular mechanism by which channel function is disrupted, has not

been unequivocally identified. Thus far, all except one (12) of the mutant HERG channels characterized, cause a net reduction in outward potassium current during repolarization through loss of function. The latter may result from different mechanisms including non-functionality (13,14), altered gating properties (15,16,17) or abnormal trafficking from the endoplasmic reticulum (ER) to the cell membrane (18-22). Especially this last category of mutants has recently become a focus of interest, because such mutant subunits may display WT properties, once they are inserted in the cell membrane. Defective protein processing has been described for other congenital human diseases, including cystic fibrosis and nephrogenic diabetes insipidus (23-25). In these diseases, the mutation causes very subtle differences in protein folding, which lead to ER retention. However, lower cell incubation temperatures or chemical chaperones could restore trafficking in these cases. Thus far, six trafficking-deficient mutations have been described in the *KCNH2* gene (Figure 1, 18-22). These mutations reside in different regions of the HERG protein and seem to determine temperature or chemical chaperone dependent rescue. For example, trafficking of mutant channels G601S, N470D and R752W (19, 20, 21) was restored by lower incubation temperatures, but only G601S and N470D channels were restored by chemical chaperones. A561V (22) was not rescued by either temperature or chemical chaperones and even expressed a dominant negative effect on wild type current.

In this study, we describe the identification of a novel missense mutation 193 A>C in exon 2 of the *KCNH2* gene in a case of congenital long QT syndrome. This mutation is located in the amino-terminal Per-Arnt-Sim (PAS) domain of the HERG protein and leads to an amino-acid substitution T65P (threonine to proline). Previous studies have focussed on this N-terminal PAS-domain, since it may interact with the S4-S5 linker, thereby controlling channel deactivation (15, 26). Using functional analysis, we demonstrate that the T65P HERG protein also displayed an accelerated deactivation, but only if it was transported to the cell membrane. The primary dysfunction of the T65P mutation was a trafficking deficiency and therefore may provide new information on additional functions of this protein domain.

Figure 1. HERG α -subunit. Representation of a single HERG protein subunit with six trans membrane domains (S1-S6), the pore region between S5 and S6, and the intracellular N- and C-terminal ends. Black dots indicate reported trafficking-deficient mutations. The novel T65P mutation described in this study is marked with a black square.

Experimental procedures

Study subjects

The pedigree of the Caucasian family in which the congenital long QT syndrome (LQTS) was diagnosed is shown in Figure 3A. Five of the family members (subjects 1, 2, 3, 4, and 6) are being treated with beta-blockers because of a prolonged QT interval. Subject 2 has suffered from a cardiovascular collapse at the age of 25, but was successfully resuscitated. Apart from an aberrant ECG pattern (sinus tachycardia, diffuse repolarisation disturbances) subject 3 is asymptomatic. Because of the abnormal ECG pattern and the highly extended QT intervals, subject 4 and her daughter (subject 6) are being treated with beta-blockers. Moreover, subject 6 suffered numerous syncopic episodes in the past. Subject 5 died suddenly at the age of 31 shortly after the start of an antibiotic treatment for otitis, which is highly suggestive for drug- induced LQTS. Subject 7, a sister of subject 1, has died suddenly at the age of 42 years. Finally, no information is available for two additional sisters of subject 1. Informed consent for genetic analysis was given prior to the collection of blood samples for DNA extraction from subjects 1, 2 and 3. Samples from other family members were not available for genetic testing analysis.

Screening of *CLQTS* genes

The DNA of subject 1 was screened for all currently known LQT genes *KCNQ1* (LQT1, KvLQT1), *KCNH2* (LQT2, HERG), *SCN5A* (LQT3, SCN5A), *KCNE1* (LQT5, MinK) and *KCNE2* (LQT6, MiRP1). PCR primers used for amplification of individual exons were described previously (8, 27-29). PCR products were directly sequenced and aligned with the respective wild type sequences in order to detect genetic variations.

Detection assay for the T65P mutation

A PCR-RFLP assay was designed for the detection of the identified mutation 193 A>C (T65P) in exon 2 of the *KCNH2* gene (Figure 3B and C). Primers used were 5'-CAC-GCG-CAC-TCT-CCT-CAC-CG-3' (MG430, forward) and 5'-CGC-CCC-GGC-CCG-CTC-CTA-C-3' (MG431, reverse). The PCR mix of 30 µl consisted of 1x PCR buffer II, 200 nM of dNTP's, 1.5 mM of MgCl₂, 250 nM of each primer, 0.75 units of AmpliTaq Gold polymerase and approximately 100 ng of genomic DNA. PCR conditions were 95°C for 10 min, 35 cycles of 95°C (45 sec), 60 °C (45 sec), 72°C (45 sec) and a final extension step of 72°C for 10 min. The resulting PCR product of 278 bp was digested with restriction enzyme *Bsp*-MI. A control group of 90 anonymous, healthy volunteers (180 alleles) was screened with the PCR-RFLP test for the T65P mutation.

Site-directed mutagenesis and constructs

The wild type HERG/pcDNA3 (HERG-WT) expression construct was kindly donated by Dr. Craig January and Dr. Zhengfeng Zhou (University of Wisconsin-Madison). Mutant T65P-HERG/pcDNA3 (HERG-T65P) was created using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega, Madison, US) according to the manufacturers instructions. Primer 5'-AGT-CGC-AGG-**GCG-AGG-GTC-G**-3' was used as the synthetic oligonucleotide to create the mutant HERG cDNA (mutation in bold and underlined). The introduction of mutation 193 A>C (T65P) in the wild type HERG cDNA was verified by direct sequencing. Wild type (WT-GFP) and mutant T65P HERG cDNA (T65P-GFP) were sub cloned downstream of the coding region of a green fluorescent protein (GFP) in expression vector pcDNA3.1_NT_GFP (Invitrogen, Paisley, UK) using restriction sites *Kpn*-I and *Eco*-RI. The ATG startcodon of the *KCNH2* cDNA was 24 bp downstream of the final GFP codon. The complete sequence of the constructs, encoding the in-frame fusion GFP-HERG protein, was verified using direct sequencing.

Stable transfection of HERG WT and T65P constructs in HEK293 cells

Human embryonic kidney 293 cells (HEK293) were cultured in DMEM supplemented with 10% foetal bovine serum, 1% L-glutamine /penicillin/streptomycin and 1% non-essential amino acids under a 5% CO₂ atmosphere. HEK293 cells were transfected stably with FUGENE Transfection Reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturers instructions. Between five and ten HERG WT/pcDNA3 or HERG T65P/pcDNA3 stable cell lines were selected by their G-418 resistance and the level of HERG protein expression was determined by western blot analysis using a HERG specific antibody (Alomone labs, Jerusalem, Israel). Cell lines with the highest HERG protein expression were used for follow up experiments.

Western blot analysis

Membrane protein preparation and Western blot analysis were performed as previously described (30). Membrane proteins isolated from the stable cell lines, cultured at 37°C, were subjected to 4-12% SDS-polyacrylamide gel electrophoresis and

electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated overnight at 4°C with anti-HERG antibody at the recommended dilution. Membranes were incubated with HRP-conjugated donkey anti-rabbit antibody and HRP-bound protein was detected with an ECL+Plus detection kit. In order to study defects in biosynthetic processing of the T65P mutant protein, Western blot procedures were repeated at incubation temperature of 27°C. The effects of channel blocking agents cisapride and E-4031 on HERG protein trafficking were studied at 37°C in a concentration range between 0 and 10 μ M.

Confocal imaging:

HEK293 cells were cultivated as described in the previous section, but on cover slips. HEK293 cells were transiently transfected with 0.5 μ g of either WT-GFP or T65P-GFP constructs. In addition, 0.1 μ g of a red fluorescent marker for the endoplasmic reticulum (DsRed-ER) was cotransfected to distinguish between ER retained and membrane bound proteins (31). Confocal images were obtained 48 hours after transfection on a Zeiss CLSM 510, equipped with an argon laser for the visualization of GFP and DsRed tagged proteins.

Whole cell current recordings and data analysis

HEK293 cells were cultured at 37°C or at 27°C, when indicated. Current recordings were made with an Axopatch-200B amplifier (Axon instruments, Union City, CA) in the whole cell configuration using suction pipettes as described previously (32). Glass patch pipettes were pulled with a P2000 laser puller (Sutter Instruments, Novato, CA) from 1.2 mm Starbore borosilicate glass (Radnoti, Monrovia, CA) and had resistances between 1.5 and 3.5 M Ω . All currents were recorded at room temperature (20-22°C) and were sampled at 1 to 10 kHz with a Digidata 1200A data acquisition system (Axon instruments), after appropriate low-pass filtering. Command voltages and data storage were controlled with pClamp8 software (Axon instruments). Cells were perfused with HEPES-buffered Tyrode's solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose and 10 mM HEPES (pH 7.35 with NaOH). The internal pipette solution contained 110 mM KCl, 2 mM MgCl₂, 5 mM K₂ATP, 5 mM K₄BAPTA and 10 mM HEPES (pH 7.2 with KOH). After seal formation and establishing the whole cell configuration, the access resistance varied between 2.5 and 8 M Ω . Series resistance was compensated to ensure that voltage errors were <5 mV. No leak subtraction was applied and the holding potential was -80 mV, unless noted otherwise. The voltage protocols are presented in the figure legends. The voltage dependence of channel activation was fitted with a Boltzmann equation according to $y = 1 / \{1 + \exp[-(E - V_{1/2})/k]\}$, where $V_{1/2}$ represents the voltage at which 50% of the channels are open and k the slope factor. Activation, deactivation and recovery from inactivation kinetics were fitted with a single or a double exponential function using a non-linear least-squares (Gauss-Newton) algorithm. Data are presented as mean $\pm$

SEM and the Student's *t*-test was used for statistical analysis; *n* represents the number of cells.

Results

Screening of LQT genes

Mutation screening by direct sequencing revealed a novel mutation in exon 2 of the *KCNH2* gene in the DNA of subject 1. This missense mutation, changing base pair 193 from an A to C, leads to an amino-acid change from a threonine to a proline (T65P) and is located in the N-terminal PAS domain of the *KCNH2* gene (Figure 1,2).

A PCR-RFLP assay was developed, based on the loss of recognition site by restriction enzyme Bsp-MI due to the mutation. In the presence of the 193 A>C (T65P) mutation, a recognition site for restriction enzyme Bsp-MI is lost and results in a 278 bp product. In the absence of the mutation, the PCR product is cut in two fragments of 155 and 123 bp, respectively. All three available LQTS family members (subjects 1, 2, and 3) were found to be heterozygous carriers of this mutation (Figure 3), while the mutation was not found in a control population of 90 healthy unrelated volunteers.

Western blot analysis

It has been shown previously that expression of the wild type HERG protein in HEK293 cells results in two distinct bands on Western blot analysis: the upper band of 155 kDa represents the complexly glycosylated mature form of the HERG channel inserted the cell membrane while the lower band (135 kDa) represents the immature core-glycosylated form of the HERG channel retained in the ER (30). Upon Western blot analysis, the T65P protein incubated at 37°C for 24 hours resulted only in the lower 135 kDa band, while the wild type protein resulted in both bands (Figure 4A). However, the mature 155 kDa band was detected after a decrease in incubation temperature to 27°C for at least 24 hours (Figure 4A), indicating that trafficking was restored. The stable HERG T65P cell line was also incubated with 2, 5 and 10 µM of HERG channel blocking agents cisapride and E-4031. Both agents were able to restore trafficking of the T65P protein in a concentration dependant manner (Figure 4B), as assessed from the appearance of the 155 kDa protein.

Figure 3. Pedigree of LQTS family and PCR-RFLP assay for mutation detection. A, family pedigree, indicating the index numbers of the family members. *Black symbols*, clinically affected subjects with LQTS; *symbols with black dot*, asymptomatic subjects carrying the LQTS gene mutation; *question mark*, suspected gene carrier; *squares*, males; *circles*, females. B, Schematic overview of the PCR-RFLP assay used for the detection of the 193 A>C (T65P) mutation in the *KCNH2* gene. C, results from the PCR-RFLP assay tested on family members. Lane numbers at the top of the lanes of the 12.5% polyacrylamide gel indicate the subject numbers (1-3) or control subjects (C). bp=basepairs.

Figure 4. Western blot analysis of WT and T65P mutant HERG channels. *A*, Influence of incubation temperature on protein trafficking. Incubations were performed at 37°C and 27°C for 24 hours. *B*, Influence of HERG blocking drugs cisapride and E-4031 on T65P protein trafficking. Crude membrane fractions were isolated 24 hrs after transfection. 35 μg of protein was loaded in each lane. Upper bands represent the glycosylated, membrane bound, mature form of HERG protein (155 kDa), lower bands represent immature HERG protein retained in the endoplasmic reticulum (135 kDa).

Confocal microscopy

To confirm Western-blotting results, GFP was tagged to the N-terminus of HERG subunits and expressed in HEK293 cells. Sub cellular trafficking of WT-GFP and T65P-GFP was investigated after incubation for 48 hrs at 37°C or 27°C. A red fluorescent endoplasmic reticulum (DsRed-ER) vector was co-transfected to distinguish between HERG protein retained in the ER and membrane bound HERG proteins. Confocal images (Figure 5) clearly show the presence of WT channels in the plasma membrane (Figure 5A, B) as well as in the ER (37°C), while the T65P mutant protein (37°C) was exclusively present in the ER (Figure 5C, D). Upon incubation at 27°C, however, trafficking of mutant T65P HERG protein was restored and the protein was incorporated in the cell membrane, as evidenced by the selective green fluorescence at the level of the plasma membrane (Figure 5E, F).

Figure 5. Subcellular distribution of WT HERG and T65P channels. Confocal imaging of GFP tagged WT (WT-GFP) and T65P (T65P-GFP) HERG channels in HEK293 cells. GFP tagged constructs were transfected transiently together with an endoplasmic reticulum targeted DsRed marker (DsRed-ER). Left panels, GFP fluorescence images for WT (37°C), T65P (37°C) and T65P (27°C) HERG channels. Right panels, superimposed images of double stained (green GFP and red DsRed) HERG WT or T65P channels. Note the absence of (green) membrane bound protein of T65P at 37°C (C and D) and the restoration of T65P protein trafficking at 27°C (E and F).

Biophysical properties of HERG WT and T65P channels

Because Western blot analysis and confocal imaging suggested dysfunctional trafficking of T65P mutant to the cell membrane, we performed whole cell patch clamp recordings on stable HERG WT and T65P cells, in order to study the functional expression. Figure 6 shows representative current recordings for HERG-WT and HERG-T65P channels. T65P stable cells only generated small currents (Figure 6C) as compared to WT stable cells after incubation at 37°C. Incubating T65P cells at 27°C for 24 hours significantly increased T65P current density (Figure 6D), suggesting temperature dependent trafficking of T65P proteins. The voltage dependence of

activation was determined for WT and T65P channels (grown at 37°C and 27°C) by plotting the maximum tail current amplitude at -40 mV (Figure 6) as a function of the prepulse voltage. These data were fit with a Boltzmann function, which revealed no significant differences in the mid-point potential ($V_{1/2}$) and slope factor (k) between WT and T65P channels (Figure 7A).

Figure 6. Voltage clamp recordings of HERG WT and T65P channels. A, voltage protocol, HERG currents were activated by 5 sec depolarising steps between -60 and +60 mV (in 10-mV increments) from a holding potential of -80 mV. Cells were clamped to -40 mV for 10 sec to elicit tail currents. Representative HERG currents elicited from WT stable cells after incubation at 37°C (B, $n=10$), T65P stable cells after incubation at 37°C (C, $n=9$) and T65P stable cells after incubation at 27°C (D, $n=7$).

To determine the time constants of activation, deactivation and recovery from inactivation, current traces were fit with a single or double exponential function as appropriate. The kinetics for T65P cells grown at 27°C were analyzed over the whole voltage range, whilst for cells grown at 37°C, the current amplitudes were too small for full analysis. At voltages resulting in the highest current amplitudes, the current kinetics of T65P cells grown at 37°C were comparable to the kinetics of T65P cells grown at 27°C (data not shown). Time constants for activation and deactivation are presented in Figure 7C. T65P channels (27°) activated and deactivated faster than WT channels. The time constants for recovery from inactivation are presented in Figure 7D. Recovery from inactivation was also faster for T65P channels than WT channels. Co-expression of WT and mutant T65P was performed by transient transfection of the T65P-pcDNA3 construct on the WT stable cell line. Maximum tail current amplitudes, as obtained according to the pulse protocol in Figure 6, were compared to maximum tail current amplitudes from stable WT cells alone. In cells grown at 37°C, no increase in HERG current density was observed while cells grown at 27°C displayed a significant

increase in HERG current density (Figure 8). These results suggest that WT subunits do not interact with T65P subunits at 37°C.

Figure 7. Voltage dependence and kinetics of HERG WT and T65P channels. **A**, Voltage dependence of steady-state HERG activation. Relative tail currents were derived from current recordings as shown in figure 5. HERG WT and T65P (cell culture at 37°C and 27°C) currents were fit with a Boltzmann function to determine midpoint of activation ($V_{1/2}$) and slope factor (k). WT ($n=10$): $V_{1/2}=2.53 \pm 1.47$, $k= -7.3 \pm 0.13$, T65P 37°C ($n=9$): $V_{1/2}=-1.19 \pm 2.39$, $k= -7.56 \pm 0.36$, T65P 27°C ($n=7$): $V_{1/2}=-0.57 \pm 2.82$, $k= -7.73 \pm 0.32$. **B**, pulse protocol eliciting deactivating tail currents. HERG channels were fully activated by a 1-second prepulse to +50 mV and then clamped to voltages between 0 and -120 mV in 10 mV increments for 4 sec. **C**, Time constants of current activation were determined by fitting activating currents between -20 and 60 mV as shown in Figure 5 to a single exponential function. Slow and fast component of current deactivation were determined by fitting deactivating tail currents (as in panel B) to a double exponential function. **D**, time constant for recovery from inactivation was determined by fitting the first 15 ms after the 1-second activating pulse (as in panel B) to a single exponential function.

Figure 8. Co-expression of HERG WT and T65P mutant channels. As a measure for channel density at the plasma membrane level, maximal tail amplitudes were determined from current recordings as in Figure 5 after 24 hours of transfection. Tail currents from WT and T65P cell lines, cultivated at 37°C or 27°C are shown in column 1 to 4. To study co-expression, 5 μ g of HERG-T65P was transiently transfected on the HERG-WT stable cell line (column 5 and 6). After transfection, cells were either incubated at 37°C or at 27°C. Error bars represent $\pm$ S.E.M.

Discussion

A novel cLQTS causing mutation, T65P in the *KCNH2* gene, was detected in a Caucasian LQTS family. Two sudden deaths occurred in this family indicating a high risk for development of lethal arrhythmias. Three members participating in the study were all heterozygous carriers of this mutation while the mutation was not detected in a control group of 90 individuals (Figure 3).

Previously, characterized *KCNH2* mutations have been shown to cause a reduction in I_{Kr} current in the repolarization phase of the cardiac action potential. This reduction in I_{Kr} current can be the result of different mechanisms. Mutant channels, such as A561V, S818L and Y667X, can cause a complete loss of function (9, 13, 33), while other mutated channels can form functional channels, but with altered gating kinetics (16). Recently, a third mechanism for dysfunctional behaviour of mutant HERG proteins was described involving protein trafficking (18, 19). It was proposed that these mutations prevented correct protein folding such that these proteins fail the “quality control” and are retained in the endoplasmic reticulum (ER). In the present study, the Western blot analysis of the T65P mutant protein showed a single 135 kDa band, while WT HERG protein normally shows two bands (Figure 4A): the immature, core-glycosylated protein synthesized in the ER (135 kDa) and the further developed, fully glycosylated, mature protein that is incorporated in the cell membrane (155 kDa). Such aberrant trafficking was initially described for the HERG mutants Y611H and

V822M by Zhou *et al.* (18). A later study by the same group (20) demonstrated that defective trafficking could be restored by lowering incubation temperatures (27°C) of the cell culture or by treatment with HERG channel blocking drugs such as cisapride or E-4031. Also in the case of T65P we observed that trafficking of the mutant protein could be restored by both lower incubation temperatures as well as pharmacological drugs (Figure 4B). The latter pharmacological chaperone “rescue” of mutant HERG proteins has opened the first door towards therapy. Ficker *et al.* demonstrated that rescue of trafficking deficient HERG proteins can be domain-restricted and that potency of drug related rescue was directly linked to channel block potency (34). However, Rajamani *et al.* showed very recently that at least for some misprocessed HERG proteins, fexofenadine had a more than 300-fold higher affinity towards HERG channel rescue than block (35). In this study it appeared that only misprocessed proteins that showed small currents at 37°C could be rescued but not the ones that showed no current at all at 37°C. Since the T65P protein also showed small currents at 37°C, fexofenadine might also restore trafficking of this mutated channel.

Interestingly, the novel T65P mutation resides in the N-terminal end of the HERG protein, which was shown to possess a so-called Per-Arnt-Sim (PAS) domain (36). Although this domain displays large amino acid sequence diversity in several proteins, it was however proposed as a domain family (36, 37). In HERG the N-terminal residues from S26 to K135 were crystallized (26). A database search revealed a high similarity with the three-dimensional structure of the PAS domain containing photoactive yellow protein (PYP) (37) although the sequence similarity is low. The crystal structure contains five antiparallel β -sheet strands pressed against a long strand, composed of a coil and one turn of a 3_{10} helix with three α -helices on the sides. The substitution of the threonine by a proline could have several consequences on HERG channels. 1) The T65P mutation is located just before the single turn of the 3_{10} -helix, which might affect the formation of this helix. 2) A putative hydrogen bond between the T65 with D67 would not be possible with the proline substitution and could destabilize the structure of the N-terminus. 3) Although residue T65 is located in a more or less loosely packed region of the N-terminus, it can not be excluded that steric hindrance induced by the rigid proline side chain could cause misfolding. 4) Any of the above effects could affect the local structure of the long strand. The latter, containing the 3_{10} helix and T65, connects the α -helices located at both sides of the sheet of β -strands. A distortion of this connector could then change the 3-dimensional structure in other parts e.g. the sheet of β -strands. The PAS domain in HERG is highly conserved among species (Figure 2).

SPECIES	GENE	SWISS PROT	PROTEIN SEQUENCE ALIGNMENT
Human	ERG	Q12809	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F I I A N A R V E N C A V I Y C N
Dog	ERG	Q9TS23	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F I I A N A R V E N C A V I Y C N
Rabbit	ERG	Q8WNY2	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F I I A N A R V E N C A V I Y C N
Rat	ERG	O08962	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F I I A N A R V E N C A V I Y C N
Mouse	ERG	O35221	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F I I A N A R V E N C A V I Y C N
Human	ERG2	Q9H252	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F L I A N A Q M E N C A I Y C N
Rat	ERG2	O54853	M E V Y R R G H V A P Q N T F L D T I T A R R F E G Q S R K F L I A N A Q M E N C A I Y C N
Mouse	ERG3	Q9ER47	M E V Y R R G H V A P Q N T F L G T I T A R R F E G Q N K K F L I A N A R V Q H C A I Y C N
Zebra fish	ERG	AAM95975	M E V Y R R G H V A L D N T Y L D T T I R R F D E G Q N R K F L I A N A Q M K M G I I Y C N
Human	ERG	Q12809	D G F C E L C G Y S R A E V M Q R F C T C D F L H G E R T Q R K A A A Q I A Q A L L G A E
Dog	ERG	Q9TS23	D G F C E L C G Y S R A E V M Q R F C T C D F L H G E R T Q R K A A A Q I A Q A L L G A E
Rabbit	ERG	Q8WNY2	D G F C E L C G Y S R A E V M Q R F C T C D F L H G E R T Q R K A A A Q I A Q A L L G A E
Rat	ERG	O08962	D G F C E L C G Y S R A E V M Q R F C T C D F L H G E R T Q R K A A A Q I A Q A L L G A E
Mouse	ERG	O35221	D G F C E L C G Y S R A E V M Q R F C T C D F L H G E R T Q R K A A A Q I A Q A L L G A E
Human	ERG2	Q9H252	D G F C E L F G Y S R V E V M Q R F C T C D F L T G E N T P S S A V S R L A Q A L L G A E
Rat	ERG2	O54853	D G F C E L F G Y S R V E V M Q R F C T C D F L T G E N T P S S A V S R L A Q A L L G A E
Mouse	ERG3	Q9ER47	D G L C E M T G F S R P D V M Q R F C T C D F L H G E R T E T K R H D I A Q A Q A L L G S E
Zebra fish	ERG	AAM95975	E G F C Q M F G F S R A E I M Q Q S C T C Q F L V G E G T M K S A L G Q L A Q A L L G S E
Human	ERG	Q12809	E R R V E I A F Y R K D G S C F L C L V D V V P V R N E D G A V I M F I N F E V V M E R
Dog	ERG	Q9TS23	E R R V E I A F Y R K D G S C F L C L V D V V P V R N E D G A V I M F I N F E V V M E R
Rabbit	ERG	Q8WNY2	E R R V E I A F Y R K D G S C F L C L V D V V P V R N E D G A V I M F I N F E V V M E R
Rat	ERG	O08962	E R R V E I A F Y R K D G S C F L C L V D V V P V R N E D G A V I M F I N F E V V M E R
Mouse	ERG	O35221	E R R V E I A F Y R K D G S C F L C L V D V V P V R N E D G A V I M F I N F E V V M E R
Human	ERG2	Q9H252	E C K V D I L Y Y A K K D A S S F R C L V D V V P V R N E D G A V I M F I N F E D L - - -
Rat	ERG2	O54853	E C K V D I L Y Y A K K D A S S F R C L V D V V P V R N E D G A V I M F I N F E D L - - -
Mouse	ERG3	Q9ER47	E R R V E I T Y Y N N E S T F I C N T H I I F V K N Q E G V A H M F I N F E Y V T D E
Zebra fish	ERG	AAM95975	E R R V E I L Y Y S N E S T G R P C I D V I F V K N E E G V V I N F I N F E L L D P

Figure 2. Alignment of N-terminal amino-acid eag domain in different species. Each amino-acid sequence is preceded by its Swiss Prot accession number. Grey shaded areas mark the identical residues. A rectangular box indicates the newly identified T65P mutation that has been characterized in this study. Mutated amino acids in the PAS domain of the human HERG protein that have been reported previously are indicated by white coding on a black background. The thick black line indicates the core PAS domain as described by Pellequer *et al.* (38). The crystal structure as obtained by Morais-Cabral *et al.* (26) extends from S26 to K135.

