Clinical and genetic spectrum of hereditary cardiac arrhythmia syndromes

Bhuiyan, Z.A.

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Molecular Diagnostics of Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) by use of Denaturing High-Performance liquid chromatography (DHPLC) and sequencing

Alex V. Postma,¹ Zahurul A. Bhuiyan,² Hennie Bikker.²

Heart Failure Research Centre,¹ Academic Medical Center, Amsterdam, The Netherlands
Department of Clinical Genetics,² Academic Medical Center, Amsterdam, The Netherlands

Clinical and Genetic Spectrum of Hereditary Cardiac Arrhythmia Syndromes
Abstract
Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disease characterised by adrenergic induced arrhythmias in the form of bi-directional and polymorphic ventricular tachycardia (PVT). CPVT is a distinct clinical entity associated with a high mortality rate of up to 50% by the age of 30 years. Recently the molecular diagnostics of this disease have become increasingly important as underlying mutations can be found in more than 60% of the identified CPVT patients. Coupled to the fact that β-blocking treatment has a favorable outcome in CPVT patients, and given the risk of sudden death, the identification of causative mutations in CPVT is important as it can greatly augment early diagnosis and subsequent preventive strategies. In this chapter we will describe the molecular diagnostics of the three genes, the cardiac ryanodine receptor, the cardiac calsequestrin and Kir2.1, known to be involved in CPVT as performed in our lab.

Keywords: CPVT, RYR2, CASQ2, Kir2.1, DHPLC, sequencing

Introduction
Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an arrhythmogenic disease characterised by adrenergic induced arrhythmias in the form of bi-directional and polymorphic ventricular tachycardia (PVT). CPVT is a distinct clinical entity associated with a high mortality rate of up to 50% by the age of 30 years. Its key features include childhood onset of syncope and collapse triggered by exercise and other stressful scenarios, with reproducible polymorphic and/or bi-directional ventricular tachycardia demonstrated during exercise testing and catecholamine infusion. Cardiac investigations show no evidence of structural heart disease and the resting 12-lead electrocardiogram is unremarkable including the QT interval. Cases have been reported throughout the world and both sexes appear to be susceptible. Recently, the molecular diagnostics of this disease has become increasingly important, as underlying mutations can be found in more than 60% of the identified CPVT patients. Coupled to the fact that β-blocking treatment has a favorable outcome in CPVT patients, and given the risk of sudden death, the identification of causative mutations in CPVT is important as it can greatly augment early diagnosis and subsequent preventive strategies. In this chapter we will describe the molecular diagnostics of the three genes currently known to be involved in CPVT, as performed in our lab.

Autosomal dominant CPVT, the cardiac ryanodine receptor (RYR2)
Many cases of CPVT are familial, and the first extensive report with a definite linkage analysis was published by Swan et al., showing linkage of CPVT with chromosome 1q42-43. This was followed by publications which associated mutations in a gene from
the 1q42-43 region, the cardiac specific ryanodine receptor type 2 (RYR2), with CPVT.\textsuperscript{1,5,6} Ryanodine receptor channels are intracellular Ca\textsuperscript{2+} release channels forming a homotetrameric membrane-spanning calcium channel on the sarcoplasmic reticulum (SR). The RYR2 gene is located on chromosome 1q42 and consists of 105 exons, which translate into 15kb of cDNA. It encodes a protein of 4967 amino acids, making it the largest known ion channel to date. At present about 50 mutations have been reported in over 52 families.\textsuperscript{1,2,5-8} The various mutations cluster within four regions within the RyR2 protein: the N-terminal side, around the binding site of FKBP12.6, a protein that stabilizes the RyR2 channel,\textsuperscript{9} the calcium binding site, and the channel-forming transmembrane domains.

**Autosomal recessive CPVT, the cardiac calsequestrin gene (CASQ2)**

In contrast to the majority of CPVT patients with an autosomal dominant mode of inheritance (RYR2), two studies have recently demonstrated a recessive form of CPVT.\textsuperscript{3,10} The affected patients of these four families all