Previously it was shown that removal of the N-terminal end, including the PAS-domain, accelerates channel deactivation (38, 39). Furthermore, splice variants that lack a part of the N-terminal domain display a faster deactivation rate (6,7). A similar effect on the deactivation rate was observed with mutations in the S4-S5 linker and it was suggested that this linker and the PAS domain might interact (17, 39) as was demonstrated for *eag* channels (40). Using an oocyte expression system Chen *et al.* (1999) investigated the effects of several LQT associated PAS domain mutations on deactivation kinetics, and showed for eight mutations that HERG channel deactivation was accelerated, although to a different extent. The novel T65P mutation was analyzed with a mammalian expression system at 37°C and also revealed some accelerating effect on channel deactivation, but we consider this effect of secondary importance compared to the trafficking deficiency displayed by the T65P mutant protein. Chen *et al.* did not report trafficking deficiency in their study as the typical low incubation temperature of oocytes might have concealed trafficking deficiency. It is therefore conceivable that some of these eight mutant proteins may be deficient in trafficking at 37°C. This hypothesis is supported by the results of Ficker *et al.* (21), who showed large currents for a trafficking deficient HERG protein (R752W) in the oocyte expression system as compared to the lack of current in the mammalian expression system.

A functional HERG channel is composed of four α -subunit proteins, encoded by the *KCNH2* gene, forming a tetramer. Most LQTS patients have the dominant Romano-Ward form, inheriting one wild type allele and one mutant allele. Therefore, the best approach to mimic the phenotypic result of the genetic profile of these patients is the study of co-expression of WT cDNA with mutant cDNA. In some cases the ability of mutant HERG proteins to form hetero-tetramers with wild type subunits is lost, leading to haplo-insufficiency. In other cases, interaction of WT subunits with mutant subunits leads to alterations in the biophysical properties or amplitude of the assembled ion channel, causing a dominant negative effect. Although the gating kinetics of T65P channels were slightly different from WT channels, it is most likely that the prolonged QT interval is caused by a reduction of I_{Kr} by ER retention of the T65P subunits. The co-expression of T65P with WT subunits at 37°C, mimicking the patients' actual genotype, showed no significant differences in current density or kinetics as compared to WT expression alone (Figure 8). This result indicates that T65P and WT subunits do not form hetero-tetramers; indeed, a decreased current density should have been observed upon ER retention of WT/T65P hetero-tetramers or an increased current density should have been observed upon incorporation in the cell membrane of these hetero-tetramers. Whether the increased current density of co-transfected WT and T65P subunits at 27°C is caused by hetero-tetramers or separate WT and T65P homo-tetramers remains to be investigated.

In summary, genetic analysis revealed a novel mutation (T65P), located in the PAS domain of HERG, which causes trafficking deficiency of the protein, and clinically leads to the long QT syndrome. This study suggests that a correctly folded PAS-domain has a major role in proper trafficking of the (tetrameric) channel complex. Furthermore, these results indicate that analysis of *KCNH2* mutations in oocytes may at best be insufficient to delineate all possible molecular mechanisms by which the mutation can cause the disease.

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CHAPTER 3

Mutation analysis in congenital long QT syndrome – a case with missense mutations in *KCNQ1* and *SCN5A*

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Abstract

Long QT Syndrome (LQTS) is a cardiac disease characterised by a prolonged QT interval on a surface ECG and by clinical symptoms such as seizures, syncope and cardiac sudden death. At present, causal mutations of LQTS have been identified in five cardiac ion channel genes. Because a causal mutation is usually unique to a specific family and can be located at any region of one of these five genes, a mutation analysis effort may require screening of the complete coding region of each of these genes. The causative nature of a detected mutation can then be determined either by family history or by functional studies, such as an electrophysiological signature of the mutation. Here we describe a mutation analysis of a LQTS patient who carries two heterozygous missense mutations in two different LQTS genes. The first mutation identified, A572D in *SCN5A*, was not linked with clinical LQTS features in the two other mutation carriers in the family, but was not identified in 90 healthy controls either. Therefore, this mutation most likely has either a mild effect on cardiac ion channel function or represents a very rare polymorphism. The second mutation, V254M in *KCNQ1*, co-segregated with higher QT-intervals and symptoms in other family members, and was previously reported in another LQTS family. Because the clinical LQTS symptoms are most pronounced in the proband, a combined effect of both mutations cannot be excluded, although no functional data are available to support such hypothesis. We conclude that, for newly presented LQTS cases, a mutation analysis strategy should routinely screen the complete coding region of all LQTS genes, followed by an evaluation of the identified mutation(s) in conjunction with family or functional data.

Introduction

Congenital long QT syndrome (LQTS) is a potentially lethal cardiac disease, visible on a surface ECG by a prolongation of the QT interval. Clinically, the disorder is manifested by syncope and eventually by sudden cardiac death due to polymorphic ventricular tachycardia, also called Torsade de Pointes (1). Depending on the mode of genetic transmittance, the disorder can be divided in two forms: the autosomal dominant Romano-Ward syndrome representing the majority of the cases (2,3), and the autosomal recessive Jervell-Lange-Nielsen syndrome that is characterized by both QT prolongation and congenital deafness (4). Our understanding of the genetic basis of LQTS has improved significantly over the last decade, with the demonstration of mutations that cause LQTS in five different genes, and the identification of one additional genetic locus on chromosome 4 that is associated with LQTS but for which the responsible gene has not been discovered yet. These known genes encode cardiac ion channel proteins that are involved in the depolarisation (*SCN5A*) and the repolarisation (*KCNQ1*, *KCNH2*, *KCNE1*, and *KCNE2*) of the cardiac action potential.

Typically, a new mutation in one of the LQTS genes is identified in each LQTS family. Once a new mutation is identified in a patient, only functional studies can truly confirm its causal relationship with LQTS. It is, however, not feasible for many genetic laboratories to perform such functional studies, including electrophysiological experiments. For molecular diagnostic purpose, therefore, the functional relevance of a new mutation can often only indirectly be assumed.

Mutation analysis of all five LQTS genes is time and labour consuming, because of the large number of exons to be screened. Apart from *KCNE1* and *KCNE2* that are intronless, *KCNH2* harbours 15 different exons, *KCNQ1* comprises 19 exons, and *SCN5A* consists of 28 exons (5-8). Because, in addition, some large exons require screening of multiple DNA fragments, a full mutation analysis of all five LQTS genes involves the screening of 65-75 different DNA fragments (approximately 21 kb in total). Obviously, it is very attractive to perform mutation screening of the LQTS genes only up to the point when a new or known mutation is found that is likely to explain the disorder in a patient and his family. In this respect, genetic variations that cause an alteration in the amino acid sequence of the ion channel protein are potentially good candidates to explore further. During evaluation of an identified mutation within a family, the variation in penetrance of LQTS mutations may complicate the link between a new mutation and the disease (9). In such case, functional studies are required to confirm the causal relationship between a mutation and LQTS.

Although typically only one mutation is responsible for the disease in a Romano-Ward form of LQTS (10,11), it has been hypothesized recently that affected subjects may carry multiple heterozygous mutations in different genes, whereby some mutations could have only mild functional consequences (12). In the present study, we report on a complete mutational screening in a Romano-Ward LQTS family, in which we detected two heterozygous missense mutations in two different LQTS genes in the proband.

Materials and Methods

Subjects

The Caucasian proband was an 11-year-old boy who was brought to our attention with clinical symptoms indicative for LQTS. The patient experienced syncope at the age of 6 and 7. QTc interval was measured to be 483 msec at rest. Since the age of 7, the boy was treated with β -blockers and did not experience further syncope since then. Family members were invited to participate in the study. Three family members from the maternal side and nine members from paternal side participated in the study (Figure 1). Among the paternal side of the family, one relative died of unexplained sudden death and another family member experienced syncope at childhood (index

number 2). All family members gave informed consent prior to genetic analysis. An anonymised group of 90 healthy Caucasian volunteers was used to screen for the presence of the novel identified LQTS gene mutation. All volunteers consented prior to genetic analysis.

Mutational screening

Genomic DNA from the proband was used for mutational screening of all five known congenital LQTS genes by direct sequencing. Oligonucleotides used for PCR amplification of the individual exons, including intron-exon boundaries, have been previously described by Itoh *et al.*, (*KCNH2*, [7]) Abbott *et al.*, (*KCNE2*, (6)), Wang *et al.*, (*SCN5A*, (8)) and Splawski *et al.*, (*KCNQ1*, *KCNH2* and *KCNE1*, (5)). PCR products were verified on 1.5% agarose, purified (Qiagen PCR purification kit), directly sequenced using the big dye terminator kit on an ABI-Prism 3700 DNA Analyzer (Applied Biosystems Inc, Foster City, CA), and aligned with the respective wild type sequences.

Detection assay for A572D in SCN5A

A PCR-RFLP detection assay was developed for the 1715 C>A (A572D) mutation in exon 12 of the *SCN5A* gene. PCR primers used were 5'-TGG-CCC-CTG-CGC-CGG-AC-3' (primer 1305, forward) and 5'-AGG-AGG-TGG-CTT-CCT-GGG-G-3' (primer 1306, reverse). The 30 µl PCR mix contained 1x GeneAmp PCR buffer II, 200 nM dNTPs, 1.5 mM MgCl₂, 250 nM of each primer, 0.75 units of *AmpliTaq* Gold polymerase and approximately 100 ng of genomic DNA. PCR conditions were 10 min 95°C, followed by 35 cycles of 95°C (45 sec), 55°C (45 sec) and 72°C (45 sec), and a final extension step at 72°C for 10 min. The PCR product was digested with *BmyI* for 3 hours at 37°C and the resulting DNA fragments were separated on 12.5% polyacrylamide gels. In the absence of the 1715 C>A mutation, the PCR product is cut into two fragments of 140 bp and 24 bp, respectively. In the presence of the mutation, the 164 bp PCR product remains undigested (Figure 2).

Detection assay for V254M in KCNQ1

A PCR-RFLP assay was developed for the detection of the 760 G>A (V254M) mutation in exon 5 of the *KCNQ1* gene. PCR primers used were 5'-AGG-GGC-AGG-GGC-AGG-GAC-AC-3' (primer 288, forward) and 5'-CGG-GGC-CTC-AGC-GCA-TCT-CA-3' (primer 289, reverse). The 30 µl PCR mix contained 1x GeneAmp PCR buffer II, 200 nM dNTPs, 1.5 mM MgCl₂, 250 nM of each primer, 0.75 units of *AmpliTaq* Gold polymerase and approximately 100 ng of genomic DNA. After an initial period of 10 min 95°C, 35 cycles were performed consisting each of 95°C (1 min), 63°C (1 min) and 72°C (1 min), and finally followed by an extension step at 72°C for 10 min. The 300 bp PCR product was digested for 3 hours at 37°C with *NcoI*; the resulting DNA fragments were separated on 12.5 % polyacrylamide gels. The 760 G>A mutation creates a *NcoI* restriction site in the DNA sequence of the PCR product; restriction enzyme digestion of the mutated fragment therefore results in two fragments of 120 and 180 bp, respectively (Figure 3).

Results

Mutation screening of cLQTS genes in the LQTS proband

Two missense mutations were detected in two different genes by direct sequencing in the DNA of the proband with the baseline QTc of 483 ms. One mutation, A572D, was detected in exon 12 of the *SCN5A* gene: a base substitution of C to A at position 1715, altering codon 572 from alanine to aspartic acid. Subsequently, a second mutation, V254M, was identified in exon 5 of the *KCNQ1* gene: a base substitution of G to A at position 760, altering codon 254 from valine to glutamic acid. The proband was heterozygous for both mutations.

Figure 1. Family tree of the LQTS proband. The clinical phenotype, QTc interval and mutation carrier status for *SCN5A* A572D and *KCNQ1* V254M are indicated for individual family members. Arrow, indicates the proband; yrs, years of age; squares, males; circles, females; upper half area in grey, heterozygous carriers of the A572D mutation in *SCN5A*; lower half area in black, heterozygous carriers of the V254M mutation in *KCNQ1*. An asterisk indicates a high or borderline QTc interval according to the diagnostic criteria for LQTS by Schwartz *et al.* (13).

*A572D mutation in the *SCN5A* gene*

The PCR-RFLP designed to detect the A572D mutation in the *SCN5A* gene (Figure 2) was used to screen the proband's parents and subsequently all available family members (Figure 1). In addition to the proband, two family members from maternal side (index numbers 4 and 13) were also heterozygous carriers of the A572D mutation. Both carriers, however, had normal QTc values and did not have a history of clinical symptoms related to LQTS. The mutation was not detected in 90 healthy volunteers, and can therefore be excluded as a common polymorphism.

Figure 2. PCR-RFLP for mutation A572D in *SCN5A*. (A) Design of the PCR-RFLP assay for the rapid detection of the A572D mutation in *SCN5A*. (B) Example of 12.5% polyacrylamide gel with different individual genotypes. Lane 1: 100 bp ladder; lane 2, 3, 4 and 6: subjects with homozygous wild type (A/A) genotype; lane 5: subject with the heterozygous mutant (A/D) genotype.

*V254M mutation in the *KCNQ1* gene*

The PCR-RFLP designed to detect the V254M mutation in the *KCNQ1* gene (Figure 3) was used to screen the proband's parents, and subsequently all available members from the paternal side of the family (Figure 1). In addition to the proband, three other family members (index numbers 2, 3 and 5) were heterozygous carriers of the V254M mutation. The QTc interval of these three individuals was higher than normal, but only one of them had suffered from syncope at young age. The two other mutation carriers were clinically asymptomatic. It can be assumed that at least one other family

member who died by sudden death at the age of 8 years, also carried the mutation, but no DNA was available to confirm this hypothesis.

Figure 3. PCR-RFLP for mutation V254M in *KCNQ1*. (A) Design of the PCR-RFLP assay for the rapid detection of the V254M mutation in *KCNQ1*. (B) Example of 12.5% polyacrylamide gel with different individual genotypes. Lane 1: 100 bp ladder; lane 2, 5 and 7: subjects with homozygous wild type (V/V) genotype; lane 3, 4 and 6: subject with the heterozygous mutant (V/M) genotype.

Discussion

Congenital long QT syndrome is caused by an abnormal prolongation of the cardiac action potential duration, leading to susceptibility of developing ventricular arrhythmia. Prolongation of the action potential duration can be caused by dysfunction of ion channels. Today, five genes that all encode cardiac ion channel have been associated with LQTS by the identification of causal mutations. The present study demonstrates that mutational screening in a newly presented case of congenital LQTS should be done thoroughly, including the exons of all known LQTS genes, and complemented with the evaluation of the pattern of inheritance in the family and/or functional studies for any newly identified mutation.

During the analysis of the reported LQTS family, we firstly detected the heterozygous A572D mutation in the *SCN5A* gene of the proband. This mutation is located in the cytoplasmic connector between the first and second domain of the sodium channel, where no mutation has been reported yet. The mutation was not found in 180 alleles from unrelated healthy controls, suggesting that A572D is indeed a causal mutation for LQTS or, alternatively, a very rare coding polymorphism. Apart from the proband, two other family members (mother and grandmother) carried the mutation, but were asymptomatic and had normal QTc intervals. Thus, surprisingly, the *SCN5A* mutation was inherited from the maternal family side that had no history of LQTS, whilst the mutation was not found at the paternal side that had a LQTS family history. Therefore, the other LQTS genes were screened in a search for additional mutations that might explain the family history at the paternal side. As a result, mutation V254M was detected in the *KCNQ1* gene. This mutation was reported earlier in a large LQTS family by Wang *et al.* (14) and was later also functionally characterized in an oocyte expression system (15).

This is to our knowledge the first report that describes two heterozygous missense mutations in two different LQTS genes in a single LQTS patient. It clearly exemplifies that the identification of a new, rare missense mutation in a LQTS gene of a patient alone is not sufficient evidence to claim a causal relationship with the disease. The availability of substantial clinical data on family members, and the discordance of these data with the inheritance pattern of the initially discovered mutation in the *SCN5A* gene, led to the correct hypothesis that another mutation - eventually in another LQTS gene - should be present.

Interestingly, the clinical symptoms and QT prolongation appeared to be more profound in the proband than in the other tested carriers of the *KCNQ1* mutation within the family. Such a low penetrance of phenotypic symptoms is a common feature in many LQTS families (9). Alternatively, one could speculate that the

additional *SCN5A* missense mutation might partially contribute to the LQTS phenotype, but no functional data are available to support such hypothesis. Mutations in *KCNQ1* have been shown to delay the repolarisation phase of the action potential, whilst mutations in *SCN5A* may sustain inward sodium current during cardiac depolarisation (15,16). In combination, both mutations could lead to the significant prolongation of the QTc interval in the proband, and consequently increase the risk for ventricular arrhythmia. Heterozygous carriers of one of the mutations, on the other hand, could remain asymptomatic with a normal or borderline QTc interval. This can, however, not explain the sudden death of a family member at the age of 8 years who presumably carried only the V254M *KCNQ1* mutation. We therefore hypothesize that the additional missense mutation in the *SCN5A* gene might possibly facilitate but is not required for the development of the clinical symptoms of LQTS in patients with a V254M mutation in *KCNQ1*.

In conclusion, this is to our knowledge the first study demonstrating that symptomatic Romano-Ward LQTS patients can carry multiple heterozygous missense mutations in different LQTS genes. It further demonstrates that complementary data (e.g., family or functional studies) are required to confirm the causal nature of a newly identified mutation in a LQTS gene. Our results are supportive for a mutation analysis strategy that routinely screens the coding sequence of all LQTS genes in any newly presented case of congenital LQTS in a molecular diagnostic laboratory.

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CHAPTER 4

Characterization of novel mutations E698X and P872fs877 in the C-terminus of the human potassium channel (HERG) that cause the long QT syndrome

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Background

Mutations in the *KCNH2* (*HERG*) gene are responsible for the chromosome 7-linked long QT syndrome. Mutations are associated with a reduction in the fast component of the delayed rectifier current I_{Kr} , thereby delaying repolarisation of cardiac cells and rendering patients vulnerable to ventricular arrhythmias and sudden death.

Methods and Results

We describe the identification and characterisation of two mutations (P872fs877 and E698X) in the C-terminus of the *KCNH2* gene in two large Dutch LQTS families. Both mutations lead to premature stop codons causing C-terminal truncations of the HERG protein. Biochemical and confocal microscopy techniques were used to investigate protein expression and trafficking. Electrophysiological methods were performed to assay potassium ion channel function. Only the P872fs877 truncated protein was expressed and functional. The P872fs877 protein showed aberrant trafficking due to accumulation of the protein in the ER. Homologous expression of P872fs877 channels produced similar currents as wild type channels although with reduced amplitudes. Heterologous co-expression of wild type and P872fs877 subunits changes the typical inward rectification behaviour of HERG likely because of the formation of heterotetramers.

Conclusions

C-terminal HERG mutations result in LQTS and the mechanisms leading to aberrant channel function in this region of the protein are a combination of aberrant trafficking and altered gating properties.

Introduction

The long QT syndrome (LQTS) is an inherited cardiac disease, which usually becomes apparent through symptoms like syncope, loss of consciousness and sudden death (1). The clinical manifestation of the disease shows itself by prolongation of the QT interval on the surface ECG, which is indicative for aberrant lengthening of the cardiac action potential (2). Mutations in the genes *KCNQ1* (KvLQT1, LQT1), *KCNH2* (HERG, LQT2), *SCN5A* (LQT3), *KCNE1* (MinK, LQT5) and *KCNE2* (MiRP1, LQT6) have been identified to cause the disease (3-7). The human *ether-à-go-go* related gene *KCNH2* (HERG) encodes the α -subunit of the rapidly activating, delayed rectifier potassium ion channel I_{Kr} , an important component of the action potential duration (8,9). Functional studies performed by various groups have indicated that mutations in *KCNH2* may result in reduced outward potassium current due to multiple mechanisms. The reduced outward current can be the result of non-functionality (10), altered gating properties (11), or abnormal protein trafficking (12) of mutated channels.

Based on the protein structure, HERG belongs to the *eag* voltage gated potassium channel family (13). Important functional regions in this family of proteins include six membrane-spanning domains (S_1 to S_6), flanked by intracellular amino (N)- and carboxyl (C)-terminal regions and an ion-conducting pore between transmembrane domains S_5 and S_6 . Because the six membrane domains and the pore constitute important parts of the channel subunits form and function, initial genetic screening focussed on these important regions of HERG. Over time however, also mutations in the N- and C-terminal regions have been identified in LQTS families. Currently, more than twenty mutations have been detected in the N-terminus of HERG. Functional characterization of nine N-terminal mutations has demonstrated that accelerated deactivation is a common factor in these mutated channels (14). Recently, trafficking deficiency has also been implicated in this N-terminal region (15), indicating that assignment of specific functional defects to protein regions is not always obvious. At least twenty different mutations have been identified in the C-terminal region of HERG, but functional data on the effects of these mutations are rather limited. Two mutations (R752W and V822M) in the C-terminus resulted in defective trafficking of the mutated proteins to the cell membrane (16,17) and mutation S818L caused complete non-functionality, albeit with some dominant negative effect on wild-type channel function (10).

In order to elucidate additional mechanisms that may be involved in the incorrect functioning of HERG proteins with C-terminal mutations, we studied the functional consequences of two newly identified C-terminal *KCNH2* mutations. The mutations E698X and P872fs877 were identified in two distinct Dutch LQTS families, and resulted both in truncated HERG proteins. We studied protein expression and trafficking with

biochemical methods and we used a whole cell patch clamp system to characterise channel function.

Methods

LQTS families

Families #1718 and #2714 are two large Dutch families with diagnosed long QT syndrome (Figures 1 and 2). Based on the clinical symptoms of the proband, other family members were initially evaluated by 12 lead ECG registration. Informed consent for participation in this study was obtained from 31 family members (13 females and 18 males) of family #1718 and from 21 members (14 females and 7 males) of family #2714. Both families originated from a Caucasian population.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples as described previously. All exons, encoding the entire region of the *KCNH2* gene, were amplified by PCR and mutation screening was performed using SSCP or DHPLC as previously described (18,19). Aberrant SSCP conformers and/or abnormal DHPLC elution profiles were subsequently analysed by automatic sequencing on an ABI377 (Applied Biosystems). After identification of the causative mutation in the proband, other mutation carriers in the LQTS families were identified by direct sequencing (P872fs877) or a PCR-RFLP test developed specifically for the mutation (E698X).

Site-Directed Mutagenesis and KCNH2 constructs

The wild type cDNA HERG/pcDNA3 expression construct was kindly donated by Dr. Craig January and Dr. Zhengfeng Zhou (University of Wisconsin-Madison). The HERG E698X and P872fs877 mutations were generated by site-directed mutagenesis of the wild type HERG/pcDNA3 construct using the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega) according to the manufacturers instructions. Primers 5'-AGT-ACT-CCT-**AGA**-GGC-GCT-G-3' (E698X) and 5'-CGT-ACT-GCC-GG-GGA-GCC-C-3' (P872fs877) were used as the synthetic oligonucleotides to create the mutant constructs (mutations in bold and underlined). The introduction of mutation 2092 G>T (E698X) and the deletion of basepair 2616 (P872fs877) in the wild type HERG cDNA were verified by direct sequencing. Wild type (WT-GFP), E698X (E698X-GFP) and P872fs877 (P872fs877-GFP) HERG cDNAs were also subcloned downstream of the coding region of a green fluorescent protein (GFP) in expression vector pcDNA3.1_NT_GFP (Invitrogen) using restriction sites *Kpn* I and *Eco* RI. The ATG start codon of the HERG cDNA was 24 bp downstream of the final GFP codon. In addition, wild type (WT-HisB), E698X (E698X-HisB) and P872fs877 (P872fs877-HisB) HERG cDNAs were also subcloned downstream of a polyhistidine region (His) in expression vector pcDNA3_HisB (Invitrogen) using restriction sites *Bam* HI and *Eco* RI. The ATG start codon of the HERG cDNA was 84 bp downstream of the last of six histidine

codons. The complete sequence, coding for the in-frame fusion GFP-HERG or HisB-HERG proteins, including restriction sites, was directly sequenced.

Western blot analysis

Both mutations E698X and P872fs877 result in truncated HERG proteins lacking the last C-terminal 282 or 461 amino acids respectively (Figure 3A). Protein expression and/or stability were investigated using Western blot analysis. Human embryonic kidney 293 cells (HEK293) were cultured and transfected as described earlier (20). Equal amounts of WT-HisB, E698X-HisB or P872fs877-HisB constructs were transiently transfected with FUGENE transfection reagent and subsequent isolation of crude membrane fractions and membrane protein preparation were performed as previously described (15,20). For Western blot analysis, the membranes were incubated overnight at 4°C with 6xHis monoclonal antibody (Clontech) at a dilution of 1:5000. Subsequently, membranes were incubated with HRP-conjugated anti-mouse antibody (Amersham Biosciences) for one hour at a dilution of 1:5000. HRP-bound protein was detected with an ECL+Plus detection kit (Amersham Biosciences)

Confocal imaging

HEK293 cells were cultivated on cover slips as described (20). HEK293 cells were transiently transfected with WT-GFP, E698X-GFP or P872fs877-GFP constructs. A red fluorescent endoplasmic reticulum (DsRed-ER) marker was cotransfected to distinguish ER retained from membrane bound proteins as described previously (15,21). Because all family members are heterozygous carriers of the P872fs877 mutation, coexpression studies were performed to investigate potential dominant negative effects on protein trafficking. For this purpose, equal amounts of WT and P872fs877 constructs were transiently transfected, with either one of the constructs tagged with GFP. Confocal images were obtained 48 hours after transfection on a Zeiss CLSM 510, equipped with an argon laser for the visualisation of GFP and DsRed tagged proteins.

Voltage clamp and data analysis

HEK293 cells were cultured at 37°C and transiently transfected with wild type, P872fs877 or E698X constructs (in pcDNA3). A GFP tagged expression vector was cotransfected to discriminate between transfected and untransfected cells. Potassium current recordings were made with an Axopatch-200B amplifier (Axon instruments, Union City, CA) in the whole cell configuration using suction pipettes as described previously (22). Glass patch pipettes were pulled with a P2000 laser puller (Sutter Instruments) from 1.2 mm Starbore borosilicate glass (Radnoti) and had resistances between 1.5 and 3.5 MΩ. All current recordings were performed at room temperature (20-22°C) with an axopatch 200B amplifier (Axon Instruments) and were low pass filtered and sampled at 1 to 10 kHz with a Digidata 1200A data acquisition system (Axon instruments). Command voltages and data storage were controlled with

pCLAMP8 software (Axon instruments). Cells were perfused with HEPES-buffered Tyrode's solution containing 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.35 with NaOH). The internal pipette solution contained 110 mM KCl, 2 mM $MgCl_2$, 5 mM K_2ATP , 5 mM K_4BAPTA and 10 mM HEPES (pH 7.2 with KOH). The access resistance varied between 2.5 and 8 M Ω . Series resistance was compensated to ensure that voltage errors were <5 mV. No leak subtraction was applied and the holding potential was -80 mV. HEK293 cells displayed a small-amplitude of endogenous current during depolarising steps, that was not present during the recordings of the tail current (20). The voltage dependence of channel activation was determined by fitting maximal tail currents to a Boltzmann equation according to $y = 1 / \{1 + \exp[-(E - V_{1/2})/k]\}$, where $V_{1/2}$ represents the voltage at which 50% of the channels are open and k the slope factor. Deactivation kinetics were fitted with a double exponential function using a non-linear least-squares (Gauss-Newton) algorithm. The inward rectification behaviour was quantified by normalizing the steady-state current amplitude at different voltages to the peak tail current amplitude at -40mV. Data are presented as mean $\pm$ SEM and the Student's *t*-test was used for statistical analysis.

Results

Clinical characteristics

A pedigree of family #1718 is presented in Figure 1. Clinical investigation identified seven family members with clinical features of LQTS and/or an aberrant ECG. These family members included 5 females (subjects II3, II4, III3, III14 and III15) and 2 males (III2 and III12). Of all events (syncopes, cardiac collapse) that occurred in these individuals, 4 were triggered by arousal, 3 by emotion or stress, 2 at rest or during sleep and 1 by exercise. An implantable cardioverter defibrillator (ICD) was implanted in subject III15 at age 18 as a result of persistent symptoms under beta-blocker therapy. A pedigree of family #2714 is presented in Figure 2. Of the 22 patients included in the study, 10 individuals presented with variable phenotypes. Aberrant ECGs (dubious T-top, disappeared U-wave, prolonged QT interval) were observed in subjects II1, II2, II5, II7, II8, III1, III2, III3, III5 and III6. Subject II7 (proband) experienced syncopes immediately after giving birth to her first child. Initially, torsades de pointes were registered in the ambulance, which later deteriorated into cardiac arrest. The patient was successfully resuscitated and an ICD was implanted. Subject II2 experienced several syncopes during menstruations or without any recognisable trigger.

Figure 1. Pedigree of long QT family #1718 carrying mutation 2092 G>T (E698X) in the KCNH2 gene. The arrow indicates the proband of the family in whom mutational screening of all LQTS genes was performed. Circles indicate females, squares indicate males. Filled circles or squares indicate heterozygous carriers of the E698X mutation, open circles and squares represent non-gene carriers. QT_c intervals (in msec) are indicated below the symbols.

Figure 2. Pedigree of long QT family #2714 carrying mutation 2616delC (p872fs877) in the KCNH2 gene. The arrow indicates the proband of the family in whom mutational screening of all LQTS genes was performed. Circles indicate females, squares indicate males. Filled circles or squares indicate heterozygous carriers of the E698X mutation, open circles and squares represent non-gene carriers. QT_c-intervals (in msec) are indicated below the symbols. ICD= patient with implantable cardioverter defibrillator.

Genetic analysis

Genetic screening resulted in the identification of a nonsense mutation in exon 8 of the *KCNH2* gene in the proband of family #1718 (Figure 1, subject III2). The mutation 2092 G>T changes codon 698 from glutamic acid into a stop codon (E698X). This stop codon truncates the HERG protein with 461 amino acids and ends the protein between S6 and the cyclic nucleotide-binding domain (cNBD). Of 31 individuals, participating in the study, 19 members were heterozygous carriers of the E698X mutation (Figure 1). A deletion of one nucleotide (2616delC) in codon P872 was detected in exon 9 in the proband of family #2714 (subject II7). This single base pair deletion introduces a frame shift, which leads to a premature stop codon at amino acid position 877 (P872fs877). The mutation is located downstream of the cNBD and causes a truncation of the HERG protein by 282 amino acids. Of 21 members screened, 11 individuals were heterozygous carriers of this mutation (Figure 2).

Western blot analysis

6xHis monoclonal antibody was used to study expression of the truncated proteins (Figure 3). Computational calculations (VNTi) estimate the molecular sizes of WT, E698X and P872fs877 HERG proteins at 131, 81 and 101 kDa, including the His-tag. Figure 3B shows all expected protein sizes, although expression levels were lower for the mutant proteins. Particularly expression of the E698X protein was very low. Only for the WT HERG protein both the core-glycosylated (135 kDa) and the fully-glycosylated (155 kDa) form were detected (20); for the mutant proteins only the core-glycosylated form was visible.

Confocal imaging

To study subcellular transport of mutant channels, HEK293 cell were transiently transfected with GFP tagged WT, P872fs877 or E698X HERG constructs. Confocal images show the presence of WT protein in the ER and plasma membrane (green, Figure 4A), whilst the red fluorescence is purely situated in the ER (Figure 4B). The P872fs877 protein was present within the plasma membrane (Figure 4C, D), although the protein was also partly accumulated in clusters and retained in the ER (green dots). Upon co-expression of equal amounts of WT and P872fs877 constructs (Figure 4 E,F,G and H) partial retainment of heterotetramers was still observed, indicating a dominant negative effect of mutant subunits on WT channels. In line with highly decreased expression levels of the E698X protein with Western blotting, no E698X protein was detected in the plasma membrane (Figure I, J).

Figure 3. Western blot analysis of WT, P872fs877 and E698X HERG proteins. A, graphic representation of HERG proteins and C-terminal truncations caused by mutations 2616delC (P872fs877) and 2092 G>T (E698X). B, Western blot analysis of HisB-tagged HERG proteins. Crude membrane fractions were isolated 24hrs after transfection and 35 μ g of total protein was loaded in each lane. Note the reduced length and reduced expression of both the truncated proteins. Especially, the E698X HERG protein expression was very low.

Current recordings of homomultimeric and heteromultimeric WT and mutant channels

Figures 5 A to D show representative current recordings of HERG WT, P872fs877 and E698X channels. The voltage clamp protocol is presented in Figure 5A. HERG currents were activated by 5 sec depolarising steps between -60 and $+60$ mV (in 10 mV-increments) from a holding potential of -80 mV. Cells were clamped to -40 mV to record tail currents. P872fs877 mutant channels produced currents resembling WT currents, although with reduced amplitudes. E698X mutant channels did not induce currents, even not after transfection with 10 μ g of E698X expressing construct (data not shown). In these experiments, only a small endogenous current was recorded. Peak-amplitude I -V plots (Figure 5E) were determined by plotting the current level at the end of the 5 sec depolarising step versus command voltage. The I -V plots indicated no significant shifts for the P872fs877 channels, but only reduced amplitudes. The voltage dependence of steady-state activation was determined by fitting the maximum tail amplitudes to a Boltzmann function. These plots (Figure 5F) indicated

no significant shifts between WT and P872fs877 channels in the midpoint of activation nor was there a difference in slope factor. The inward rectification behaviour was quantified by normalizing the steady-state current level to the peak tail amplitude at -40 mV (Figure 6). For WT HERG it is clearly shown that the steady state current level at $+20$ mV only amounted $\sim 40\%$ of the peak tail amplitude. However, upon transfection of P872fs877 on a WT stable cell line, the inward rectification was clearly decreased and was even absent in some cases. The latter drastic changes are somewhat obscured in the data presentation (Figure 6) by averaging; as a consequence of transient expression some cells only express a little amount of transfected P872fs877 but still show WT HERG as the transfection is performed on a WT stable cell line.

Figure 4. Subcellular distribution of WT, P872fs877 and E698X channels. Confocal imaging of GFP tagged WT and mutant channels in HEK293 cells. Cells were transfected with GFP tagged constructs in combination with an DsRed tagged, ER-targeted construct. Upper panels, GFP fluorescence images of WT-GFP (A), P872fs877-GFP (C), P872fs877-GFP + WT (E), WT-GFP + P872fs877 (G) and E698X-GFP (I) channels. Lower panels, super-imposed images of double stained (green GFP and red DsRed) channels. Note the accumulation of partly retained P872fs877 homotetrameric and WT/P872fs877 heterotetrameric HERG channels.

Figure 5. Voltage clamp recordings of HERG WT, P872fs877 and E698X channels. A, Voltage-clamp protocol and representative current recordings from transiently transfected WT (B, $n = 7$), P872fs877 (C, $n = 8$) and E698X cells (D, $n = 3$). E, peak amplitude I - V plots for HERG WT, P872fs877 and E698X channels. I - V plots were determined by plotting the current level at the end of the 5 sec depolarising step versus the command voltage. Current amplitudes were reduced in P872fs877 currents. No current was recorded from E698X channels. F, voltage dependence of steady-state activation. Relative tail currents were derived from the currents as shown in C and D. Tail currents were fitted with a Boltzmann function to determine midpoint of activation ($V_{1/2}$) and slope factor (k). HERG WT ($n = 7$): $V_{1/2} = -3.01 \pm 1.5$, $k = 8.09 \pm 0.34$, HERG P872fs877 ($n = 8$): $V_{1/2} = -0.03 \pm 2.22$, $k = 8.13 \pm 0.17$. The mutant P872fs877 protein did not cause any significant differences in either the voltage dependence or the slope factor.

Figure 6. Inward rectification of HERG WT and WT+P872fs877. The inward rectification was determined by normalizing the steady-state current amplitude at the end of the voltage step as shown in figure 5A by the peak tail amplitude at -40 mV. The inward rectification of WT channels was maximal at -60 mV as the steady-state current level amounted only ~20% of the peak tail amplitude. This behaviour was clearly changed upon transfection of P872fs877 on the WT stable cell line. All values above 0 mV were significantly different (n=5).

Discussion

In this study, we investigated the genetic origin of LQTS in two large families. Genetic screening revealed two previously unknown mutations in the C-terminus of the *KCNH2* gene, which both co-segregated with prolonged QT-intervals in gene carriers. In one family, a single base pair mutation 2092 G>T in exon 8 of the *KCNH2* gene was identified that leads to a premature stop codon at amino acid 698 (E698X). This mutated gene encodes a HERG protein lacking the last 461 amino acids. In a second family, a single base pair deletion in exon 9 of *KCNH2* was identified, resulting in a frameshift and a premature stop codon at amino acid 877 (P872fs877). This stop codon truncates the HERG protein by 282 amino acids.

Mutations in LQTS genes that lead to truncated proteins have been reported previously in the literature. Especially in the *KCNH2* gene many frame shifts and nonsense mutations have been reported, but very few of them have been functionally characterised. An explanation may be that many of these mutations lead to a deletion

of important regions of the HERG protein, such as the pore region or parts of the transmembrane domains, and may therefore appear of less interest for further studies as these proteins are expected to be non-functional. Indeed, some are non-functional with no influence on wild type channel function (23). Yet, at least one other nonsense mutation has been reported to be non-functional with a negative effect on wild type function (24). Moreover, it is also possible that truncated proteins may be functional, albeit with altered gating properties. Therefore, in this study, expression and functional behaviour of C-terminal truncated proteins were investigated.

Molecular techniques were applied to study protein expression and intracellular transport. Despite the deletion of a significant part of the C-terminus in both mutated HERG proteins, the truncated proteins were both detected. The expression of the E698X protein was however hardly visible, indicating that this truncation leads to either inefficient translation or early mRNA/protein degradation. It has been demonstrated for some diseases, such as cystic fibrosis and nephrogenic diabetes insipidus that some mutations lead to misfolding of proteins and retention in the endoplasmic reticulum. This trafficking deficiency was later also observed for some mutated HERG proteins (12,17). We hypothesised that the HERG proteins with a truncated C-terminus may also show aberrant trafficking to the cell membrane and used confocal imaging to study subcellular trafficking of GFP-tagged WT and mutant proteins. In line with Western blotting results, no membrane bound proteins were detected for E698X channels. P872fs877 channels were only partly transported to the cell membrane, whilst part of the proteins were clustered together and retained in the ER. This partially retainment of mutant proteins may be the result of several mechanisms. Upon post- or cotranslational insertion of newly synthesized proteins in ER membranes, proteins bind with ER chaperone molecules whose role it is to facilitate protein maturation and folding (25). Incorrectly folded and/or assembled proteins are retained in or degraded from the ER into secretory pathways. One explanation for this retainment is that the proteins are immobilized through extensive interactions with chaperones, and are unable to move to ER exit sites (25). The observed reduction in current amplitudes of homotetrameric P872fs877 channels may therefore be caused by the partial retainment of proteins in the ER. Upon co-expression of WT and mutant P872fs877 channels, transport still showed partial clustering of proteins in the ER, indicating that heterotetramers are formed and that the presence of mutant subunits in a tetramer has a dominant negative influence on the trafficking of channels.

To study functional properties of mutant channels, we performed whole cell patch clamp experiments. Some electrophysiological structure-function studies of HERG channels have been performed to investigate effects on channel function. Aydar *et al.* (26) performed sequential deletion analysis of the HERG C-terminus to delineate the minimum sequence necessary for channel function. They concluded that of the total

length of the HERG protein (1159 amino-acids), at least 881 amino acids are necessary for channel function, since a deletion of 278 amino acids still produced functional channels and a deletion of 311 amino acids resulted in non-functional channels. They further concluded that truncations generally lead to reduced current amplitudes, although not in a linear relation to the protein length. In addition, truncations greater than 236 amino acids resulted in accelerated deactivation rates. The P872fs877 mutation detected in our study has a C-terminal deletion of 282 amino acids. This truncated protein, however, did produce functional channels similar to WT channels, and therefore the minimum protein length needed for functional expression can be somewhat smaller than the 881 (Δ 278) amino acids. In accordance with this study, the homotetrameric P872fs877 channels produced currents with reduced densities as compared to wild type channels, but an acceleration of deactivation was not detected. This reduced current amplitude may, as indicated above, be the result of partial retainment of proteins in the ER. Kupersmidt *et al.* (27) functionally characterised a protein with a C-terminal deletion of 462 amino acids, which is only one amino acid less than the E698X protein. This mutated channel resulted in a non-functional channel in line with our results from E698X channels.

Co-expression studies of WT with P872fs877 channels produced some unexpected results because the typical inward rectification was absent in several of the analysed cells. This strongly indicates the formation of heterotetramers. At present, the origin of this alteration is not completely elucidated but may eventually be caused by changes in inactivation kinetics because the activation kinetics are not changed in the voltage range 10 to 60 mV (data not shown).

In conclusion, the explanation behind LQTS diagnosis in these families is the result of mutated HERG channels causing (1) partial ER retention of mutant and or heterotetrameric subunits and (2) changes in the inward rectification behaviour of heterotetrameric channels.

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CHAPTER 5

Analysis of the Human *KCNH2* (*HERG*) gene: Identification and characterization of a novel mutation Y667X associated with Long QT Syndrome and a non-pathological 9 bp insertion

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Abstract

Long QT (LQT) syndrome is a potentially life-threatening disorder, characterised by a distinct cardiac arrhythmia known as *torsades de pointes*. Mutations within a number of genes linked to the familial form, including that coding for a cardiac potassium channel called KCNH2 (HERG), have been described based on the characterized genomic organisation. A standardized method was developed to screen the entire gene for gene variants. We report a single base pair substitution, introducing a premature STOP codon at codon 667 of the gene in a healthy individual with an extended QTc interval (460 msec). *In vitro* expression of the codon Y667X variant in *Xenopus* oocyte suggests that the autosomal dominant variant does not function in a dominant/negative manner and cannot co-assemble to form a channel, resulting in a reduction of the KCNH2 current, and an extension of the QT interval. This indicates that pathogenic LQT gene variants exist in the apparently normal population, the prognosis and clinical consequences of which remain to be determined. The assays described should facilitate future studies into this area.

Introduction

Long QT syndrome (LQT) is a cardiac disorder that typically affects young, previously healthy individuals. The frequency of LQT is unknown but may be as common as 1 in 5,000 and may be responsible for 3,000-4,000 deaths per year in the United States alone (1,2). Using affected families the inherited form of the disease has been linked to 5 chromosomal regions. Five genes responsible for the syndrome have been identified within four of these chromosomal regions, and mutations within these genes have been shown to associate preferentially within affected families (3,4). Here we describe the identification and electrophysiological characterization of a novel mutation Y667X in exon 8 of the KCNH2 gene (LQT2; MIM#152427), detected by single stranded conformation polymorphism analysis as well as a non-pathological 9 base-pair insertion in exon 4 of this gene.

Materials and Methods

Subjects

A volunteer in a Phase I clinical trial was brought to our attention, presenting with a prolonged QT_c interval of 460 msec. All available family members of the proband were recruited for inclusion in our study. Eight volunteer subjects with a normal QT_c interval (<440 msec) and no history of cardiac arrhythmia during trials were recruited as control subjects for the study.

PCR-SSCP analysis

DNA was extracted from peripheral leucocytes of all subjects. PCR primers^{\$} were designed based on the sequence obtained from a BAC clone (EMBL, accession numbers AJ010538 through AJ010551) and published data (5). To maximize detection efficiency, SSCP analysis was performed under two conditions: a 15% Excelgel run at 4°C; a 12% Excelgel run at 15°C (gels from Pharmacia). Gels were run for 1.5 to 2.5 hours at 600 mV. Those gene fragments showing variant SSCP patterns were reamplified, purified (Qiagen PCR purification kit), sequenced directly on both sense and antisense strands using the ABI Big Dye Terminator cycle sequencing kit, and analyzed on an ABI 377. The obtained gene sequence was compared with the published sequence of Itoh *et al.* (5).

Site-directed mutagenesis

The full-length wild type KCNH2 cDNA was sub-cloned into the oocyte expression vector SP64T.GL. PCR amplification with primers 5'-GCAACATGGAGCAGCCACACATG-3' and 5'-CCCCTCGAGTTACAGCCGCTGGATGATGGCC-3' (Restriction site for Xho I underlined, point mutation in bold) was performed using the full length KCNH2 construct as a template to create the STOP codon in the wild type sequence. The amplified PCR product and KCNH2 FL/pSP64T.GL were digested with *Bgl* II and *Xho* I, gel purified, and ligated to create a final KCNH2-STOP/pSP64T.GL construct. Introduction of the stop codon and the absence of any PCR induced base pair changes were verified by full sequence analysis. Messenger RNA was synthesized from the wild type and mutant cDNA SP64T.GL constructs using the Ambion mMessage mMachine SP6 in vitro Transcription kit, according to the manufacturers instructions.

Electrophysiological characterization of the codon 667 variant

Defolliculated *Xenopus laevis* oocytes were injected with 46 nl of mRNA, containing either KCNH2 wt mRNA, mutant mRNA or combinations of both. Recordings were made at 19-22 °C with a two-electrode voltage clamp amplifier (Turbo TEC 01C or 10CD, Npi electronics). Microelectrodes were filled with 3 M KCL and the holding

^{\$} Primer sequences and amplification conditions can be obtained from the authors.

potential was -80 mV. Stimulation and data acquisition were controlled with the Pulse software (HEKA elektronik), which was connected to the amplifier via an ITC-16 interface (Instrutech Inc.). Data analysis was performed by means of the programs Pulsfit (HEKA) and Igor Pro (Wavemetrics Inc.).

Results

Analysis of variant sequences

The subject with the QTc of 460 msec showed a variant band pattern on SSCP analysis of exon 8. Sequence analysis revealed that this subject was heterozygous for a base substitution of C to A at position 2001, altering codon 667 from TAC, encoding tyrosine, to a STOP codon (TAA). A specific PCR-RFLP detection assay was developed, based on the loss of a recognition site for restriction enzyme Ava I associated with the mutation; Screening of available family members indicted that this mutation had been inherited from the maternal line. The probands mother was also a heterozygous carrier of the mutation with a prolonged QTc (479 msec). A second variant band pattern was observed on SSCP analysis of exon 4 of a control subject. Direct sequencing of the double stranded PCR product indicated that the subject was heterozygous for a 9 base pair insertion, GCGCGGGCG, beginning at position 568 of the cDNA sequence. This insertion leads to an in-frame insertion of three amino-acids, Gly Ala Gly, between codons 189 and 190 of the published gene sequence. This subject had a QTc of 367 msec and no medical history associated of cardiac arrhythmias or associated problems.

Electrophysiological characterization of the codon 667 KCNH2 variant

The first test was to investigate the influence of the Y667X mutation on channel function (Figure 1). Oocytes injected with wt mRNA expressed a normal KCNH2 current (Figure 1A), while oocytes injected with up to 400 ng/ μ l of the KCNH2 mutant did not express functional channels at all, even up to 6 days after injection of the mRNA (Figure 1B). The generated currents with the mutant mRNA were identical to those in the non-injected oocytes (Figure 1C). The hypothesis that KCNH2 mutant subunits are translated equally well as wt subunits, assemble equally well with the wt subunits and that the presence of at least one mutant subunit in a hetero-tetrameric channel would make the channel non-functional, was rejected upon co-injection of 100 ng/ μ l of wt with 300 ng/ μ l of mutant KCNH2. The current after co-injection was much higher than the expected 1/64 part of the current after injection of only 100 ng/ μ l of KCNH2 wt.

Figure 1. Electrophysiological characterization of the codon 667 *KCNH2* gene variant. **A.** Oocyte injected with 200 ng/ μ l of *KCNH2* wt. From a -80 mV holding potential, a 100-msec pulse to -60 mV was given to measure the leak current. After a 100-msec interval at -80 mV, 500-msec depolarizing test pulses from -60 mV to $+60$ mV were given, followed by a 600-msec period at -60 mV, which elicited the *KCNH2* tail current. **B.** Oocyte injected with 400 ng/ μ l of the mutant. The mutant does not produce functional *KCNH2* channels. **C.** Non-injected oocyte.

In order to explain why the heterozygous patients display a prolonged QT-interval, the *KCNH2* “normalized” tail current amplitude after injection of oocytes with 400 ng/ μ l wt was compared to that of oocytes co-injected with 200 ng/ μ l wt + 200 ng/ μ l mutant. Because the current amplitude increased over time, the tail current amplitude was divided by the average tail current of the 400 ng/ μ l *KCNH2* wt-injected oocytes measured at the same experimental day. The normalized tail current amplitude was significantly smaller in the group of oocytes co-injected with 200 ng/ μ l wt + 200 ng/ μ l mutant than in the group of oocytes injected with 400 ng/ μ l *KCNH2* wt (Table 1). In addition, there were no significant differences in normalized tail current amplitudes, half-activation potentials, rate of activation and deactivation and in inactivation between the oocytes co-injected with 200 ng/ μ l wt + 200 ng/ μ l mutant and the oocyte group injected with 200 ng/ μ l wt alone (Table 1). All these electrophysiological experiments together strongly suggest that co-injection of this *KCNH2* mutant does not influence the properties of the *KCNH2* current induced by the *KCNH2* wt subunits.

Table 1. Influence of co-injection of equal amounts of wild type and mutant mRNA. The batches of oocytes 1 and 2 (first column) are independent experiments with oocytes obtained from different animals. The second column shows the type of injection. The third column gives the amplitude of the tail current in μA , measured at a potential of -60 mV . The fourth column represents the normalised tail current, obtained by division of the tail current amplitude of each oocyte by the mean tail current of the WT $400\text{ ng}/\mu\text{l}$ injected oocytes on the same day, to take into account the increase in expression with time. The fifth column represents the number of oocytes in each group.

	Injection	Amplitude of tail current (μA)	Normalized amplitude of tail current	N
Oocyte batch 1	WT $400\text{ ng}/\mu\text{l}$	2.772 ± 0.468	1.000 ± 0.095	27
	WT $200\text{ ng}/\mu\text{l}$ + Mut $200\text{ ng}/\mu\text{l}$	1.723 ± 0.318	$0.609 \pm 0.089^{**}$	27
Oocyte batch 2	WT $400\text{ ng}/\mu\text{l}$	3.466 ± 0.435	1.000 ± 0.117	20
	WT $200\text{ ng}/\mu\text{l}$ + Mut $200\text{ ng}/\mu\text{l}$	2.452 ± 0.364	$0.575 \pm 0.075^{**}$	28
	WT $200\text{ ng}/\mu\text{l}$	2.382 ± 0.388	$0.535 \pm 0.080^{**}$	24

Results are expressed as mean $\pm$ S.E.M. $^{**} P < 0.01$ (two-sided Student's t-test for independent samples) for the comparison with the group of oocytes injected with $400\text{ ng}/\mu\text{l}$ of the same batch of oocytes.

Discussion

Previous work has linked the *KCNH2* gene, which encodes the channel responsible for the rapidly activating delayed rectifier K^+ phase of the cardiac cycle, to inherited LQT (6,7). In this study we have identified two novel variations in the *KCNH2* gene; One variant, a 9 bp insertion between codons 189 and 190 in the amino terminal portion of the gene, produces an in frame insertion of 3 amino acids, and appears to be non-pathological in effect on QTc interval.

Conversely, however, the single base substitution (C>A) at position 2001 in exon 8 of the gene, was associated with a prolongation of the QTc interval in the proband (460 msec) as well as the mother of the proband (479 msec), both subjects being heterozygous for the mutation. The fact that the substitution introduces a STOP codon in the gene at position 667, terminating translation of the protein 5' to the coding region for the nucleotide-binding domain and truncating the protein by 492 amino acids from the carboxy-terminal end of the protein provides further evidence for a causal association.

In order to fully characterize the effects of this mutation on channel function, electrophysiology experiments were performed, comparing both wild type and mutant forms of the channel. Knowing that *KCNH2* belongs to a superfamily of K^+ channels which are believed to assemble as tetramers (8), all data from the electrophysiological experiments obtained, can be explained by assuming that the *KCNH2* mutant subunit does not assemble, neither with the mutant congeners nor with the *KCNH2* wt subunits. This explains 1) that injection of the mutant alone does

not form functional channels (Figure 1), 2) that this mutant has no influence on the amplitude and properties of the KCNH2 current after coinjection with wt and 3) that the normalized current with the "heterozygous" combination (200 ng/ μ l wt + 200 ng/ μ l mutant) was smaller than that in the 400 ng/ μ l KCNH2 wt group. The longer QT-interval in the patients heterozygous for this mutant can therefore probably be explained by the reduced amount of KCNH2 channels incorporated in the heart cell membrane, since a smaller amount of KCNH2 wt subunits is produced and the mutant subunits do not contribute to channel formation and to the KCNH2 current. Ludwig *et al.* (9) proposed that the C-terminal portion of the rat *KCNH2* gene is important for coassembly. Our results suggest that this may also be the case in humans. It is apparent that the STOP (Y667X) mutation is acting in an autosomal dominant manner.

The subjects in this study were in excellent health and were identified only as a result of routine screening for entry into phase I clinical trials. One may thus question whether the prognosis and clinical implications for these individuals will be different to those having a family history or having presented with specific symptoms associated with an prolonged QTc. Presumably there exist within the population other asymptomatic individuals with similar variants which have not yet been described. Since we suspect that mutations within the same genes responsible for the familial form of the disease could also determine susceptibility to the acquired form of the disease, we suspect that these individuals may be at increased risk of drug induced QT prolongation. In this case diagnostic testing via genotyping may be useful to identify susceptible individuals. Until the majority of causative mutations are discovered and functionally characterised, molecular diagnosis still requires screening of the entire *KCNH2* gene for mutations.

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CHAPTER 6

Two linked variations in transcriptional regulatory elements of the *CYP3A5* gene constitute the major genetic determinant of polymorphic activity in humans

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Abstract

Cytochrome P450 3A subfamily members (CYP3A) are the most abundant liver cytochrome P450 forms, responsible for the biotransformation of over 50% of all drugs. The expression and activity of isoforms CYP3A4 and CYP3A5 show wide inter-individual variation, influencing both drug response and disease susceptibility. The molecular basis for this variation has never been defined. In this study, we use probe drug midazolam to determine the CYP3A5 phenotype in a panel of liver samples. A clear bimodality in metabolism was observed. Analysis of the 5'-flanking region of the CYP3A5 gene identified two linked polymorphisms, T⁻³⁶⁹G and A⁻⁴⁵G, located in transcriptional regulatory elements, which are associated with increased expression and activity of the gene. A polymerase chain reaction based detection assay is described facilitating future studies into both the metabolic consequences of this variation and disease association studies relating to CYP3A5.

Introduction

The CYP3A enzymes represent one of the most important subfamilies of the cytochrome P450 (CYP) superfamily. The CYP3A subfamily comprises the most abundantly expressed cytochrome P450 enzymes in human liver, and is responsible for the metabolism of over 50% of all clinically used drugs, including the dihydropyridines, cyclosporine, erythromycin and barbiturates (1). Wide inter-individual variation in the metabolism of CYP3A substrates has been noted and is a factor in determining individual drug efficacy. Evidence also exists for the metabolism of an array of lipophilic environmental pollutants, including the activation of pro-carcinogens such as aflatoxin B1 by members of this subfamily (2). Consequently, inter-individual variation in activity may also represent a host susceptibility factor to a number of diseases with an environmental component.

To date, four CYP3A cDNAs have been identified in humans: CYP3A3, CYP3A4, CYP3A5 and CYP3A7. It is believed that CYP3A3 represents an allelic variant of CYP3A4, whilst CYP3A7 is the major foetal hepatic cytochrome and CYP3A4 is the predominant cytochrome P450 found in human adult liver (3). Initial experiments suggested that a polymorphism exists in CYP3A4 (4). However other studies, whilst confirming a wide range of inter-individual variation in CYP3A4 expression have failed to confirm the original bimodality (5,6). Overlapping substrate specificities between CYP3A4 and CYP3A5 have previously made it difficult to separate metabolism by these isoforms; consequently little phenotyping data have been produced to study variation in CYP3A5 activity in humans. However, there is evidence for the polymorphic expression of CYP3A5. Both immunoblotting and Northern blot analysis have detected CYP3A5 expression in only 10-30% of human livers (7-9). More recently, analysis of 30 human

liver samples using immunoblotting found that only 3% showed no detectable CYP3A5, whilst a large number had trace amounts, suggesting that a polymorphism in this enzyme may be regulatory as opposed to structural (10).

Comparisons of the 5' flanking regions from the *CYP3A4*, *3A5* and *3A7* genes have identified putative binding sites for several transcriptional regulatory factors common to all isoforms (11-13). However, the molecular basis, if any, for the inter-individual variation in expression of the CYP3A sub-family members has so far remained unclear. Indeed, it has been suggested that the host cellular environment may be a greater determinant of inducibility than gene structure (14). This would render prediction of CYP3A activity virtually impossible since it is difficult to identify (all) relevant inducers in the environment, and to quantify their levels.

The delineation of CYP3A4 and CYP3A5 metabolism has been shown to be possible using the sedative midazolam as a probe drug (15,16). In this case, two metabolites are formed: 1-hydroxy midazolam (1-OHM) and 4-hydroxy midazolam (4-OHM). Samples containing a higher proportion of CYP3A5 compared to CYP3A4 have their metabolism driven towards the 1-OHM route and therefore show a higher ratio of 1-OHM/4-OHM than those containing only CYP3A4. Using this information, we have measured CYP3A5-mediated metabolism in microsomes prepared from a panel of human livers and compared the 5' flanking sequences of the corresponding *CYP3A5* gene. Here we report the identification of two linked polymorphisms, located in putative transcriptional regulatory regions, which appear to cause increased *CYP3A5* gene transcription leading to increased metabolic activity. The description of PCR-based assays for their detection will allow prediction of inter-individual variability in response to drugs metabolised by this isoform, as well as facilitating disease association studies.

Materials and Methods

Liver microsome preparation

Human liver samples were obtained from kidney transplant donors, and flash-frozen immediately on removal. Ethical committee approval for the study was obtained. Human liver microsomes were prepared according to previously described protocols (17), and protein content was determined by the method of Lowry as modified by Miller (18).

Midazolam hydroxylase assay

The rates of midazolam overall metabolism and of the formation of 1- and 4-OH-midazolam were determined as follows. Each incubation vessel contained an aliquot of the microsomal suspension (containing 1 mg of microsomal protein) in 1.15% KCl-0.01 M phosphate buffer pH 7.4; 10 μ l of a stock solution of 6 mM midazolam dissolved in dimethyl sulphoxide (DMSO) to reach a final midazolam concentration of 60 μ M; 500 μ l of a cofactor mixture containing 0.5 mg of glucose-6-phosphate, 0.5 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 U of glucose-6-phosphate dehydrogenase dissolved in 0.5 M Na-K-phosphate buffer, pH 7.4 and a 1.15% KCl-0.01 M phosphate buffer pH 7.4 to bring the incubation volume to 0.9 ml. After a pre-incubation for 5 min at 37 °C, the incubations were started by adding 100 μ l of a solution of 1.25 mg/ml NADP to reach a final concentration of 0.125 mg/ml. Tubes were continuously shaken at 100 oscillations/min in a Heto shaking waterbath (Heto Laboratory Equipment, Allerød, Denmark). Blank incubates with boiled microsomes were incubated under identical conditions as the control incubates. The incubations were stopped after 30 min by immersing the tubes in dry ice. Samples were stored at -18 °C until analysis. The incubation samples were analysed for unchanged midazolam and for its metabolites 1'- and 4-hydroxymidazolam by high-performance liquid chromatography (HPLC) with ultraviolet (UV)-detection.

HPLC determination of midazolam metabolites

The 1-ml samples of midazolam were thawed and diluted with 1 ml DMSO. Samples were sonicated for 10 min, centrifuged and an aliquot of the supernatant was injected directly onto the HPLC-column. The HPLC apparatus consisted of a Waters 600 MS pump. The samples were injected automatically, using a WISP 717 (Waters, Milford, USA) plus automatic injector. Stainless steel columns (30 cm (4.6 mm inner diameter) were packed with Kromasill 18 (5 μ m, Akzo Nobel, Eka Chemicals, Bohus, Sweden) bound phase by a balanced density slurry procedure (Haskel DSTV 122-C pump, 10 7 Pa, Haskel, Burbank, USA). UV-detection at 230 nm was performed using a Waters 996 Diode Array Detector (Waters, Milford, USA). Elution at 1-ml/min started with a short gradient from 100% 0.1 M ammonium acetate, pH 7.0 (solvent system A) to 50% of solvent system A and 50% of solvent system B containing 1 M ammonium acetate pH

7.0, methanol and acetonitrile (10/45/45), over a 1-min period, followed by a second gradient to 100% solvent system B in 15 min. This solvent composition was held for 2 min before equilibration with the starting conditions. The identity of the metabolites of midazolam was confirmed using mass spectroscopy. The conversion of UV-peak areas into ng was performed by a Millenium 2020 CDS system (Waters, Milford, USA) on a calibration curve of midazolam. This calibration curve was obtained after injection of known amounts of the drug (0, 1059, 2117, 3176 and 5028 ng) and linear (weighted by $1/x$) regression analysis of the corresponding UV-peak areas. The equation of the calibration curve was $ng = 0.000333 \text{ (area (} r^2 = 0.9997, n = 5 \text{))}$. The metabolic activity was expressed as pmol metabolite formed/min mg protein, and a metabolic ratio was determined for each sample according to the ratio of 1OHM/4OHM in each sample.

Genomic DNA preparation

DNA was isolated from frozen liver samples using a QIAmp Tissue Kit (QIAGEN, Hilden, Germany).

RNA preparation

RNA was isolated from the liver samples using a QIAGEN RNAeasy Midi Kit (QIAGEN). Twenty μg of RNA was treated with RNAase-free DNAase I (Boehringer Mannheim, Brussels, Belgium), for 30 min at 37 °C in 20mM Tris-HCl, pH 8.0, 100 mM MgCl₂. Samples were phenol/chloroform extracted, ethanol precipitated and resuspended in 30 μl of TE buffer. Two and a half μg of the treated sample was reverse-transcribed for 50 min at 42 °C in 1x First Strand Buffer (Life Technologies, Merelbeke, Belgium), 0.01 M DTT and 0.5 M dNTPs using 0.5 μg of Oligo(dT) random primers and 200 U SuperScript II Reverse Transcriptase (Gibco BRL, Life Technologies, Merelbeke, Belgium) for use on the ABI Prism 7700 Sequence Detection System (Foster City, CA, USA).

Sequencing of the CYP3A5 5' flanking region

A 1343 bp 5'-flanking region of CYP3A5 was PCR-amplified from genomic DNA isolated from liver samples, using primers 3A51 (5'-GGAAGCAACCTACATGTCCATC-3') and 3A52 (5'-ATCGCCACTTGCCTTCTTC-3') based on the published sequence of Jounaidi *et al.* (13). PCR conditions were: one cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 57°C for 30 s, 72°C for 2.5 min, and one final cycle of 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). Sequencing primers were designed (Table 1), and used to sequence directly the PCR product on both sense and antisense strands by cycle sequencing using the ABI BigDye Terminator cycle sequencing kit (Perkin Elmer, Nieuwerkerk, The Netherlands). Sequencing reactions were analysed on an ABI 377 automated sequencer (Foster City, CA, USA). Contig sequences were aligned and compared using the Sequence Editor version 1.0.3 software packages (Perkin Elmer) and manually edited for identification of heterozygous positions.

Table 1. Primers used for sequencing the 5'-flanking region of CYP3A5 from the polymerase chain reaction product 3A51/3A52 (see text).

Primer	Orientation ^a	Position ^a	Sequence (5' to 3')
3A51	F	-1238 to -1217	GGAAGCAACCTACATGTCCATC
3A5p01	F	-978 to -963	AGTACAGGGAGCACAG
3A5p08	R	-917 to -932	CACCTATTCACTCTG
3A5p02	F	-698 to -684	TGCTATCACCACAGAC
3A5p07	R	-689 to -704	GGTGATAGCAATAGAC
3A5p03	F	-364 to -349	AGGATGTGTAGGAGTC
3A5p06	R	-417 to -434	CCTCACACAGATGTAACC
3A5p04	F	-176 to -161	TAAGAACTCAGGTTCC
3A5p05	R	-178 to -194	CAGAACTGAAGTGGAG
3A52	R	+105 to +87	ATCGCCACTTGCCTCTTCT

^aF = 5' to 3', R = 3' to 5'. Primer locations are based on CYP3A5 sequence data of Jounaidi *et al.* (13).

PCR detection assays for the A⁻⁴⁵G and T⁻³⁶⁹G mutations

All PCR assays were performed utilizing a 1 in 100 dilution of the original 3A51/3A52 PCR product as template, under the following conditions: one cycle of 95°C for 1 min, 30 cycles of 95°C for 1 min, 55°C for 30 sec, 72°C for 1 min, and 1 final cycle of 72°C for 10 min. All products were sequenced to confirm the identity of the product as CYP3A5. Oligonucleotide mismatched primers utilized in the assays were: 3A5F1 (5'-GGGTCTGTCTGGCTGCGC-3'), 3A5F2 (5'-GGGGTCTGTCTGGCTGAGC-3'), and 3A5R1 (5'-TTTATGTCTGGAGAAGGACG-3'), where positions of mismatch(es) are underlined. For the A⁻⁴⁵G mutation, a PCR was performed using primer pair 3A5F2 and 3A5R1. Twenty µl of PCR product was digested for a minimum of 3 h at 65°C using 15 U of *Tai* I, and the restriction fragments visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose gel. For the T⁻³⁶⁹G mutation a PCR was performed using primer pair 3A5F2 and 3A5R1 as described above. Twenty µl of PCR product was digested with 15 U of *Alu* I for a minimum of 3 h, and restriction fragments were separated by electrophoresis on a 12.5% ExcelGel on a Pharmacia Multiphor Electrophoresis system (Pharmacia, Amersham Pharmacia, Uppsala, Sweden). Fragments were visualized by silver staining in a Hoeffer Automatic Gel Stainer (Pharmacia). To detect the presence of mutations on the same chromosome, the PCR was performed using primers 3A5F1 and 3A5R1. Twenty µl of PCR product was digested for a minimum of 3 h at 65°C using 15 U of *Mvn* I, and the resulting restriction fragments were visualized by ethidium bromide staining after electrophoresis on a 1.5% agarose gel.

Relative quantification and comparison of CYP3A5 RNA

Relative levels of CYP3A5 mRNA were determined by real time quantitative PCR using the ABI 7700 Sequence Detection System (Perkin Elmer). Optimal primers and probes for the detection of CYP3A5 were designed using the PrimerExpress program, and subsequently checked to ensure specificity for CYP3A5. Primers utilized for the

quantification PCR were: forward 5'-AAGTGGCGATGGACCTCATC-3'; reverse 5'-GAGGAGCACCAGGCT GACA-3'. The TaqMan probe was labelled with the 5' reporter dye 6-carboxy-fluorescein (FAM), and had the sequence 5'-CAAATTTGGCGG TGGAAACCTGGC-3'. Optimal primer/probe ratios and concentrations were determined and the experiments run according to standard protocols for the ABI 7700 Sequence Detection System. CYP3A5 mRNA expression for all samples was normalized against the expression of β -actin mRNA. The threshold cycle (C_t) is the PCR cycle number where the ABI 7700 begins to detect an increase in fluorescent signal associated with the linear amplification of PCR product. The C_t value is dependent on the initial amount of template copy. Quantities of CYP3A5 in each sample were determined by averaging the C_t from three separate PCR reactions of each sample. Relative differences in C_t between samples were calculated by subtracting the C_t of each sample from the highest C_t within the samples (lowest expression). Since the amount of PCR product doubles with every cycle in the linear range of a PCR, the differences in C_t were converted into estimated differences of mRNA quantity between the samples by calculating $2^{\delta C_t}$, where δC_t is the difference in cycle threshold between two samples. Negative controls were performed on each run to ensure that no signals were due to DNA contamination. Control samples consisted of RNA samples, which had been treated in exactly the same manner as for the quantitative PCR, but without the addition of the reverse transcriptase.

Statistical analysis

Statistical analysis was performed on the JMP Statistical program version 3.2.2 (SAS Institute Inc., Cary, NC, USA). Metabolic ratio and CYP3A5 mRNA expression data ($2^{\delta C_t}$) were checked to ensure that they conformed to a normal distribution. CYP3A5 mRNA expression data did not conform to a normal distribution and were log-transformed, after which the data were normally distributed. Metabolic ratios and expression levels were compared between groups using a *t*-test.

Western blot analysis

Forty μ g of microsomal protein prepared from each liver were solubilized in an equal volume of Laemmli sample buffer (BioRad, Eke, Belgium) by four cycles of freezing and boiling for 10 min. Samples were loaded onto pre-cast 10% SDS-PAGE Ready Gels (BioRad) and electrophoresed for 1 hour at 180 V. Separated proteins were transferred onto Hybond-P membranes (Amersham, Amersham Pharmacia, Uppsala, Sweden) using a Trans-blot SD apparatus (BioRad). Membranes were blocked by an overnight incubation at 4°C in 1x PBS containing 5% (w:v) nonfat milk and 0.1% (v:v) Tween 20. Membranes were incubated at ambient temperature for 1 hour in a 1:3000 dilution of specific human CYP3A5 antibody (Gentest Co, MA, USA) in 1x (PBS, 2.5% (w:v) nonfat milk, then rinsed four times in 1x PBS, 2.5% (w:v) nonfat milk, 0.1% (v:v) Tween. Membranes were incubated at ambient temperature for 1 h in a 1:5000 dilution of Anti-Rabbit IgG peroxidase conjugate (Sigma, Bornem, Belgium) in 1x PBS, 2.5% (w:v)

nonfat milk, and rinsed as previously. The membranes were developed using the ECL Plus Western Blotting Detection System (Amersham) according to manufacturer's instructions, and visualized by autoradiography using Kodak X-Omat film (Sigma, Bornem, Belgium).

Results

Midazolam phenotyping

A panel of 39 human liver samples was phenotyped for CYP3A5 activity, using the metabolism of midazolam to its 1-OH metabolite as a marker of activity. Human liver microsomal samples containing CYP3A5 in addition to CYP3A4 exhibit a significantly greater ratio of 1-OHM to 4-OHM compared to samples containing only CYP3A4. For microsomes containing both CYP3A4 and CYP3A5, 1-OHM/4-OHM ratios between 5 and 9 were observed. Samples containing only CYP3A4 showed 1-OHM/4-OHM ratios < 4 (Gorski *et al.*, 1994). Analysis of the CYP3A5 phenotypes in our data set showed a clear bimodal distribution, with six samples (15%) having metabolic ratios greater than 5, and the remaining samples having metabolic ratios between 1.5 and 3.5 (Fig. 1a). Of the 39 liver samples from which microsomes were prepared for metabolic analysis, sufficient tissue was available for full DNA, RNA and protein analysis for 26, which included six samples lying in the higher metabolic ratio range. In addition to these 26 samples, microsomes for protein analysis were available for a further three samples, all of which had metabolic ratios of < 4.

A)

B)

Figure 1. (a) Relationship between midazolam metabolic ratio and genotype for the linked A⁻⁴⁵G and T⁻³⁶⁹G mutations in the 5' flanking region of the *CYP3A5* gene. Midazolam metabolic ratio = 1-OHM/4-OHM. (b) Relationship between *CYP3A5* mRNA expression and the linked A⁻⁴⁵G and T⁻³⁶⁹G linked mutations in the 5' flanking region of *CYP3A5*. Relative Ct difference = difference in threshold cycle between samples, as described in the Materials and methods section. wt = samples with the wild type sequence in the 5' flanking region as previously published (13) Het = samples heterozygous for the linked polymorphisms, A⁻⁴⁵G and T⁻³⁶⁹G.

Analysis of CYP3A5 gene 5' flanking region

The 5' flanking region of *CYP3A5* was PCR-amplified from genomic DNA of all 26 samples and sequenced in full, extending from -1238 to +105 (counting from the presumed transcription start site). Alignment showed that the region was well conserved. Only a small number of inter-individual variations were identified in addition to a few differences with the published sequence (Table 2). All nucleotide positions are based on the published sequence of Jounaidi *et al.* (13). All variants detected were heterozygous, and all samples heterozygous for the more frequent A⁻⁴⁵G mutation were also heterozygous for the T⁻³⁶⁹G mutation, suggesting that the two mutations were linked. These two mutations fall within two separate putative regulatory elements, a basic transcription element (BTE: A⁻⁴⁵G, also called Sp1 site) and an activator protein-3 motif (AP-3: T⁻³⁶⁹G). None of the remaining variants fell within putative regulatory domains. PCR assays were developed to confirm the presence of the A⁻⁴⁵G and T⁻³⁶⁹G mutations in each individual sample, and to ascertain if the two mutations were on the same, or on separate chromosomes. The PCR assay for the A⁻⁴⁵G mutation was based on the creation of a recognition site for the restriction enzyme *Tai*-I by utilizing an oligonucleotide mismatch primer (3A5R1). This primer introduces a *Tai*-I recognition site only when the wild type 'A' nucleotide is present at position -45.

Digestion of the 369 bp PCR product with *Tai*-I yields fragments of 349 and 20 bp for the wild-type sequence, whilst the product remains undigested if the mutant 'G' nucleotide is present (Fig. 2). Similarly, for the detection of the T⁻³⁶⁹G mutation a second oligonucleotide mismatch primer was used (3A5F2). This primer introduces a recognition site for the restriction enzyme *Alu*-I when the wild-type 'T' nucleotide is present at position -369, digesting the wild-type PCR product to yield fragments of 318, 33 and 18 bp. This site is lost when the mutant 'G' nucleotide is present; *Alu*-I then yields digestion products of 336 and 33 bp (Fig. 3). To determine if the mutations were present on the same chromosome, a PCR assay was developed utilizing two oligonucleotide mismatch primers (3A5F1 and 3A5R1). Both primers introduce recognition sites for the restriction enzyme *Mvn*-I when the mutant nucleotides are present at positions -45 and -369. If the mutations are present on different chromosomes then the original 369 bp product is digested to yield products of 349/350 bp and 20/19 bp (inseparable by gel electrophoresis), whilst if present on the same chromosome the fragment is digested to yield products of 330 and 20/19 bp. In addition to confirming the individual genotypes of the samples as determined by sequencing, the two mutations were in all cases linked on the chromosome (data not shown).

Table 2. Variations found in the 5' flanking region of the *CYP3A5* gene by sequencing.

Mutation	Nucleotide position*	Samples containing variant
A to G	-45	10,11,16,20,32,37,38
G to A	-160	36
T to G	-369	10,11,16,20,32,37,38
T to C	-881	10,11
G to T	-1212	12

All mutations were detected in the heterozygous state only.*Nucleotide numbering is based on the sequence of the *CYP3A5* flanking sequence published by Jounaidi *et al.* (13).

Figure 2. Polymerase chain reaction (PCR) assay for the detection of the A⁴⁵G mutation. (a) Diagram of relative position of primers, and of the recognition site for the restriction enzyme *Tai*I, which is introduced into the PCR product utilizing mismatched primer 3A5R1 when the wild-type 'A' nucleotide is present at position -45, and is lost when the mutant 'G' nucleotide is present. (b) Diagrammatical representation of expected restriction fragments for each possible genotype for the A⁴⁵G mutation, i.e. homozygous wild type, heterozygous and homozygous mutant. (c) 1.5% agarose gel of *Tai*I restriction digest of 3A5F2/3A5R1 PCR product for detection of the A⁴⁵G mutation. Lane 1, 100 bp ladder; lanes 2 and 7, reference undigested PCR products; lane 3, sample homozygous for the wild-type 'A' nucleotide at position -45; lanes 4, 5 and 6, samples heterozygous for the A⁴⁵G mutation; last lane is a negative control.

Figure 3. Polymerase chain reaction (PCR) assay for the detection of the T³⁶⁹G mutation. (a) Diagram of relative positions of primers, and of the recognition site for the restriction enzyme *Alu*-I. The forward recognition site is introduced into the PCR product utilizing mismatched primer 3A5F2 when the wild-type 'T' nucleotide is present at position -369, and is lost when the mutant 'G' nucleotide is present. (b) Diagrammatical representation of expected restriction fragments for each possible genotype for the T³⁶⁹G mutation, i.e. homozygous wild type, heterozygous and homozygous mutant. (c) 12.5% polyacrylamide ExcelGel of *Alu*-I restriction digest of 3A5F2/3A5R1 PCR product for detection of the T³⁶⁹G mutation. Lane 1, 100 bp ladder; lanes 2, 5, 6, 7 and 8, samples homozygous for the wild-type 'T' nucleotide at position -369; lanes 3, 4 and 9, samples heterozygous for the T³⁶⁹G mutation. Fragment X, additional digestion product resulting from reamplification of original template by primers 3A51/3A52.

Relationship between CYP3A5 allelic variants, CYP3A5-mediated metabolism, CYP3A5 mRNA and protein expression

Samples were grouped according to genotype: 'wild-type' or 'mutant' (containing the linked polymorphisms), and the 1-OHM/4-OHM metabolic ratios (mr) were compared between the groups (Fig. 4a). With the exception of one outlier (liver sample number 38, mr = 2.08), all individuals carrying the linked mutations had metabolic ratios > 5.0, whilst the wild-type group all possessed metabolic ratios of < 3.5. The mean metabolic ratios for the mutant group were significantly higher than those from the wild-type group (6.0 ± 2.0 versus 2.7 ± 0.42 , mean $\pm$ SD; $P < 0.001$). Quantitative PCR was used to ascertain if the mutations in the 5' flanking region were affecting gene expression. Whilst mRNA levels showed greater variation than the metabolic ratio data, a degree of bimodality was observed (Fig. 4b). The mutant group had CYP3A5 mRNA levels skewed towards the higher end of the expression range, showing significantly higher average levels of CYP3A5 mRNA than the wild-type group (mean $\ln$ Ct = 4.03, SD = 0.97, against mean $\ln$ Ct = 2.06, SD = 1.2, $P < 0.006$) (Fig. 4b). In this case the outlier (presenting with the mutant genotype, but wild-type metabolic ratio) also fell within the lower range of expression ($\ln$ Ct = 2.9). The level of CYP3A5 protein expression levels was estimated for 29 liver samples by Western blot analysis using a CYP3A5-specific antibody. A single band of 52 kDa corresponding to CYP3A5 was clearly apparent in some samples. With the exception of the single outlier with the high expression genotype (mutant) and low metabolic ratio phenotype (wild-type), all samples which possessed the high expression genotype, a high metabolic ratio and high CYP3A5 mRNA expression level clearly show high levels of CYP3A5 protein expression when compared to those samples with the low expression genotype and phenotype (Fig. 5). The single outlier with the high expression genotype, but low expression phenotype showed levels of CYP3A5 expression similar to those in the low expression genotype group. Longer exposure of the Western blot indicated that a very low level of CYP3A5 expression was apparent in most samples (data not shown).

Population allele frequencies

Three hundred normal healthy Caucasian volunteers for phase I clinical trials were screened for the presence of the CYP3A5 gene mutations. The variant allele frequency was 0.092 (55 out of 600) with the wild-type allele frequency being 0.908 (545/600). The homozygous wild-type frequency was 0.820 (246/300), the heterozygous frequency was 0.177 (53/300) and 1 individual was homozygous for the variant sequence (frequency 0.003). These values are in Hardy-Weinberg equilibrium ($P = 0.292$).

Figure 4. Comparison of OH-midazolam metabolic ratios and CYP3A5 expression in samples with and without the linked mutations. (a) Comparison of 1-OHM/4-OHM metabolic ratios between samples with the linked mutations (HET group) and those wild-type for the mutations at positions -45 and -369 (WT group). Means and quartiles are shown for each group, as is overall mean for the combined groups (central line). (b) Comparison of CYP3A5 expression (log transformed) between samples with the linked mutations (HET group) and those wild type for the mutations at positions -45 and -369 (WT group). Means and quartiles are shown for each group, as is overall mean for the combined groups (central line).

Figure 5. Western blot analysis of CYP3A5 protein expression in liver samples. Western blot of microsomes prepared from liver samples and probed with a CYP3A5-specific antibody. Liver samples of subjects with the linked polymorphisms at base pair positions -45 and -369 (HET group) are marked with an asterisk. Sizes indicated in kDa from Wide Range Colour marker (Sigma).

Discussion

CYP3A subfamily members are the predominant CYP proteins expressed in the liver and play a major role in the metabolism of an ever-expanding list of therapeutic compounds and environmental toxins (2,19,20). Inter-individual variability in activity of the subfamily members represents a major determinant of individual drug response/toxicity and may also play a role in susceptibility to environmental toxins. In this respect, the ability to predict metabolism levels, based on a simple diagnostic test, would allow not only safer use of drugs metabolised by the specific family member, but also facilitate disease association studies. To date, the genetic basis for any CYP3A variation has never been proven. In the present study, we describe the major genetic determinant of increased CYP3A5 activity, resulting from two linked polymorphisms in the 5' flanking region of the gene, which result in increased levels of gene transcription. The 5' flanking sequences of CYP3A5 obtained in this study show high levels of homology to those published by Jounaidi *et al.* (13), and show little inter-individual variation. Interestingly, Jounaidi *et al.* (13) sequenced two human genomic clones, one of which contained the two linked mutations described in detail in this report. This would suggest that one clone was derived from an individual in the low expression group, and one from an individual in the high expression/metabolism group.

Previous studies have suggested that CYP3A5 is expressed in only 10-30% of livers (7-9), whilst another study has stated that some expression is constitutive in all samples (10). The present study supports the findings that some CYP3A5 expression is constitutive, with some metabolic activity and mRNA being detected in all livers studied, although CYP3A5 protein was not convincingly demonstrated in all samples using the procedures described. We detected enhanced RNA and protein expression in 23% of the samples for which tissue was available (six out of 26), which is similar to the fraction of the population showing liver expression in previous studies. This supports the findings of Boobis *et al.* (10), that some low level of expression is constitutive in all liver samples, although this can only be detected convincingly using more sensitive detection techniques (such as PCR), and not by Western or Northern blot analysis. Whilst both polymorphisms detected lie within putative transcriptional regulatory elements, we suspect that the variant within the BTE (Sp1 site) is more likely to be responsible for altered expression since it has been reported that a BTE flanking the TATA box accounts for the constitutive expression of CYP1A1, and a similar region has been found in several other CYP genes including CYP2B1, CYP2B2, CYP2E1 (21) CYP3A4 (12) and CYP3A7 (11). In the case of the CYP3A4 gene, this element has been shown to bind nuclear extracts (12) and a basic transcription element binding factor (BTEB) has been shown to interact with the 5' regulatory region of the CYP3A7 gene (11), indicating a role of this region in the general control of cytochrome P450 expression.

The exact mechanism of upregulation of CYP3A5 expression in the allelic variant described here remains to be determined, although the presence of one of the mutations within the BTE, and the relevance of this element for the expression of other P450s indicates a possible mechanistic link. Using methylation interference footprinting, it has been shown that all guanine residues within the BTE, and other guanine residues in the vicinity, interacted with the transcriptional factors BTEB (BTE-binding protein) and Sp1 (22). Given that the mutation within the BTE described in this paper alters an adenine residue to a guanine residue, increasing the percentage identity to the Sp1 binding site from 80% to 90%, this could facilitate binding of transcription factors to the variant form of the BTE. Whilst preliminary data indicate that this is in fact the biochemical mechanism for the altered expression of CYP3A5 described here (unpublished observations), at this stage we cannot definitively rule out linkage disequilibrium with another mutation, which may be affecting gene expression or protein/RNA stability.

Although there is considerable overlap in the range of CYP3A5 mRNA levels seen in the homozygous and heterozygous group, the distribution of metabolic ratios is clearly bimodal, as is the amount of CYP3A5 protein (even allowing for the semi-quantitative nature of Western blots). We cannot exclude the presence of other polymorphisms that may affect the translation efficiency or protein stability of CYP3A5. Given the better correlation between DNA polymorphism and protein level and the notorious lability of RNA, the simpler explanation is that differential RNA degradation or yield (due to differences in sample handling) has blurred the distinction between high and low expressors. Whatever the explanation for the discrepancy at the mRNA level, it does not in any way diminish the predictive value of the DNA polymorphism described. There is, however, one individual whose genotype (heterozygous mutant) is not predictive of his metabolic phenotype (low expression). The fact that CYP3A5 protein as well as mRNA levels were low in this outlier indicates that the explanation must be sought at the transcriptional level (e.g. in transcription factors controlling CYP3A5 expression). An AUG element in the 5'-untranslated region of the BTEB gene has been shown to be partly responsible for cell specific translational control of BTEB (23). Mutations within this region were shown to affect BTEB translation. Therefore, whilst the outlier in our study has a high expression genotype for CYP3A5 expression, this individual may have a 'low' expression phenotype for BTEB. Additionally, it is possible that a mechanism similar to that responsible for inducing CYP1A1 expression may also affect CYP3A5 expression. In addition to the BTE, CYP1A1 expression is mediated by a xenobiotic responsive element (XRE). In this case, inducers enhance expression by binding to a cytosolic receptor (Ah receptor), which is translocated into the nucleus (possibly in association with an accessory protein coded for at the Arnt gene), where it binds the XRE (24,25). Although variations in these and other transcription factors could further modulate CYP3A5 expression, this does not detract

from the fact that the polymorphism described here seems to be the major determinant of CYP3A5 expression, at least in liver.

Despite the relatively small number of samples available for analysis in the present study, strong associations have been found between the two linked polymorphisms on the one hand and CYP3A5 mRNA, protein and activity levels in the liver on the other hand. The unravelling of genetic mechanisms for the polymorphic metabolism by CYP3A5 will have important consequences in the field of pharmacogenetics. The ability to predict metabolism by genotyping will greatly facilitate disease association studies and may also help to explain adverse reactions or poor response to therapeutics that are metabolised by this cytochrome P450 isoform. It will also help in delineating which factors affecting CYP3A5 activity are genetic and which are environmental; for both, further work will be required to understand fully the complex variation in expression observed with this enzyme.

Note added after publication

Shortly after publication of this article, another research group discovered that upstream of the *CYP3A5* gene a smaller pseudogene, called *CYP3A5P1*, was present (Finta *et al.*, *Gene* 2000; 260: 13-23). The *CYP3A5P1* consists of an exon 1, identical to exon 1 of the *CYP3A5* gene, and a novel exon 2 and 13. Furthermore, sequence identity in the 5' untranslated promoter region between the two genes was only 89%. Subsequently, Kuehl *et al.*, discovered that the two *CYP3A5* polymorphisms described in our article above, were in fact located in this pseudogene, and could therefore not be functionally responsible for the polymorphic CYP3A5 expression (Kuehl *et al.*, *Nat Genet* 2001; 27: 383-391). They detected complete linkage disequilibrium of the polymorphisms with another polymorphism located in intron 3 of the *CYP3A5* gene, named *CYP3A5*3*, which causes an alternatively spliced mRNA and a truncated protein. Nevertheless, also in their study as well as in subsequent studies by several other groups, the correlation of the *CYP3A5P1* genotype with CYP3A5 expression levels in the liver has proven to be highly predictive (Chou *et al.*, *Drug Metab Dispos* 2001; 29: 1205-1209, Zhu *et al.*, *Acta Pharmacol Sin* 2002; 23: 567-571).

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CHAPTER 7

Genetic susceptibility to acquired long QT syndrome: the impact of congenital long QT genes and drug metabolising enzyme genes

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Abstract

Acquired Long QT Syndrome (aLQTS) is a disorder that predisposes to ventricular arrhythmias, typically of the *Torsade de Pointes* (TdP) type, and sudden death. One of the major causes of aLQTS is drug administration, with concurrent drug therapy indicated in many reports. "Forme fruste" cases of congenital LQTS (cLQTS) and variations in drug concentrations have been implicated as risk factors. In this study we evaluated the role of genetic predisposition to aLQTS by mutational screening of cLQTS genes and mutation analysis of known variations in drug metabolising enzyme genes. Thirty-two unrelated aLQTS cases were recruited, all of whom had confirmed TdP resulting from drug administration. The entire coding regions of all known cLQTS genes (*KCNH2*, *KCNQ1*, *SCN5A*, *KCNE1*, and *KCNE2*) were screened for mutations. Mutations that have previously been reported to be functionally relevant accounted for 2 out of 32 of aLQTS cases. Eleven novel polymorphisms were detected in the cLQTS genes. In addition, known polymorphisms in the drug metabolising enzyme genes *CYP2D6*, *CYP3A4* and *CYP3A5P1* were assayed. Of note, all patients on drugs that undergo CYP3A mediated metabolism carried the genotype predictive of low CYP3A5 expression, while none of the aLQTS subjects with the genotype predictive of high CYP3A5 activity were treated with a drug that is metabolised via CYP3A.

In conclusion, exonic mutations in the cLQTS genes explain only a limited proportion of aLQTS cases. We hypothesise that inter-individual variation in CYP3A5 expression may also affect the risk for aLQTS when prescribing (combinations of) drugs metabolised by CYP3A.

Introduction

Congenital long QT syndrome (cLQTS) is a rare, yet in some cases lethal, cardiac disease caused by malfunctioning of ion channels in cardiac cells (1). The syndrome is characterised by symptoms such as syncope and seizures and typically manifests itself with specific, potentially fatal, ventricular arrhythmias of the *Torsade de Pointes* (TdP) type. The molecular basis of cLQTS is becoming increasingly understood and mutations in 5 genes have been identified as causative for the disease in cLQTS families. Mutations in the potassium ion channel genes *KCNH2* (HERG, LQT2) and *KCNE2* (LQT6) affect the rapid component of the delayed rectifier I_{Kr} , whilst mutations in the genes *KCNQ1* (KvLQT1, LQT1) and *KCNE1* (LQT5) affect the slow component of the delayed rectifier I_{Ks} . Mutations in the sodium ion channel gene *SCN5A* (LQT3) affect the current I_{Na} responsible for the depolarisation of the cardiac action potential (2,3). Linkage analysis suggests that at least one further susceptibility gene is present at locus 4q25-27 (LQT4) (4). In total, over 200 different disease-causing mutations in these genes have been identified in cLQTS families. Only a minority of these mutations has been electrophysiologically characterised; for most other mutations there is a

good deal of genetic evidence that the identified mutations are the causative basis of the disease in the respective families.

Acquired long QT syndrome (aLQTS) presents with symptoms that are very similar to those for cLQTS, although the conditions, risk factors and triggers for their occurrence differ. The major cause of aLQTS is through drug administration, with drug overdose and concomitant medications indicated in many case reports. Drug induced QT prolongation and TdP are rare side effects and as such may not become apparent during the drug development process. The idiosyncratic manner in which only some individuals develop QT prolongation and TdP in response to similar doses of the same drugs is not fully understood. Drugs from many different classes with diverse chemical structures have been reported to be capable of prolonging the QT interval and causing TdP (5-7). Many of the drugs that have been proven to cause aLQTS have been shown to interact directly with and block the cardiac ion channels coded for by the cLQTS genes (see also <http://www.qtdrugs.org>).

It has been proposed that some cases of aLQTS represent instances of “forme fruste” of cLQTS, i.e. these patients carry normal or borderline QT intervals resulting from “mild” mutations in one of the congenital LQTS genes and are, under normal circumstances, asymptomatic. On challenge with certain drugs, however, these individuals appear to be more susceptible to cardiac ion channel blockade and QT prolongation. In this respect, variations in the *KCNE2* and *KCNQ1* genes have been identified previously as causative in aLQTS case reports (8, 9). This suggests that some aLQTS patients may be identifiable by mutational screening of cLQTS genes, and may thus be preventable. Whilst some cLQTS gene variants have been reported in the literature for individual aLQTS cases, the importance of “forme fruste” as a risk factor for TdP in a larger population of aLQTS cases remains to be demonstrated.

Overdose with a trigger drug and concurrent medication(s) have been indicated in many cases of aLQTS, suggesting that variations in the concentration of trigger drugs at the cardiac myocyte level is an important determinant of increased susceptibility to aLQTS. The circulating plasma concentration of a drug depends largely on the activity of the enzymes responsible for its metabolism. Together, the cytochrome P450 (CYP) enzymes CYP2D6 and CYP3A are responsible for the hepatic metabolism of the majority of all clinically used drugs (10). CYP2D6 is involved in the metabolism of more than 50 drugs. The CYP3A subfamily consists of three members that show high levels of homology: CYP3A4, CYP3A5, and CYP3A7, the latter being expressed in foetal and newborn liver only. CYP3A4 is often regarded as the major isoform responsible for the drug metabolism, but CYP3A5 has wide overlapping substrate specificity and might therefore also be important. Inter-individual differences in CYP activity are partially due to variations within the genes coding for the enzymes. The genetic basis of inter-individual variations in drug metabolism is becoming increasingly understood for the CYP enzymes, and has shown relevance in influencing the pharmacokinetic profile of a

drug. In this respect it is now possible to reliably predict over 95% of CYP2D6 poor metabolisers on the basis of routine genotyping, whilst we have also recently described two linked variations in the *CYP3A5P1* gene that predict CYP3A5 expression and activity in vitro. Genetic variations have also been described in CYP3A4, although the functional consequences of these remain to be determined.

Here we investigate the role of genetic variation in the cLQTS genes and the impact of inter-individual variations in CYP genes as risk factors in 32 patients presenting with acquired QT prolongation and TdP as a consequence of drug administration.

Subjects and methods

Patient and control subjects

A total of thirty-two unrelated aLQTS subjects were recruited from three centres: the Academic Hospital Maastricht, The Netherlands (n=11), the Westfalian Wilhelms-University Muenster, Germany (n=20) and the Ottawa Hospital, Ontario, Canada (n=1). All subjects experienced TdP as a result of drug administration. Details of individual subjects are presented in Table 1. TdP was defined as either non-sustained or sustained ventricular tachycardia showing phasic variation in the electrical polarity of the QRS complex and a “short-long-short” initiating sequence (11). In addition, all subjects showed a QTc prolongation ($QTc > 440 \text{ msec}^{1/2}$) and changes in the configuration of the T(-U) wave (i.e., biphasic T waves, beat-to-beat changes in T wave morphology, post-extrasystolic T wave abnormalities (i.e., an increased post-extrasystolic T-U wave amplitude) or emergence of new large U waves) compared to an electrocardiogram recorded prior to drug exposure implicated in the generation of TdP. In case no baseline and on-drug ECGs could be retrieved from the referring physicians, subjects were classified only as demonstrating TdP if the qualifying ECG showed the typical TdP pattern. Electrocardiographic intervals were measured from tracings as close as possible to the event. The QT interval was measured from electrocardiogram lead II or the available rhythm strip. For rate-correction, the Bazett equation was applied.

Genotype/allele frequencies of variations in drug metabolising enzyme genes identified in the group of aLQTS subjects were compared with a control group of 300 anonymous healthy Caucasians. This group of control subjects was recruited from a large pool of clinical trial volunteers available at the Janssen Research Foundation (Belgium). For genotype/allele frequency comparisons in the cLQTS genes, a randomly selected subset of 32 individuals of the control group was used. Because demographic and clinical information was not available for the anonymised control group, matching with the aLQTS group was impossible. Full IRB approval and subject informed consent was obtained from all participants.

Mutational screening of long QT genes

Mutational screening of all individual exons and intron/exon boundaries of *KCNH2*, *KCNQ1*, *SCN5A*, *KCNE1* and *KCNE2* was performed by PCR amplification and direct sequencing on an ABI-Prism-3700 sequencer (PE-Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Oligonucleotides (Eurogentec, Seraing, Belgium) used for amplification have been previously described by Itoh *et al.* (*KCNH2*) (12), Splawski *et al.* (*KCNQ1*, *KCNH2* and *KCNE1*) (13), Wang *et al.* (*SCN5A*) (14), and Abbott *et al.* (*KCNE2*) (15). All sequences were aligned with their respective wild type gene sequences reported in GenBank under accession numbers U04270 (*KCNH2*), AF071002 (*KCNE1*), NM_005136 (*KCNE2*), AF000571 (*KCNQ1*) and NM_000335 (*SCN5A*), using the Sequencher software package (Gene Codes Corporation, Ann Arbor, MI). Frequencies of known and novel genetic variations were determined in the panels of 32 aLQTS subjects and 32 control subjects.

CYP450 genotyping

Oligonucleotides were ordered from Eurogentec (Seraing, Belgium), restriction enzymes from New England Biolabs (Hertfordshire, UK), GeneAmp PCR buffer II, MgCl₂ and AmpliTaq Gold from PE-Biosystems (Nieuwerkerk a/d IJssel, The Netherlands), and dNTPs from Amersham-Pharmacia-Biotech (Roosendaal, The Netherlands).

CYP2D6

Genotyping was performed for the inactivating gene variations CYP2D6*3, *4, *5, *6. The *5 inactivating gene deletion assay was performed as described previously (16). PCR-RFLP assays were developed for the *3, *4, and *6 variations and PCR conditions were 10 min 95°C, followed by 35 cycles of 95°C, 45 sec, 58°C (*4/*6) or 55°C (*3), 45 sec and 72°C, 45 sec. The reaction was terminated with a final extension step at 72°C for 10 min. The PCR mix consisted of 1x GeneAmp PCR buffer II, 200 nM dNTPs, 1.5 mM MgCl₂, 250 nM of each primer, 0.75 units of AmpliTaq Gold polymerase and approximately 100 ng of genomic DNA, in a volume of 30 µl. For the CYP2D6*3 variation 10% DMSO was added to this PCR mix. The *4 and *6 variations were analysed in the same RFLP assay, using PCR primers 5'-GCA-AGA-AGT-CGC-TGG-AGC-CG-3' (forward, mismatch underlined) and 5'-CTC-GGT-CTC-TCG-CTC-CGC-AC-3' (reverse). The resulting PCR product (342 bp) was digested with *Scr* FI at 37°C for 3 hours, and fragments were separated on 12.5% polyacrylamide. For the *3 variation, PCR primers 5'-GCC-TCG-GAA-GAG-CAG-GAT-TTG-CA-3' (forward) and 5'-GGC-TGG-GTC-CCA-GGT-CAT-AC-3' (reverse, mismatch underlined) were used. The PCR product (495 bp) was digested with *Bsa* AI (37°C, 3 hours), and analysed on 12.5% polyacrylamide.

CYP3A4

PCR assays were developed for the detection of CYP3A4 allelic variants CYP3A4*1B, *2, *3. PCR conditions were 95°C 10 min, followed by 35 cycles of 95°C, 45 sec, 59°C (*1B)

or 58°C (*2) or 56°C (*3), 45 sec and 72°C, 45 sec. The PCR was terminated with a final extension step of 72°C for 10 min. PCR mixes were as described for CYP2D6 genotyping. For the CYP3A4*1B PCR, 10% DMSO was added to the mix. Primers used for the CYP3A4*1B PCR were 5'-AGG-CAC-ACT-CCA-GGC-ATA-GG-3' (forward) and 5'-TTC-TCC-ACC-TTG-GAA-GTT-GGC-3' (reverse).

Table 1. Clinical information of the examined aLQTS cases.

Case reference number	Age (years)	Sex	QTc at rest (msec ^{1/2})	QTc after drug treatment (msec ^{1/2})	Suspected trigger drugs* and concurrent medication(s)
5	68	F	400	575	Amiodarone*, Sotalol*, Digoxin
7	63	M	400	510	Amiodarone*, Carbasalatecalcium, Metoprolol
8	76	F	450	562	Ibutilide*, Verapamil, Omeprazole
9	84	M	440	531	Digoxin*, Fexofenadine, Verapamil
10	81	F	440	527	Amiodarone*, Furosemide, Captopril, Bumetadine*, Digoxin
11	79	F	408	568	Furosemide*, Doxazosin, Lisinopril, Salbutamol, Acenocoumarol
12	24	F	400	496	Ketanserin*
13	76	F	430	585	Sotalol*
14	65	M	400	580	Sotalol*
15	82	F	391	502	Amiodarone*
16	12	M	420	n.m.†	Amiodarone*
17	70	F	454	477	Quinidine*
18	77	F	440	640	Cisapride*, Clarythromycin*
19	75	F	405	637	Sotalol*
20	82	F	400	510	Sotalol*, Enalapril, Acenocoumarol
21	77	F	440	560	Sotalol*, Mirtazapine, Risperidone*, Digoxin, Captopril*
22	80	F	400	630	Sotalol*, Furosemide, Digoxin, Tamoxifen*, Venlafaxine*
24	65	M	490	504	Sotalol*
25	60	F	378	428	Sotalol*
27	64	F	440	600	Amiodarone*
28	73	F	420	560	Amiodarone*, Sotalol*
29	66	M	397	498	Sotalol*
30	75	M	450	600	Sotalol*
31	38	F	402	459	Sotalol*
32	71	M	n.m.†	564	Quinidine*
33	75	F	430	550	Sotalol*
34	75	M	n.m.	456	Sotalol*
35	71	M	420	550	Amiodarone*
36	57	F	400	515	Sotalol*
37	70	M	444	540	Sotalol*
38	69	F	470	471	Sotalol*
42	51	F	454	477	Quinidine*

*Drugs that have been implicated in QT prolongation and TdP (www.qtdrugs.org). †Not measurable due to pacing. F= female, M= male; n.m.= not measured.

Primers used for the CYP3A4*2 PCR were 5'-AGC-GTC-TTT-GGG-GCC-TAC-AG-3' (forward) and 5'-TGA-TGA-TGG-TCA-CAC-ATA-TCT-TC-3' (reverse). PCR products (280 bp and 298 bp for *1B and *2, respectively) were purified and directly sequenced. The CYP3A4*3 PCR-RFLP assay was performed using PCR primers 5'-ATC-TAC-CAA-CGT-GGA-ACC-AG-3' (forward) and 5'-TGT-TCA-TGA-GAG-CAA-ACC-AC-3' (reverse, mismatch underlined). The 121 bp PCR product was digested with *Nsp* I (37°C, 3 hours) and the DNA fragments were separated on 12.5% polyacrylamide.

CYP3A5 - CYP3A5P1

We reported earlier on the discovery of two linked polymorphisms in the promoter region of the CYP3A5 gene that are associated with a higher in vitro CYP3A5 expression and activity (17). Further studies have shown that these variations were in fact polymorphisms in the 5' flanking region of the CYP3A5P1 pseudogene (18, 19). Nevertheless, these polymorphisms remain predictive of higher in vitro CYP3A5 activity and expression (19). Genotyping for the linked polymorphisms in the CYP3A5P1 gene was performed as described previously (17).

Results

Subject Data and medication

In addition to detailed patient information, Table 1 provides an extensive summary of trigger drugs, pre- and post-dose QTc, and concurrent medications for all aLQTS subjects. With exception of four patients, all subjects were older than 50 years. There were approximately twice as many female patients than male patients in this study (21 and 11, respectively). The average QTc interval for the aLQTS subjects prior to drug treatment was 425 msec^{1/2} (males = 433 msec^{1/2}, females = 422 msec^{1/2}), rising to 540 msec^{1/2} post-treatment (males = 533 msec^{1/2}, females = 542 msec^{1/2}). Table 2 shows for each of these drugs the route of metabolism in the body and the enzymes that are involved (where known).

Mutational screening in LQT genes

The LQT gene sequences of the aLQTS patients were compared to the wild type sequences deposited in GenBank. Table 3 summarises the 25 variations that were detected in the studied congenital LQTS genes, 14 of which had previously been reported in the LQT database². Only two of these variations, however, have been found and confirmed to be the causal mutations for congenital LQTS (3,8). Subject 16 was heterozygous for base pair substitution 22 A>G in exon 1 of the *KCNE2* gene, leading to a T8A amino acid substitution. This mutation has been described previously in a case report of an individual presenting with antibiotic drug-induced cardiac arrhythmia whilst taking bactrim (trimethoprim and sulfmethoxazole), and has been functionally characterised as the causative mutation for the reported case (8). The suspected trigger compound for subject 16 was the antiarrhythmic drug amiodarone.

This drug is a CYP3A substrate (20), and this subject carried a genotype predictive of low CYP3A5 expression (see below). Together, a picture of interacting risk factors may be constructed for this subject: a mutation in the ion channel KvLQT1, a trigger compound that is metabolised by CYP3A and low CYP3A5 mediated metabolism. Subject 18 was heterozygous for base pair substitution 1039 C>T in exon 5 of the *KCNH2* gene, resulting in amino acid substitution P347S, that was reported earlier as the causative mutation in a cLQTS family (4). The suspected trigger compound for subject 18 was cisapride (21), although the patient was also taking clarithromycin. Cisapride has been implicated in aLQTS cases and has the proven ability to block the HERG channel (22,23). In addition, both cisapride and clarithromycin are metabolised by the CYP3A family, and this subject carried the genotype predictive of low CYP3A5 activity (24,25). Thus, also for subject 18, one can build up a picture of interacting risk factors: a mutation in the *KCNH2* gene; a trigger compound which can block the HERG channel; concurrent medication competing for metabolism by CYP3A and a low activity CYP3A5 phenotype, the latter which can conspire to raise the circulating plasma levels of cisapride to a level at which it can interact with a susceptible HERG channel.

Four further variations were found in the aLQTS patients that resulted in altered amino acid sequences (Table 3). All of these variations have been reported previously (26-29). Three of these variations, namely G38S in *KCNE1*, K897T in *KCNH2*, and H558R in *SCN5A*, were also frequently present in our control population, suggesting that these variations are non-functional polymorphisms rather than pathogenic mutations. The D85N variation in the *KCNE1* gene was detected in two of the aLQTS subjects but not in the control subjects. Sixteen out of 19 of the other known or novel intragenic variations that were detected in our set of 32 aLQTS patients were also detected in our control population. None of these changed the amino acid coding sequence, nor is literature available that would suggest a possible role for one or more of these variations as the causal factor for the disease. The variations T377T and G628G in *KCNQ1* and variation IVS16-6 C>T in *SCN5A* were detected only once in the aLQTS subjects but not in the control group. Because of the conservative nature of these variants, it might be expected that these variations represent low frequency polymorphisms with little or no effect on the functionality of the genes.

Table 2. Mechanisms of drug biotransformation and metabolite formation of the drugs taken by aLQTS subjects in the study.

Drug	Biotransformation / major metabolite formation	Enzymes involved in metabolism	Inhibitor	Inducer	Reference
Acenocoumarol	(R/S) 6- and 7-hydroxylation	CYP2C9, CYP2C19			Br J Pharmacol 1993;110:482-490
Amiodarone	N-deethylation	CYP3A			Drug Metab Disp 1993;21:978-985
Bumetadine	Unchanged (50%), metabolism (50%)	Unknown			Goodman & Gilman 1999, 8 th ed.
Captopril	Unknown	CYP2D6			Drug Met Rev 1997;29:413-580
Carbasalatecalcium	Unknown				
Cisapride	N-dealkylation	CYP3A , CYP2A6			Br J Pharmacol 2000;129:1655-1667
Clarithromycin	N-demethylation and hydroxylation	CYP3A			Drug Met Disp 1997;25:623-630
Digoxin	Unchanged	Not applicable			
Doxazosin	6- and 7-hydroxylation	Unknown			
Enalapril	Unknown	CYP3A			Biochem Pharmacol 1992;44:2089-2098
Furosemide	Unchanged	Not applicable			Ned Tijdsch Geneesk 1986;130:1588-1590
Ibutilide	ω -oxidation	Unknown			Drugs 1997; 54: 313-330
Ketanserine	Ketone reduction and oxidative N-dealkylation	Unknown			Arzneim-Forsch Drug Res 1988;38:789-794
Lisinoprilate*	Unchanged	Not applicable			Goodman & Gilman 1999, 8 th ed.
Metoprolol	O-demethylation	CYP2D6			Biochem Pharmacol 1992;44:2089-2098
Mirtazapine	8-hydroxylation, N-demethylation, N-oxidation	CYP3A , CYP2D6 , CYP1A2			Drug Met Disp 2000;28:1168-1175
Omeprazole	N-oxidation, O-demethylation	CYP3A , CYP2C19		CYP1A2	Eur J Gastroent Hep 1996;8:521-525
Quinidine	Hydroxylation, N-oxidation	CYP3A	CYP2D6		Drug Met Rev 1997;29:413-580
Risperidone	9-hydroxylation	CYP3A , CYP2D6			Arch Pharmacol 1999;359:147-151
Salbutamol	O-sulphation	Sulphotransferase(s)			Xenobiotica 1997;27:279-286
Sotalol	Unchanged	Not applicable			Acta Pharmacol Toxicol 1976;39:118-128
Tamoxifen	N-demethylation, 4-hydroxylation	CYP3A , CYP2D6 , CYP2C9			Biochem Pharmacol 1997;53:171-178
Vanlafaxine	O- and N-demethylation	CYP2D6			Neuropsychopharmacol 1999;20:480-490
Verapamil	N-dealkylation	CYP3A , CYP1A2			Drug Met Rev 1997;29:413-580

* Lisinopril (pro-drug) is converted by serum esterase(s) to lisinoprilate.

Table 3. Genetic variations identified in cLQTS genes in 32 aLQTS patients and 32 healthy control subjects.

Gene	Nucleotide variation	Location	Coding effect	Known/ novel*	aLQTS case reference numbers carrying the variation (heterozygote // homozygote)	aLQTS cases (number)		Controls (number)	
						Het.	Hom.	Het.	Hom.
KCNF1	84 G>A	Exon 1	S28S	novel	21 // -	1	0	0	0
KCNF1	112 G>A	Exon 1	G38S	known	10,11,13,14,15,16,17,28,30,34,35,38// 5,9,21	12	3	11	5
KCNF1	253 G>A	Exon 1	D85N	known	22 // 32	1	1	0	0
KCNF2	22 A>G	Exon 1	T8A	known	16 // -	1	0	0	0
KCNH2	1039 C>T	Exon 5	P347S	known	18 // -	1	0	0	0
KCNH2	1467 C>T	Exon 6	I489I	known	7,8,13,15,17,28,33,36,38 // 14,31,34,35	9	4	8	2
KCNH2	1539 C>T	Exon 6	F513F	known	7,8,13,15,17,28,33,36,38 // 14,31,34,35	9	4	8	2
KCNH2	1692 A>G	Exon 7	L564L	known	5,7,8,9,13,15,16,17,18,22,24,25,33,36,42 // 14,21,28,31,34,35,38	15	7	17	3
KCNH2	1956 C>T	Exon 8	Y652Y	known	5,7,8,13,17,18,19,20,22,24,27,32 // 12,30,37	12	3	16	5
KCNH2	2690 A>C	Exon 11	K897T	known	9,10,15,16,19,20,25,29,32,36,42 // 11	11	1	13	1
KCNQ1	C1131G	Exon 8	T377T	novel	20 // -	1	0	0	0
KCNQ1	IVS11+14 T>C	Intron 11	-	novel	7,9,11,14,15 // -	5	0	5	0
KCNQ1	1638 G>A	Exon 12	S546S	known	8,13,16,20,22,28,30,42 // 21,29	8	2	8	2
KCNQ1	IVS12+36 G>A	Intron 12	-	novel	13,15,16,17,20,22,27,29,30,32,34// 8,18,21,28,42	11	5	13	4
KCNQ1	1884 C>T	Exon 15	G628G	novel	19 // -	1	0	0	0
KCNQ1	1986 C>T	Exon 15	Y662Y	known	9,10,11,12,15,16,21,25,34 // 7,8,30	9	3	12	2
SCN5A	87 G>A	Exon 2	A29A	novel	7,12,19,21,24,25,28,30,32,34,35,36,42 // -	13	0	16	1
SCN5A	IVS3-24 C>T	Intron 3	-	novel	10,22 // -	2	0	1	0
SCN5A	IVS9-3 C>A	Intron 9	-	novel	11,14,15,25,33,34,35 // 5,7	7	2	10	0
SCN5A	1673 A>G	Exon 12	H558R	known	11,14,15,25,34,35,37 // 5,7	7	2	12	1
SCN5A	IVS16-6 C>T	Intron 16	-	novel	42 // -	1	0	0	0
SCN5A	3183 G>A	Exon 17	E1061E	novel	15,19,24,30,32,35,42 // 37	7	1	8	0
SCN5A	4218 G>A	Exon 23	G1406G	novel	21 // -	1	0	1	0
SCN5A	IVS24+53 T>C	Intron 24	-	known	19,21,32 // -	3	0	3	0
SCN5A	5457 T>C	Exon 28	D1819D	known	8,12,15,18,19,22,28,29,30,36,38// 21,32,33,37,42	11	5	16	4

*Compared to the long QT database on October 1st, 2001 (www.serum.dk/en/forskning/lqtsdb/lqtsdb.htm).

*CYP450 genotyping***CYP2D6**

Based on genotype, one aLQTS patient out of 32 (3.1%) was predicted to be a phenotypic CYP2D6 poor metaboliser. This frequency was not statistically different from the 7% poor metabolisers identified in the control population (n=300). The trigger drug for this CYP2D6 poor metaboliser (subject 15) was the antiarrhythmic drug amiodarone, which is a substrate for CYP3A and not CYP2D6 (20).

CYP3A4

One subject (case 32) was heterozygous for the *CYP3A4* *1B allele; all other patients were homozygous wild type for the genotyped *CYP3A4* variants. The trigger drug for the patient carrying the *CYP3A4* *1B allele was quinidine, which is a known *CYP3A4* substrate and CYP2D6 inhibitor. There were no statistically significant differences in *CYP3A4* genotype or allele frequencies between aLQTS subjects (n=32) and controls (n=300), and both groups conformed to the Hardy-Weinberg equilibrium (data not shown).

CYP3A5

Four subjects (12.5%) were heterozygous for the linked polymorphisms -44 A>G and -369 T>G flanking the *CYP3A5P1* pseudogene and that predict high CYP3A5 expression and activity *in vitro* (subjects 25, 29, 33 and 36), compared to 18% in the control population (53 out of 300). One control individual was homozygous for the linked *CYP3A5P1* polymorphisms (0.3%); no cases of the latter genotype were detected in the aLQTS group. Neither the genotype frequencies nor the allele frequencies were statistically significantly different between the aLQTS group and controls, and both groups conformed to Hardy-Weinberg equilibrium (data not shown). Twelve out of 28 patients with the *CYP3A5P1* genotype predictive of normal low CYP3A5 expression were taking suspected trigger drugs that are known to undergo CYP3A5 mediated metabolism. All four subjects with the *CYP3A5P1* genotype that is predictive of high CYP3A5 expression were taking the antiarrhythmic drug sotalol, which does not undergo significant hepatic metabolism (30,31).

Discussion

We have investigated the genetic predisposition to development of TdP in a group of 32 aLQTS patients, all of whom were subjects confirmed by ECG to have experienced TdP as a consequence of drug administration. To this aim, we screened the entire coding region of all currently known cLQTS genes, and complemented these data by genotyping for well-documented variations in the cytochrome P450 genes *CYP2D6*, *CYP3A4* and *CYP3A5*.

In the present study, female patients were twice as prevalent as male patients, supporting female gender as a determinant of susceptibility. The average QTc interval before drug treatment was below a recommended cut off point for defining QT prolongation (450 and 470 msec^{1/2} for respectively men and women) (32), but was raised in most cases by more than 100 msec^{1/2} after drug intake, clearly indicating the drug-induced nature of the disease. Individual case reports have shown that some patients with drug-induced TdP carry sporadic mutations in the genes involved in cLQTS (8,9). In our group of 32 aLQTS subjects, only one case represented a true “forme fruste” of the cLQTS, while one other case carried a mutation that was reported earlier in a cLQTS family (3,8). Perhaps a reluctance to publish negative results of the screening for these genes in other aLQTS cases may have suggested that this represents a more significant risk factor than it actually is.

We cannot exclude that any of the other variations in the cLQTS genes that were detected in this study might also be contributory risk factors in the individual cases, but further *in vitro* studies would be required to demonstrate their functional significance. Literature evidence, however, suggests that none of the four other identified missense mutations is likely to be causative for LQTS, but rather represent frequently occurring polymorphisms (26-29). It has been postulated that the K897T variation in *KCNH2* is QT-shortening and therefore “protective” in female cLQTS patients carrying both the Fin and K897T variation in the gene encoding the HERG channel (26). In our study, this frequent variation was detected both in aLQTS subjects and control subjects. The QTc interval of the aLQTS subject carrying the T897T genotype was not strikingly different from the other aLQTS cases that carried the K897K or K897T genotype. The D85N variation in *KCNE1* was detected in two aLQTS cases but not in controls, and cannot, therefore, be excluded as a potential genetic risk factor. The patients in our study that carry this genetic variation received different drugs, sotalol and quinidine respectively. Literature reports, confirmed by the control group data reported here, indicated that this variation is a rare polymorphism, and excluded it as causative in the Jervell and Lange-Nielsen syndrome (27,28). However, it has been shown for at least one rare polymorphism, namely T8A in *KCNE2*, that low frequency variations in cLQTS genes might be associated with drug induced LQTS (8).

Therefore, functional studies including the electrophysiological characterisation are required to determine the clinical significance of the D85N variant.

Numerous mutations have been identified in the genes currently known to be involved in cLQTS. Most of the affected families present with a “novel” gene mutation that is responsible for the syndrome in that particular family. When considering predicting susceptible individuals prior to drug prescription, the cLQTS genes will be difficult targets, since it is unlikely that genotyping for a limited number of mutations would predict the majority of aLQTS cases. If the pattern of cLQTS were followed, then most of cases would carry different mutations making any screening assay difficult, especially until all relevant mutations have been found. In this respect, it is an important finding that the same functional variant in the *KCNE2* gene can predispose to TdP with two different drugs acting through the KvLQT1 channel (bactrim and amiodarone respectively). Non-specificity of the cLQTS gene variants in determining susceptibility to a number of drugs would certainly be an advantage in an attempt to predict susceptible individuals.

Beside changes in channel function induced by mutations the local concentration of trigger drugs at the level of the cardiac myocyte is thought to be another risk factor in drug induced LQTS. Higher levels of trigger drugs can be attained due to overdose or concurrent medication with drugs that inhibit the metabolism of the trigger drugs (33,34). Inter-individual variations in circulating plasma levels of drugs are also possible through mutations in the genes coding for the drug metabolising enzymes. Functional variations in the activity of cytochrome P450 enzymes that are responsible for the metabolic conversion of the majority of drugs, are becoming increasingly understood and several of the phenotypes are to a large extent predictable based on genotyping (35). To our knowledge, this is the first study in which genetic variations in the drug metabolising enzymes have been studied as a potential risk factor for aLQTS and TdP. Unfortunately, the study design did not allow to evaluate drug levels in serum or tissue in order to demonstrate the functional significance of the evaluated genetic variations, but our observations suggest that such analyses might reveal important information in future studies.

The genetic basis of CYP2D6 mediated metabolism is well understood; studies in Caucasians have demonstrated the ability to predict over 95% of the poor metabolizers by genotyping (36). The potential role of CYP2D6 in aLQTS has previously been eluded to, based on the expression of CYP2D6 in the human heart, the ability of psychotropics to lengthen the QTc and their metabolism by CYP2D6 (5,37,38). The present study cannot, however, confirm this hypothesis since none of the trigger compounds were metabolised by CYP2D6 or have been stated as being psychotropic. The CYP3A family is involved in the metabolism of a large number of drugs, including antibiotics, antipsychotics and antiarrhythmic drugs indicated in aLQTS cases (39).

Since CYP3A4 and CYP3A5 show a wide overlapping substrate specificity, it is likely that CYP3A mediated metabolism of drugs is an interaction of activity of both enzymes. Only a limited number of variations have been reported in the *CYP3A4* gene, and conflicting reports on their functional consequences have been published (40-42). It is therefore difficult to appreciate the significance of the *1B allele as a contributing risk factor for aLQTS. The aLQTS patient in our population who carried the *CYP3A4**1B allele also carried the *CYP3A5P1* genotype that is predictive of low CYP3A5 expression. Because of the overlapping substrate specificity of CYP3A4 and CYP3A5, one might speculate that these combined genotypes could represent a risk factor that contributes to the development of aLQTS caused by drugs metabolised by CYP3A, although this needs to be clarified further. Interestingly, none of the four aLQTS patients in our study, which carried the *CYP3A5P1* genotype predictive of high CYP3A5 activity were triggered by a drug that undergoes significant hepatic metabolism. We therefore hypothesise that this genotype might eventually represent a protective factor against the development of aLQTS caused by drugs that are metabolised by CYP3A. Further studies in a larger number of aLQTS patients whose arrhythmias were triggered by drugs that are substrates for CYP3A will be required to validate this hypothesis.

Due to the limited size of the aLQTS group and the different classes of trigger drugs, it is difficult to draw general conclusions. Nevertheless, this study represents the most in depth study of genetic predisposition to aLQTS to date. Sesti *et al.* (8) reported mutational screening in a larger population (n=98), but these authors examined only the intronless *KCNE2* gene compared to all five currently known cLQTS genes examined in the present study. Most likely, the low incidence of the disorder, as well as the difficulty to timely confirm the diagnosis by ECG, might explain why it appears to be difficult to collect a reasonably large group of aLQTS cases. Additionally, the control population does not represent a "true" control group, as it was not matched for age, sex, ethnicity, disease, or drug regime. The assembly of such control group was unfortunately not feasible within the design of our study. Finally, screening of the promoter regions of the LQTS genes could possibly add significant value to the study, but the genomic sequences of these regions were not publicly available when the present mutation screening was performed. We suggest this should be incorporated in future studies.

We initiated this study on a series of aLQTS cases in an attempt to make some generalisations that may be applied when determining susceptibility to TdP. The ability to predict susceptible individuals could save lives as well as allowing many otherwise beneficial drugs to reach or remain on the market. The picture that is developing, however, is that aLQTS results from a number of interacting risk factors, including the specific trigger drug, concurrent medication that competes for the relevant drug metabolising enzyme, a susceptible metabolic phenotype, and other

additional risk factors (e.g., a mutation in one of the cLQTS genes, an underlying heart disease, etc.), in a manner that is unique to the individual. Each of these risk factors contributes only partially to the phenotypic outcome and is usually not sufficient on its own to lead to aLQTS. In conclusion, compared to the familial cases of cLQTS that are usually caused by a specific mutation in a single gene, drug induced LQTS appears to be a complex disease with the interaction of many genes and environmental factors all contributing to an individual-specific risk level for the disease. Further work is needed to establish additional candidate genes that may influence this risk, and to study the importance of such genes in an extended population.

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CHAPTER 8

General discussion

Congenital long QT syndrome

Genetics of the congenital long QT syndrome

Genetic approaches have contributed largely to defining the molecular basis of the long QT syndrome, a disease associated with serious arrhythmias. In the past two decades it has been recognized that at least one of the causes for these arrhythmias are mutations in cardiac ion channel genes. So far, five genes have been causally linked to LQTS. Extensive genetic screening in LQTS families has indicated that these mutations are not confined to a specific location or region, but rather spread over the complete sequence of each gene. Thus, in most families LQTS is caused by a specific, or "private" mutation. Today, genetic screening of LQTS families worldwide has resulted in the identification of more than 200 different mutations in these five different genes (summarized in Appendices 1 to 5). The majority of the mutations are detected in either *KCNQ1* (approximately 40%) or *KCNH2* (approximately 50%).

Genetic testing of patients with clinically diagnosed LQTS can be very useful. Although therapy should be initiated immediately in these cases prior to genetic testing, specific modifications to therapy can be made if the affected gene is known. Genetic testing may, however, be even more useful for the diagnosis of otherwise asymptomatic family members of a proband. Based on the identified causative mutation in the proband, a simple mutation-specific test can identify other "silent" mutation carriers within the family (Chapter 2, 3 and 4). (Asymptomatic) mutation carriers within a LQTS family, who have decided to have children, may be informed about their probability of having an affected child and the accompanying risks involved. A mutation-specific test developed for this particular family can determine the genotype of a newborn shortly after birth.

An important shortcoming today is the relatively low sensitivity of genetic screening for LQTS, indicated by the fact that a mutation is identified in only 50-60% of all families that have been diagnosed with the syndrome (1). This implies that yet other LQTS genes need to be discovered before molecular diagnosis would enable the identification of the causing mutation in all newly presenting LQTS families. Today, therefore, a positive result of a genetic screening of the LQTS genes can confirm the diagnosis, but a negative result does not necessarily exclude the disease in the screened proband and his or her family members. Additionally, although a specific mutation identified in a LQTS family is generally assumed to be 100% predictive for the disease in that particular family, several individual cases (2,3) - including the patient described in Chapter 3 - have been reported to carry more than one causative mutation (compound heterozygosity) in either of the LQTS genes, each with a different contribution to the phenotype. These examples stipulate the importance of a

well set up screening strategy to optimise the outcome. The optimal strategy to follow may depend on many factors, including the applied screening methodology, the number of LQTS families to be screened simultaneously, the family size, and the available clinical data of each of the family members. In a large family with multiple symptomatic members, an identified mutation that is co-segregating with the LQTS symptoms is highly indicative for the causative nature of this mutation, and screening may be terminated at this point. In other cases (e.g. Chapter 3), an identified mutation may not co-segregate with LQTS symptoms or phenotypic characteristics, and screening should be continued. Sometimes, only the proband is symptomatic and no other family members are either available or symptomatic. When a mutation is identified in such patients, additional tests may be required to determine the causative nature of the identified mutation. These tests may include genotyping of a healthy control population to exclude the possibility that this mutation is a common polymorphism. Additionally, functional characterisation of the mutation in an electrophysiological expression system may be performed. Today, however, the latter tests are only performed in a research environment and not in clinical diagnostics.

In genetic research laboratories, one may want to expand the screening sequence to include introns and promoter regions of the LQTS genes. So far, only the coding regions and intron-exon boundaries are screened because mutations in these areas can change protein translation. However, this does not exclude the possibility that intronic or promoter region mutations can effect transcription or expression of the LQTS genes. This additional screening may be performed in LQTS families, in which no mutation was identified with traditional screening. In this respect, advances in high-throughput PCR and mutational screening technologies are expected to provide the tools to more efficiently perform such extended screenings. Also specific micro-arrays are being developed to simultaneously screen for hundreds of different mutations that cause a particular disease, such as LQTS, which may eventually decrease the need for PCR-based screening of all individual exons of genes.

Functional consequences of mutations in LQTS genes

The discovery that potassium and sodium ion channel genes are involved in LQTS has largely contributed to our current understanding of the arrhythmogenic nature of this disease. These ion channels are very important contributors to the cardiac action potential, which in its turn is directly linked to the length of the QT-interval. Therefore, the study of the functional consequences of identified mutations in these ion channel genes might reveal interesting new information to better understand LQTS. However, in contrast to the more general availability of molecular genetic laboratories that can perform mutation analysis of LQTS genes, functional screening of the identified mutations is yet only possible in relatively few specialised research laboratories with specific expertise and specialised “patch clamp” electrophysiology equipment available.

The mutations that have been characterised in oocyte or mammalian expression systems have revealed a broad scale of functional defects, including persistent inward sodium currents caused by non-inactivation (4) and reduced potassium currents caused by non-expression, non-functionality or aberrant protein trafficking (5,6), all leading to prolongation of the action potential. The T65P mutation in HERG described in Chapter 2 exemplifies non-functionality of a mutant protein due to incorrect protein folding and subsequent lack of transportation to the cell membrane. *In vitro* temperature decrease and chemical “chaperone” binding enabled correct trafficking. Because channel function of the rescued channels resembled wild type channels, options for mutation-specific therapy may be possible for this particular mutation. In this respect, an initial study with fexofenadine has shown the ability to rescue trafficking of mutant HERG channels N470D and G601S without blocking them (7). However, this method will only work for some of the mutations that show trafficking deficiency, and these mutations currently represent only a minority. The majority of mutations, such as the ones described in Chapter 4, cause (combinations of) non-expression, non-functionality, or altered function of ion channels. These functional changes pose researchers for much more difficult challenges, because restoration of protein expression or correction of channel kinetic properties is usually much more difficult to accomplish.

In the future, one might possibly find an explanation for the discordance between functional severity of mutations observed *in vitro* and phenotypes observed *in vivo*. It could be expected that patients with dominant negative mutations show more severe phenotypes, although such general genotype-phenotype correlations are not observed. Today, the overall mechanisms underlying fatal arrhythmias in a given phenotype remain rather unclear. Functional studies in either native cells or animal models might increase the insights into these mechanisms (Table 1). Especially the studies of specific mutations that make use of newly developed *in vivo* models are expected to result in more closely related human phenotypes compared to *in vitro* systems.

Table 1. Overview of reported *in vivo* models on LQTS.

Method	Species/cells	Gene/mutation	Ref
Transgenic animal model	mice	<i>KCNQ1</i> knockout	(8)
Transgenic animal model	mice	<i>KCNE1</i> knockout	(10)
Transgenic animal model	mice	<i>KCNE1</i> knockout	(11)
Viral gene transfer	guinea pig myocytes	<i>KCNQ1</i> /G306R	(12)
Transgenic animal model	mice	<i>KCNH2</i> /G628S	(13)
Viral gene transfer	guinea pig	<i>KCNH2</i> /G628S	(14)
Viral gene transfer	guinea pig	<i>KCNE1</i> /D76N	(14)
Transgenic animal model	mice	<i>SCN5A</i> /ΔKPQ	(9)

The number of LQTS mutation or gene knockout studies using *in vivo* native cells or animal models is presently still rather limited (Table 1), but these studies show promising results. For example, the *KCNQ1* knockout mouse (8) may be a good model to study mechanisms involved in JLNS, because several phenotypic characteristics of JLNS patients (deafness, abnormal T-wave morphology, and prolongation of the QT interval) are observed in this model. The *SCN5A* Δ KPQ mouse model (9) correlates well with LQT3 features observed in LQTS patients carrying this mutation.

Genotype-phenotype correlations and clinical implications

Before genetic testing was available, it was assumed that the penetrance of LQTS was 100%, implying that all genetically affected individuals would manifest the typical clinical symptoms of LQTS. However, genetic screening in many LQTS families showed that the penetrance is usually much lower. Earlier studies have shown that several LQTS family members with normal QT intervals experienced syncope or cardiac arrest (15), and that several gene mutation carriers were asymptomatic and possessed normal QT intervals (16). It was recently reported that the penetrance could be as low as 25% (17). Also the families studied in this thesis showed a low penetrance of clinical symptoms, ranging between 18% in the LQTS family with mutation P872fs877 in *KCNH2* (Chapter 4), over 33% in the families with mutations T65P in *KCNH2* (Chapter 2) and V254M in *KCNQ1* (Chapter 3), up to 37% in the family with mutation E698X in *KCNH2* (Chapter 4). An explanation for this variable expression of the phenotype in mutation carriers remains unclear, but it may be beneficial to identify the asymptomatic mutation carriers and inform them about the potential risks and treatments. Treatment of asymptomatic children, of whom it is impossible to determine if or when they will become symptomatic, may be crucial especially because the first phenotypic event of LQTS is sudden death in 30 to 40% of the cases (18).

Increasing evidence indicates that the phenotypic differences in LQTS are not only influenced by a specific mutation in a LQTS gene, but also by the variable function or expression of other modifier genes that may affect susceptibility and severity. Because of the gender differences observed in LQTS phenotypes, it is hypothesized that these modifiers may include sex hormones (19). In addition, variable expression or functioning of other genes influencing the cardiac action potential and/or its response to triggers/stressors, such as adrenergic stimulation, is also thought to play a role (20).

Acquired long QT syndrome

The list of drugs that have been reported to cause marked QT interval prolongation and *torsades de pointes* (TdP) is expanding rapidly. It is well recognized that 1-8% of the patients receiving anti-arrhythmic drugs develop TdP. In the last two decades, however, an expanding number of non-cardiovascular agents including certain antihistamines, antibiotics and antipsychotics have also been implicated in drug-induced ("acquired") LQTS. The list of risk factors contributing to acquired LQTS is also expanding and comprises gender, electrolyte disturbances, other heart disease, concomitant medication and underlying mutations in congenital LQTS genes (Figure 1). Despite the knowledge of these risk factors, the development of TdP in an individual patient remains very unpredictable. Drug action is very variable in the human population. This variability is the result of functional polymorphisms in genes encoding molecules of drug metabolism, molecules that drugs target, or molecules that modulate the overall activity of the environment in which drugs act. For a long time, underlying "forme fruste" mutations in congenital LQTS genes have been assumed often to explain TdP development, because many drugs block the ion channels encoded by these genes. This assumption can be ascribed to the number of case reports describing acquired LQTS individuals in whom such a mutation was detected. Initially, our conviction of the importance of congenital LQTS mutations in acquired LQTS patients was reinforced by the identification of an Y667X mutation in the *KCNH2* gene in a healthy volunteer participating in a clinical drug trial (Chapter 5). Somewhat surprisingly, however, genetic screening studies in larger groups of acquired LQTS patients demonstrated later that an underlying mutation in one of the congenital LQTS genes is found in only a small minority of patients (21, 22, Chapter 7). Presently, the importance of genetic polymorphisms causing inter-individual variation in drug metabolism has been largely unexplored as a risk factor for acquired LQTS. Yet it is well recognized that concomitant medication is a common factor in the majority of acquired LQTS cases. Almost all QT prolonging drugs are, at least in part, metabolised by the hepatic CYP3A family, with CYP3A4 and CYP3A5 being the most important enzymes. Overdosing of a drug, or co-administration of a second drug that competes for metabolism by the same enzyme, can eventually lead to elevated plasma levels of the drug that blocks the cardiac ion channel genes.

Figure 1. Acquired long QT syndrome - a complex genetic disorder. Schematic overview of genetic and environmental factors contributing to acquired long QT syndrome.

The highly variable expression level of CYP3A5 within the general population is well documented, while the relative contribution of the CYP3A5 enzyme to the total CYP3A metabolism is largely unknown. Therefore, we searched for genetic variations in the CYP3A5 gene and identified two DNA polymorphisms in linkage disequilibrium that were predictable for CYP3A5 expression level. A preliminary study in acquired LQTS patients indicated that patients who received drugs that are metabolized by CYP3A4/5 enzymes are more likely to be low CYP3A5 metabolizers, although the number of patients was not large enough to determine statistical significance (Chapter 7). In the future, the statistical significance of the CYP3A5 expression level might be determined if a larger group of well defined acquired LQTS patients could be collected. In this respect, several research groups that collect DNA from drug-induced LQTS patients should intensively collaborate, as it is very difficult to assemble a group with sufficient statistical power. Obviously, screening for functional variations in drug metabolising enzyme genes should not be restricted to CYP3A4/5 and CYP2D6, but should eventually include all genes involved in the metabolism of the drugs that have been taken by acquired LQTS patients.

Another hypothesis that has been proposed to explain the role of genetic predisposition to acquired LQTS is to examine genes involved in the sympathetic nervous system, because adrenergic activation is often a trigger for life-threatening

arrhythmias. Recently, the contribution of five validated non-synonymous coding region polymorphisms in genes encoding the β 1-adrenergic and β 2-adrenergic receptors has been explored in 93 patients with acquired LQTS, but none of these polymorphisms was associated with drug-induced torsades de pointes (23).

Outlook for the future

It is clear that significant progress has been made during the last decade in the elucidation of the genes and molecular mechanisms involved in congenital and acquired LQTS. Nevertheless, it might be expected that a significant amount of additional research will be required to fully understand the factors that modulate the expression of the LQTS phenotype. At this time, the trigger for TdP development in both congenital and acquired LQTS is one of the largest mysteries, and research is expected to focus on this issue. It is believed that this trigger is influenced by several factors, including the adrenergic control of cardiac electrophysiology, sex hormones, interaction of modifying factors with specific genes, and the control of differential ion channel expression. Further exploration of each of these areas may result in a better understanding of the idiosyncratic manner in which TdP develops in both syndromes.

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Summary

Chapter 1 discusses the most common characteristics of long QT syndrome (LQTS). This syndrome is responsible for the majority of unexplained sudden deaths without any recognisable heart disease. The “beating” of a heart is set in motion by a series of electrical impulses that make the heart muscle contract, pump the blood through the body, and supply it with oxygen. In LQTS patients, the balance between these electrical impulses can be suddenly disrupted, which may trigger an uncoordinated heart rhythm. The result is a shortage of oxygen which can lead to sudden loss of consciousness or, when sustained, to sudden death. The syndrome is named after the distance between two characteristic waveforms visible on an ECG, respectively the QRS-complex and the T-wave. In the majority of LQTS patients this distance is too long, resulting in a heartbeat recovery time that is too small to initiate the next one. The chapter describes how ionic currents through ion channels in the cell membrane of ventricular cells are responsible for the balance in the electrical impulses. LQTS is caused by mutations in genes encoding the ion channels and is genetically inherited. The genes that have been identified so far are potassium channel genes *KCNQ1*, *KCNH2* and modulating β -subunits *KCNE1* and *KCNE2*, and sodium channel gene *SCN5A*. The function of the genes is discussed, including the functional effects of mutations in these genes. The second part of the introduction deals with acquired LQTS, a form of LQTS caused by drug intake. All the different drug classes and some additional risk factors that can contribute to acquired LQTS are discussed.

Chapter 2 describes the detection and functional characterisation of a novel missense mutation in the *KCNH2* gene that was detected in a LQTS family. This mutation causes trafficking deficiency of the HERG protein to the cell membrane, where it should function as an ion channel. The trafficking deficiency could be restored by lower incubation temperatures or by incubation with chemical chaperones. Tetramerisation of wild type subunits with mutant subunits was not possible at 37°C, resulting in 50% non-functional channels (haplo-insufficiency) but no dominant negative effect.

Chapter 3 describes a LQTS patient with two different missense mutations in two different LQTS genes. The first detected mutation (A572D, *SCN5A*) could not explain the LQTS symptoms, as this mutation was inherited from the maternal side of the family, while LQTS characteristics were only observed at the paternal side. Further screening of other LQTS genes led to the detection of a missense mutation in another gene (V254 M, *KCNQ1*) that could explain the LQTS characteristics in family members from the paternal side. The observation that a LQTS patient can carry more than one mutation, each with a separate contribution to the phenotype, indicates the importance of a thorough screening of all LQTS genes.

Chapter 4 describes two large LQTS families, each with a different mutation that was identified in the *KCNH2* gene, respectively E698X and P872fs877. Both mutations lead

to premature stop codons resulting in truncated HERG proteins. Only the HERG P872fs877 truncated protein was expressed; the HERG E698X protein was hardly detectable. The P872fs877 HERG protein was functional although transport of the protein to the cell membrane was disrupted by partial retention of the proteins in the endoplasmic reticulum. The kinetics of homo-multimeric HERG P872fs877 were comparable to wild type homo-multimers. However, with formation of hetero-multimers, kinetic differences as compared to wild type kinetics were observed. In summary, the E698X mutation leads to 50% haplo-insufficiency and the P872fs877 mutation leads to partial haplo-insufficiency combined with a kinetically dominant negative effect on channel function.

Chapter 5 illustrates the connection between the congenital LQTS and acquired LQTS by genetic screening of a healthy volunteer participating in a clinical trial. The volunteer was screened for mutations in all LQTS genes because of an observed prolongation of the QT-interval in response to drug treatment. A mutation in the *KCNH2* gene (Y667X) was identified that was inherited from the maternal side. Both mother and son had a baseline prolongation of the QT-interval, although both were asymptomatic. This study shows that the percentage of congenital LQTS patients in the general population is underestimated, and that especially these asymptomatic, unidentified LQTS patients may be vulnerable to the development of arrhythmia by drug intake.

Chapter 6 describes the search for a genetic background in variable CYP3A5 expression. Both the CYP3A4 and CYP3A5 proteins are responsible for the metabolism of many endo- and exogenous compounds and show substantial overlapping substrate specificity. Many drugs that might induce acquired LQTS are metabolised by these enzymes. Human CYP3A5 expression in the liver is highly variable: CYP3A5 expression can be detected in only 10-30% of Caucasians. We identified two polymorphisms in the promoter region of the *CYP3A5P1* gene that were in linkage disequilibrium. The less frequent allele was associated with high expression levels of CYP3A5 mRNA and protein in the liver. The Caucasian population frequency of this allele was 18%, in line with the percentage in the population that shows high CYP3A5 expression. The observations of chapter 5 and 6 were investigated in a population of 32 acquired LQTS patients (Chapter 7). Only a minority of these patients (6%) was carrier of a mutation in one of the congenital LQTS genes. The patients in which arrhythmia were provoked by a CYP3A4/5 substrate were all carrier of the *CYP3A5P1* genotype predictive of low CYP3A5 expression. Although statistical significance needs to be explored in a larger acquired LQTS population, one factor that influences the risk for acquired LQTS might be an impaired ability of CYP3A5 metabolism.

In summary, all the observations from previous chapters are discussed in Chapter 8. The added value but also shortcomings of genetic screening in congenital and acquired LQTS are discussed as well as the novel insights into the functional consequences of identified mutations. The chapter is concluded with possible future steps in the better understanding of several features characteristic for both congenital and acquired LQTS syndrome.

Samenvatting

De voornaamste karakteristieken van het verlengde QT-interval syndroom (LQTS) worden besproken in Hoofdstuk 1. Deze aandoening is verantwoordelijk voor het grootste percentage aan plotseling overlijden zonder verdere aantoonbare hartproblemen. Het “kloppen” van het hart wordt veroorzaakt door een aaneenschakeling van specifieke elektrische impulsen die de hartspier doen samentrekken, het bloed rondpompt en hiermee het lichaam van zuurstof voorziet. Bij LQTS patiënten kan de coördinatie tussen deze elektrische impulsen verstoord raken, waardoor een ongecontroleerd hartritme ontstaat. Het gevolg is een tekort aan zuurstof dat kan leiden tot bewusteloosheid en eventueel ook tot overlijden. Een typisch kenmerk van deze aandoening is de verlengde afstand tussen het QRS-complex en de T-golf op een electrocardiogram. Door deze verlenging is bij de meeste LQTS patiënten de tijdsduur van hartslagen dusdanig lang dat de benodigde, tussenliggende herstelperiode in het gedrang komt. Hoofdstuk 1 beschrijft dat ionenkanalen in de celmembraan van ventriculaire hartcellen verantwoordelijk zijn voor de elektrische impulsbalansen. Congenitale LQTS is een erfelijke aandoening die wordt veroorzaakt door mutaties in genen die coderen voor deze ionenkanalen. De genen die tot nu toe zijn geïmpliceerd in congenitale LQTS, zijn de kaliumkanalen *KCNQ1*, *KCNH2* en hun bijbehorende, modulerende β -subeenheden *KCNE1* en *KCNE2*, en het natriumkanal *SCN5A*. De functie van deze genen wordt besproken, alsook de functionele effecten die mutaties in de genen tot gevolg kunnen hebben. Het tweede deel van de inleiding bespreekt de verworven vorm van LQTS. Er wordt een overzicht gegeven van de verschillende medicijnklassen die deze vorm kunnen veroorzaken en een aantal belangrijke additionele risicofactoren. Hoofdstuk 1 wordt afgesloten met een overzicht van de doelstellingen van dit proefschrift.

Hoofdstuk 2 beschrijft de detectie en functionele karakterisatie van een nieuwe mutatie in het *KCNH2* gen die werd gedetecteerd in een LQTS familie. Deze mutatie verhindert een normaal transport van het eiwit (HERG) naar de celmembraan, waar het zijn functie als ionenkanaal dient te vervullen. Dit transportprobleem kon gecorrigeerd worden door de omgevingstemperatuur te verlagen of door chemische stoffen aan het eiwit te binden. Omdat tetramerisatie van wild type met gemuteerde subeenheden niet mogelijk was op 37°C, is de helft van het totaal aantal kanalen niet functioneel (haploinsufficiëntie) en kan men niet spreken van een dominant negatief effect.

Hoofdstuk 3 beschrijft een LQTS patient waarin twee missense mutaties in twee verschillende LQTS genen werden gevonden. De eerst gevonden missense mutatie (A572D, *SCN5A*) kon geen verklaring geven voor de LQTS symptomen omdat die werd overgeërfd langs de maternale tak van de familie terwijl LQTS karakteristieken alleen in de paternale tak voorkwamen. Door verdere screening van andere LQTS genen werd een tweede missense mutatie gevonden in een ander gen (V254M, *KCNQ1*), die de

LQTS karakteristieken in de familieleden langs paternale kant wél kon verklaren. De observatie dat een LQTS patiënt meer dan één mutatie kan dragen, elk met een verschillende pathogene bijdrage tot het fenotype, geeft het belang van een goede strategie voor grondige screening van alle gekende LQTS genen aan.

Hoofdstuk 4 beschijft twee grote LQTS families waarin een causale mutatie werd gevonden in het *KCNH2* gen voor elke familie, respectievelijk E698X en P872fs877. Beide mutaties leiden tot een prematuur stopcodon waardoor een gedeelte van het C-terminale eindstuk van het eiwit ontbreekt. Enkel het P872fs877 gemuteerde HERG eiwit kwam tot expressie, terwijl het E698X HERG eiwit nauwelijks aantoonbaar was. Het P872fs877 HERG eiwit was functioneel, maar het transport naar de celmembraan bleek verstoord door een gedeeltelijke retentie in het endoplasmatisch reticulum. De kinetiek van P872fs877 mutante homo-multimeren was vergelijkbaar met wild type homo-multimeren, maar bij de vorming van hetero-multimere kanalen ontstaan kinetische verschillen ten opzichte van wild type kanalen. Mutatie E698X geeft dus aanleiding tot 50% haploinsufficiëntie; mutatie P872fs877 resulteert in haploinsufficiëntie gecombineerd met een kinetisch dominant negatief effect.

Hoofdstuk 5 illustreert de link tussen congenitale en verworven LQTS. Het genomische DNA van een gezonde vrijwilliger, die deelnam aan een klinische studie, werd gescreend voor mutaties in LQTS genen vanwege een verlenging van het QT interval die werd geobserveerd als respons op de toediening van medicatie. Een *KCNH2* nonsense mutatie (Y667X) werd gevonden, die was overgeërfd van zijn moeder. In beide familieleden bleek het QT interval verlengd, maar verder waren beiden asymptomatisch. Deze studie toont aan dat de frequentie van congenitaal LQTS in de normale bevolking mogelijk anders wordt en dat de asymptomatische LQTS patiënten meer gevoelig kunnen zijn voor het ontstaan van ritmestoornissen door medicatie.

In Hoofdstuk 6 werd gezocht naar een genetische achtergrond van variabele CYP3A5 expressie. De CYP3A4 en CYP3A5 eiwitten zijn betrokken in het metabolisme van vele endo- en exogene stoffen, en vertonen qua substraatspecificiteit veel overlap. Een groot deel van de medicijnen die verworven LQTS kunnen veroorzaken worden door deze enzymen afgebroken. De expressie van CYP3A5 is zeer variabel: in slechts 10-30% van de Caucasische populatie kan CYP3A5 expressie worden aangetoond in de lever. Door middel van mutatiescreening ontdekten we twee DNA polymorfismen in de promotor regio van het *CYP3A5P1* gen, die in linkage disequilibrium zijn. Het minst frequente allel van deze polymorfismen is geassocieerd met een hogere, hepatische expressie van CYP3A5 mRNA en eiwit. De Caucasische populatiefrequentie van dit allel bedroeg 18%, in lijn met het percentage van de populatie dat hogere CYP3A5 expressie vertoont.

In Hoofdstuk 7 werden de bevindingen van hoofdstuk 5 en 6 in een groep van 32 patiënten met verworven LQTS getest. Slechts een klein percentage (6%) van deze patiënten blijkt drager van een overgeërfd mutatie in een LQTS gen. De patiënten waarin LQTS was geïnduceerd door een CYP3A4/CYP3A5 substraat bezaten allen een

CYP3A5P1 genotype dat is geassocieerd met een lagere *CYP3A5* expressie. Deze observatie dient in de toekomst in een grotere patiëntengroep bekeken te worden om de statistische relevantie te kunnen berekenen.

Tenslotte worden de bevindingen van de voorgaande hoofdstukken bediscussieerd in Hoofdstuk 8. De toegevoegde waarde, maar ook de beperkingen, van mutatiescreening van LQTS genes in congenitaal en verworven LQTS patiënten wordt besproken, alsook de nieuwe inzichten in de functionele consequenties van de gevonden mutaties. Tenslotte worden mogelijkheden voorgesteld voor verder onderzoek in beide vormen van LQTS, die kunnen leiden tot een verbeterd inzicht in de aandoeningen en efficiëntere therapieën.

Dankwoord

Wanneer je kunt spreken van een aparte manier van promoveren, dan denk ik dat mijn promotieperiode daar een goed voorbeeld van is. Na mijn studie Scheikundige Technologie aan de TU Eindhoven wist ik al snel dat de genetica mijn interesse had gewekt en dat ik daarin verder wilde gaan. Als Research Assistant bij Janssen Pharmaceutica in de afdeling Experimentele Moleculaire Biologie onder leiding van Martin Armstrong en Walter Luyten, deed ik meer ervaring op in het vakgebied. Eind 1998 kreeg ik de kans om binnen Janssen mijn promotie-onderzoek te beginnen. Met steun van Martin en Walter gaf Frank Konings mij toestemming om op het long QT project mijn promotie uit te voeren. Prof. dr. Marcel Borgers, die zowel binnen Janssen als op de Universiteit Maastricht werkzaam was, stemde toe om als mijn eerste Promotor op te treden. Mede dankzij hen is dit boekwerk tot stand gekomen en ik wil al deze mensen daarom enorm bedanken voor hun aanmoediging en steun die ik de afgelopen vier jaar van hen heb gekregen.

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clamp" electrophysiology, een zeer interessante, doch moeilijke techniek. Deze techniek heeft enorm veel bijgedragen aan mijn proefschrift en ik wil Dirk en Adam dan ook heel hartelijk bedanken voor hun goede begeleiding. Ook de andere mensen van dit labo, te weten Diane, Natascha, Alain, Wim, Iris, Evy en Tine wil ik graag bedanken voor hun hulp en gezelligheid.

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Curriculum vitae

Aimée Paulussen werd geboren op 17 december 1971 te Middelburg. Van 1984 tot 1990 doorliep zij het Voortgezet Wetenschappelijk Onderwijs (VWO) aan het Newman College in Breda. Vervolgens volgde zij de opleiding Scheikundige Technologie aan de Technische Universiteit Eindhoven (TUE). Tijdens deze opleiding deed zij haar bedrijfsstage bij PURAC Biochem in Gorinchem. Haar afstudeerstage volgde zij bij het Centraal Hematologisch Laboratorium (CHL) in het Academisch Ziekenhuis St. Radboud te Nijmegen. Na het behalen van haar ingenieursdiploma eind 1996, begon zij in 1997 haar loopbaan als research assistant bij Janssen Pharmaceutica in Beerse, België. Eind 1998 begon zij binnen Janssen Pharmaceutica aan haar PhD project over long QT syndrome en werd tegelijkertijd als Assistant in Opleiding (AIO) aangesteld aan de Universiteit Maastricht (Promotor: Prof. dr. Marcel Borgers, co-promotores: Dr. ir. Jeroen Aerssens, Dr. Bert Smeets).

Sinds 1 februari 2003 is Aimée aangesteld als Post-Doc aan de Universiteit Maastricht in de Department Genetics & Cell Biology, sectie Population Genetics, Genomics and Bioinformatics.

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APPENDIX 1: *KCNQ1* mutations

Nucleotide change	Coding change	Region	#fam	Type	Functional effect	Dom. negative	Ref.
211-219del	71-73delAAP	N-term	1	RWS			(30)
332 A>G	Y111C	N-term	1	RWS			(21)
341 T>C	L14P	N-term	1	RWS			(109)
350 C>T	P117L	N-term	1	SIDS			(43)
365 T>C	L122P	S2	1	RWS			(17)
	R130C	S2	1	RWS			(35)
451delCT		S2	1	JLNS			(15)
470 T>G	F157C	S2	1	RWS			(44)
477 + 1 G>A	-	S2	1	JLNS			(21)
477 + 5 G>A	-	S2	1	JLNS			(18)
478 G>A	E160K	S2	1	RWS			(21)
500-502del (ΔTCG)	F167W/ del168G	S2	1	RWS			(1)
502 G>C/A	G168R	S2	8	RWS			(4), (8), (21), (109)
504delG	-	S2	1	RWS			(16)
513 C>G	Y171X	S2-S3	1	JLNS			(45)
520 C>T	R174C	S2-S3	1	RWS	non-functional	yes	(4), (5)
521 G>A	R174H	S2-S3	1	RWS			(21)
532 G>C	A178P	S2-S3	1	RWS			(1)
532 G>A	A178T	S2-S3	1	RWS			(19)
535 G>A	G179S	S2-S3	1	RWS			(21)
551 A>C	Y184S	S2-S3	2	RWS			(21), (34)
565 G>A	G189R	S2-S3	3	RWS			(1), (35)
567insG	G189fs/94	S2-S3		RWS			(21)
569 G>A	R190Q	S2-S3	2	RWS	non-functional	no	(1), (4), (18)
572 T>C	L191P	S2-S3	1	RWS (Chinese)			(110)
572del5	-	S2-S3	7	JLNS	non-functional	no	(31), (32), (38)
580 G>C	A194P	S2-S3	1	RWS			(21)
639 + 5 G>A	-	S3	1	RWS			(30)
674 C>T	S225L	S4	2	RWS (r)	v.d. activation: pos shift	yes	(21), (22), (37)
724 G>A	D242N	S4-S5	1	RWS			(36)
727 C>T	R243C	S4-S5	2	RWS			(21)
728 G>A	R243H	S4-S5	1	JLNS	v.d. activation/ inactivation: pos shift, d.r.↑	yes	(13), (14), (18)
742 C>T	W248R	S4-S5	1	RWS	v.d. activation/i nactivation: pos shift, a.r.↓	yes	(14), (21)
749 T>A	L250H	S4-S5	1	RWS			(36)

760 G>A	V254M	S4-S5	6	RWS			(1), (2), (4), (21)
775 C>T	R259C	S4-S5	2	RWS/ acquired LQTS	reduced amplitude	yes	(33), (109)
783 G>C	E261D	S4-S5	1	RWS	non-functional	yes	(32), (38)
781 G>A	E261K	S4-S5	1	RWS	non-functional	no	(4), (14)
797 T>C	L266P	S5	1	RWS			(21)
805 G>A	G269S	S5	1	RWS			(30)
806 G>A	G269D	S5	3	RWS	non-functional	yes	(4), (5), (21)
817 C>T	L273F	S5	3	RWS			(1), (21), (38)
824 T>C	F275S	S5	1	RWS (Chinese)			(110)
830 T>C	S277L	S5	1	RWS (Chinese)			(110)
842 A>G	Y281C	S5	1	RWS (r)	non-functional	yes	(22), (37)
898 G>A	A300T	S5-pore	1	RWS (r)	v.d.activation: neg shift	no	(23), (37)
914 G>C	W305S	pore	2	JLNS	non-functional	yes	(5), (9)
916 G>A	G306R	pore	1	RWS	non-functional	yes	(1), (7)
917 G>T	G306V	pore	1	RWS (Chinese)			(110)
921 +1 G>T	-	pore	1	RWS			(21)
922 -1 G>C	-	pore	1	RWS			(12)
922 -2 A>C	-	pore	1	RWS			(21)
926 C>G	T309R	pore	1	RWS			(4)
928 G>A	V310I	pore	1	RWS			(21)
932 C>T	T311I	pore	1	RWS	non-functional	yes	(6), (26)
935 C>T	T312I	pore	2	RWS	non-functional	yes	(1), (7), (21)
939 C>G	I313M	pore	1	RWS			(19)
940 G>A	G314S	pore	6	RWS	non-functional	yes	(4), (5), (7), (8), (25), (36)
944 A>C	Y315S	pore	2	RWS	non-functional	yes	(4), (5)
944 A>G	Y315C	pore	3	RWS, acquired LQTS	non-functional	yes	(8), (22), (37), (41)
946 G>C	G316R	pore	1	RWS			(109)
949 G>A	D317N	pore	2	RWS	non-functional	yes	(7), (26)
954 G>C	K318N	pore	1	RWS			(8)
958 C>G	P320A	pore	1	RWS			(4)
973 G>A	G325R	S6	4	RWS			(4), (19), (21)
1008delC	-	S6	1	JLNS	non-functional	no	(39), (38)
1015- 1017del	delF339	S6	1	RWS			(46)
1017- 1019del	delF340	S6	1	RWS			(21)
ΔGGT	SP/V341	S6	1	RWS			(10)

1022 C>A	A341E	S6	5	RWS			(1), (20), (21)
1022 C>T	A341V	S6	9	RWS			(1), (4), (10), (25), (27), (109)
1024 C>T	L342F	S6	1	RWS	non-functional	yes	(4), (5)
1031 C>T	A344V	S6	1	RWS			(4)
1032 G>A	A344A	S6	9	RWS			(10), (11), (12), (21), (36)
1032 G>C	A344A	S6	1	RWS			(12)
1033 G>C	G345R	S6	1	RWS			(28)
1033 G>A	G345E	S6	1	RWS			(1)
1046 C>G	S349W	S6	1	RWS			(21)
1058 T>C	L353P	S6	1	RWS			(8)
1066 C>T	Q356X	S6	1	RWS			(21)
1096 C>T	R366W	S6-Cterm	1	RWS			(8)
1097 G>A	R366Q	S6-Cterm	1	RWS			(21)
1097 G>C	R366P	S6-Cterm	1	RWS			(19)
1111 G>A	A371T	S6-Cterm	1	RWS			(4)
1117 T>C	S373P	S6-Cterm	1	RWS			(34)
1172 C>T	T391I	S6-Cterm	1	RWS			(21)
1174 C>T	W329R	S6-Cterm	1	RWS			(34)
1188delC	-	S6-Cterm	1	JLNS			(16)
1343delC	-	S6-Cterm	1	RWS			(24)
1343 C>G	P448R	S6-Cterm	2	RWS			(21)
1552 C>T	R518X	S6-Cterm	9	JLNS/RWS	non-functional	no	(16), (21), (29), (32), (38)
1557 G>A	K557E	S6-Cterm	1	RWS			(109)
1573 G>A	A525T	S6-Cterm	1	RWS (r)			(29)
1588 C>T	Q530X	S6-Cterm	5	JLNS/ RWS	non-functional	no	(21), (32), (38)
1597 C>T	R533W	S6-Cterm	1	RWS (r)	v.d.activation: pos shift, d.r.↑	yes	(18)
1615 C>T	R539W	S6-Cterm	1	RWS	v.d.activation: pos shift, d.r.↑	yes	(18)
1630 -7 +8	-	S6-Cterm	2	JLNS	non-functional	yes	(3), (5)
1663 C>T	R555C	C-term	3	RWS	v.d.activation: pos shift, deact. kin ↑	yes	(4), (5)
1697 C>T	S566F	C-term	3	RWS			(21)
1747 C>T	R583C	C-term	1	Acquired LQTS	Reduced amplitude v.d.activation.: pos. shift		(42)
1760 C>T	T587M	C-term	2	JLNS/ RWS			(24), (36)
1766 G>A	G589D	C-term	34/2	RWS/			(45)

	(Finnish founder)			JLNS			
1772 G>A	R591H	C-term	1	RWS			(24)
1781 G>A	R594Q	C-term	4	RWS	non-functional	no	(21), (38), (109)
1892del20	-	C-term	1	JLNS			(24)
1893insC	-	C-term	1	RWS			(24)
1927 G>A	G643S	C-term	6 (p)	RWS	reduced amplitude	no	(41)

Numbering from ATG startcodon in isoform 1 (Genbank accession number AF000571). RWS=Romano-Ward syndrome, JLNS=Jervell and Lange-Nielsen syndrome, SIDS= sudden infant death syndrome. (p)= polymorphism, (r)= recessive, v.d. = voltage dependance, d.r.= deactivation rate, a.r.= activation rate, pos=positive, neg=negative

APPENDIX 2: *KCNH2* mutations

Nucleotide change	Coding change	Region	#fam	Functional effect	Dominant negative	Rescue	Refs.
77 G>T	S26I	PAS	1				(71)
87 C>A	F29L	PAS	1	d.r. ↑			(21), (59)
98 A>C	N33T	PAS	2	d.r. ↑			(21), (59)
131 G>T	C44F	PAS	1				(78)
132 C>A	C44X	PAS	1				(21)
140 G>T	G47V	PAS	1				(21)
157 G>C	G53R	PAS	1	d.r. ↑			(21), (59)
167 G>A	R56Q	PAS	1	d.r. ↑	no(amp) yes(kinetics)		(21), (59)
172 G>A	E58K	PAS	1				(78)
193 A>C	T65P	PAS	1	Trafficking deficient	no	T + C	(58)
196 T>G	C66G	PAS	1	d.r. ↑			(21), (59)
209 A>G	H70R	PAS	2	d.r. ↑			(21), (59)
215 C>A	P72Q	PAS	2				(21)
221-251del	R73fs/31	PAS	1				(21)
232 G>C	A78P	PAS	1	d.r. ↑			(21), (59)
234-250dup	A83fs/37	PAS	1				(21)
241 C>T	Q81X	PAS	1				(21)
257 T>G	L86R	PAS	1	d.r. ↑			(21), (59)
260 T>C	L87P	PAS	1				(109)
296 A>C	Y99S	PAS	1				(109)
340 C>T	P114S	PAS	1				(78)
371 T>G	M124R	PAS-S1	1				(71)
422-423insC	P141fs/2	PAS-S1	1				(21)
453delC	-	PAS-S1	1				(64), (73)
453-454insC	P151fs/179	PAS-S1	1				(21)
526 C>T	R176W	PAS-S1	1				(64), (73)
558-600dup	-	PAS-S1	1				(74)
582-	G192fs328	PAS-S1	1				(109)
587delCCGTG							
724-725insC	P241fs/89	PAS-S1	1				(21)
885delC	V295fs/63	PAS-S1	1				(21)
934 C>T	R312C	PAS-S1	1				(21)
1001 C>T	P334L	PAS-S1	1				(78)
1009insT	336fs355	PAS-S1	2				(78)
1039 C>T	P347S	PAS-S1	3				(21), (109)
1128 G>A	Q376sp	PAS-S1	1				(21)
1129 -2 A>G	Q376sp	PAS-S1	1				(21)
1238 T>C	L413P	S1	1				(110)
	(Chinese)						
1261delA	Y420fs12	S1	1	non-functional	no		(47), (51)

1283 C>T	S428L	S1-S2	1				(71)
1283 C>A	S428X	S1-S2	1				(22)
1307 C>T	T436M	S1-S2	1				(22)
1332 G>T	E444D	S1-S2	1				(110)
	(Chinese)						
1341 C>A	Y447X	S1-S2	1				(109)
1352 C>T	P451L	S2	1				(64)
1408 A>G	N470D	S2	1	Trafficking deficient	yes	yes (T+C)	(47), (51), (57)
1421 C>T	T474I	S2-S3	1	v.d. act: neg shift			(19)
1468 G>A	A490T	S2-S3	1	Reduced amplitude			(77)
1479 C>G	Y493X	S2-S3	1				(52)
1498-1525del	ΔI500-F508	S3	1	non-functional	no		(47), (51)
1501 A>G	D501N	S3	1				(109)
1592 G>A	R531Q	S4	1				(21)
1600 C>T	R534C	S4	2	v.d. act.: neg shift, a.r.↑, d.r.↑, s.s.i. ↓	no		(52), (60), (62)
1631delAG	-	S4-S5	1				(64), (73)
1655 T>C	L552S	S5	3				(21), (73), (111)
	(Finnish founder)						
1671delT	T556fs7	S5	1				(21)
1672 G>C	A558P	S5	1				(34)
1676 T>A	L559H	S5	1				(110)
	(Chinese)						
1681 G>A	A561T	S5	4				(48), (21)
1682 C>T	A561V	S5	6	Trafficking deficient	yes	no (T+C)	(47), (51), (19), (21), (66), (69), (22)
1691 T>C	L564P	S5	1				(75)
1704 G>T	W568C	S5	1				(78)
1705 T>C	Y569H	S5	1				(64), (73)
1714 G>T	G572C	S5-pore	1				(8)
1714 G>C	G572R	S5-pore	1				(63)
1724 A>G	E575G	S5-pore	1				(78)
1744 C>T	R582C	S5-pore	1				(34)
1750 G>A	G584S	S5-pore	2				(21), (64), (73)
1755 G>T	W585C	S5-pore	1				(21)
1762 A>G	N588D	S5-pore	1				(8)
1777 A>T	I593X	S5-pore	1				(71)
1778 T>G	I593R	S5-pore	1	non-functional			(49), (54)
1778 T>C	I593T	S5-pore	1				(21)
1787 C>T	P596L	S5-pore	1				(71)
1801 G>A	G601S	S5-pore	2	Trafficking	no		(55), (56),

				deficient			(64), (73)
1810 G>A	G604S	S5-pore	2				(21), (34)
1825 G>A	D609N	S5-pore	2				(21), (78)
1831 T>C	Y611H	S5-pore	1	Trafficking deficient			(19), (54)
1834 G>T	V612L	S5-pore	1	non-functional	yes		953),(70)
1838 C>T	T613M	pore	4	non-functional	yes		(21),(64), (70), (34)
1841 C>T	A614V	pore	7				(8), (19), (53),(22), (76)
1843 C>G	L615V	pore	1	reduced current	yes		(21), (70)
1862 G>A	S621N	pore	1				(78)
1876 G>A	G626S	pore	1				(21)
1881 C>G	F627L	pore	1				(21)
1882 G>A	G628S	pore	2	non-functional	yes		(47),(51), (54), (21)
1885 A>G	N629D	pore	1	gain of function	yes		(53), (68)
1886 A>G	N629S	pore	1				(53)
1887 C>A	N629K	pore	1	non-functional	yes		(61)
1888 G>C	V630L	pore	1				(19)
1889 T>C	V630A	pore	1				(8)
1894 C>T	P632S	pore	1				(21)
1898 A>G	N633S	pore	1				(53)
1909 G>A	E637K	pore-S6	1	non-functional	yes		(72)
1912 A>G	K638E	S6	1				(21)
1913-1915del	K638del	S6	1				(21)
1920 C>A	F640L	S6	1				(34)
1933 A>T	M645L	S6	1				(21)
1951-	-	S6	1				(52)
1952delAT							
2001 C>A	Y667X	S6-cNBD	1	non-functional	no		(65)
2044 G>T	E682X	S6-cNBD	1				(21)
2092 G>T	E698X	S6-cNBD	1				(109)
2173 C>T	Q725X	S6-cNBD	1				(52)
2218-	H739fs/63	S6-cNBD	1				(21)
2219insT							
C>T	R744X	S6-cNBD	1				(71)
2254 C>T	R752W	S6-cNBD	1	Trafficking deficient	no	yes(T) no (C)	(21), (67)
2350 C>T	R784W	cNBD	1	Reduced amplitude v.d.a.: pos shift			(42)
2356-2376dup	31bp dup	cNBD	1				(52)
2395delC	I798fs/10	cNBD	1				(21)
2398 +1 G>C	L799sp	cNBD	2				(47), (21)
2414 T>C	F805S	cNBD	1				(21)
2414 T>G	F805C	cNBD	1				(21)

2453 C>T	S818L	cNBD	1		(20)
2464 G>A	V822M	cNBD	2	Trafficking deficient	(50).
					(54), (20)
2467 C>T	R823W	cNBD	2		(21)
2471insG	R823fs828	cNBD	1		(109)
2582 A>T	N861I	C-term	1		(21)
2592 +1 G>A	D864sp	C-term	2		(20), (21)
2616delC	P872fs877	C-term	1		(109)
2660delG	K886fs/85	C-term	1		(21)
2750 C>T	P917L	C-term	1		(21)
2762delA	R920fs/51	C-term	1		(21)
2764 C>T	R922W	C-term	1		(21)
2775-	G925fs/13	C-term	1		(21)
2776insG					
2879 G>A	S960N	C-term	1		(78)
2906delG	P968fs/4	C-term	1		(21)
2959-	P986fs/130	C-term	1		(21)
2960delCT					
-	W1001X	C-term	1		(71)
3040 C>T	R1014X	C-term	2		(21)
3094delC	G1031fs/24	C-term	1		(21)
3108insG	-	C-term	1		(20)
3303-	P1101fs	C-term	1		(21)
3304insC					

Numbering from ATG startcodon (Genbank accession number U04270), fs= last amino-acid unaffected by a frameshift (number after fs is the number of amino acids before termination), sp= last unaffected amino acid before predicted splice mutation, T=lower cell culture incubation temperature, C=incubation with chemical chaperones, v.d. a=voltage dependance of activation, a.r.=activation rate, d.r.=deactivation rate, s.s.i.=steady-state inactivation.

APPENDIX 3: *SCN5A* mutations

Nucleotide change	Coding change	Region	# fam	Functional effect	Phenotype	Refs
IVS7DS +4 InsAA	-	DI S5-S6	1		IVF	(85)
1715 C>A	A572D	DI-DII	1		RWS	(2)
1844 G>A	G615E	DI/DII	1	No effect (without drug)	acquired	(42)
1852 C>T	L618F	DI/DII	1	No effect (without drug)	acquired	(42)
2971 AA>TC	S941N	DII-DIII	1	Persistent and increased inward current	SIDS/ RWS	(96)
2989 G>T	A997S	DII S6-DIII S1	1	Persistent and increased inward current	SIDS	(94)
3340 G>A	D114N	DII-DIII	1		RWS	(21)
3694 C>T	R1232W +	DIII S1-S2	1	v.d. s.s.i.: pos shift, recov inact: ↑	BrS	(85)
4859 C>T	T1620M	DIV S3-S4	1			
3748 T>C	F1250L	DIII S2	1	No effect (without drug)	acquired	(42)
3883 G>A	E1295K	DIII S4	1	Pos. shift window current	RWS	(91)
IVS22 +2 T>C	-	DIII S4	1		PCCD	(89)
3912 C>T	T1304M	DIII S4	1		RWS	(97)
3974 A>G	N1325S	DIII S4-S5	1	Inactivation ↓, I_{Na} ↑	RWS	(80), (82)
3988 G>C	A1330P	DIII S4-S5	1	v.d. s.s.i.: pos shift, inactivation ↓, recov. inact. ↑	SIDS	(92)
4190delA	V1398X	DIII S6	1		IVF	(85)
4216 G>T	G1406R	DIII S5-S6	1	non-functional	BrS/ ICCD	(93)
	1500ΔK	DIII-DIV	1	Inactivation: neg shift, Activation: pos shift	Combi of LQTS, BrS, ICCD	(112)
4501 C>G	L1501V	DIII-DIV	1		RWS	(21)
4511-	1505-	DIII-DIV	4	Inactivation ↓, I_{Na} ↑	RWS	(79), (80), (81), (82)
4520del	1507ΔKPQ					(21)
4850- 4852del	DelF1617	DIV S3-S4	1		RWS	(21)
4868 G>A	R1623Q	DIV S4	2	Inactivation ↓↓, I_{Na} ↑↑	RWS	(83), (88), (21)
4868 G>T	R1623L	DIV S4	1		RWS	(21)
4931 G>A	R1644H	DIV S4	2	Inactivation ↓, I_{Na} ↑	RWS	(80), (82), (21)

4934 C>T	T1645M	DIV S4	1		RWS	(97)
4999 G>A	V1667I	DIV S5	1		RWS/ acquired	(98)
5280 delG	-	DIV S5-S6	1		PCCD	(89)
5298 A>C	M1766L	DIVS6- C-term	1	persistent late sodium current	RWS	(99)
5329 G>A	V1777M	C-term	1	v.d. s.s.i.: neg shift, v.d. s.s.a.: pos shift, persistent inward current	RWS(r)	(95)
5349 G>A	E1784K	C-term	2	Inactivation ↓, I_{Na} ↑	RWS	(90), (21)
5360 G>A	S1787N	C-term	1		RWS	(21)
5369 A>G	D1790G	C-term		Inactivation: neg shift interaction with β- subunit, persistent inward current	RWS	(84)
5383 T>C	Y1795H	C-term	1	channel avail: neg shift reduced ampl, inact ↑	BrS	(87)
5384 A>G	Y1795C	C-term	1	Inactivation ↓	RWS	(87)
5385 insTGA	1795 insD	C-term	1	v.d. s.s.i.: neg shift v.d. s.s.a.: pos shift, reduced window current	RWS/IVF	(86)
5474 T>C	L1825P	C-term	1	Slow I_{Na+} decay, with non-inactivating component, loss of function	acquired	(100)
5477 G>A	R1826H	C-term	1	Persistent and increased inward current	SIDS	(94)
5516 A>G	D1840G	C-term	1		RWS	(101)

Numbering from startcodon ATG (Genbank accession number M77235). PCCD= progressive cardiac conduction defect, ICCD= isolated cardiac conduction defect, IVF= idiopathic ventricular fibrillation, BrS=Brugada syndrome, RWS=Romano-Ward syndrome, SIDS= Sudden Arrhythmic Death Syndrome, (r)= recessive form. v.d.a. =voltage dependence of activation, a.r.=activation rate, s.s.i.=steady-state inactivation., s.s.a.=steady-state activation.

APPENDIX 4: *KCNE1* mutations

Nucleotide change	Mutation	#fam	Phenotype	Functional effect	Dominant negative	Refs
20 C>T+226 G>A	T71+D76N	1	JLNS			(103)
95 G>A	R32H	1	RWS			(21)
139 G>T+	V47F+	1	JLNS	Trafficking deficient (L51H), reduced amplitudes (V47F), d.r. ↑, v.d.a.: pos shift (V47F)	no yes	(105)
152 T>A	L51H					
172 A>C, 176 T>C, 177 G>T	T59P+	1	JLNS		no	(31)
221 C>T	L60P					
	S74L	1	RWS	d.r. ↑, v.d.a.: pos shift		(102)
226 G>A	D76N	2	RWS (d) JLNS(r)	Reduced amplitude, d.r. ↑, v.d.a.: pos shift	yes	(102), (104), (105)
259 T>C	W87R	1	RWS	v.d.a.: pos shift, d.r. ↑		(105)
292 C>T	R98W	1	RWS			(21)
325 G>A	V109I	1	RWS			(106)
379 C>A	P127T	1	RWS			(21)

Numbering from ATG startcodon (Genbank accession number L28168). RWS=Romano-Ward syndrome, JLNS=Jervell and Lange-Nielsen syndrome, d=dominant, heterozygous, r=recessive, homozygous. v.d. a=voltage dependance of activation, a.r.=activation rate, d.r. = deactivation rate, s.s.i.=steady-state inactivation.

APPENDIX 5: *KCNE2* mutations

Nucleotide change	Mutation	# fam	Phenotype	Functional effect	Refs
25 C>G	Q9E	1	Drug-induced arrhythmia	a.r. ↓, d.r. ↑	(107)
161 T>C	M54T	1	Inherited and drug-induced arrhythmia	a.r. ↓, d.r. ↑	(107), (108)
170 T>C	I57T	1	RWS, drug-induced arrhythmia	a.r. ↓, d.r. ↑	(107), (108)
347 C>T	A116V	1	Drug-induced arrhythmia		(108)
Polymorphisms					
22 A>G	T8A	1.4%	Drug-induced arrhythmia		(107), (108)

Numbering from ATG startcodon (Genbank accession number AF071002). a.r.=activation rate, d.r. = deactivation rate

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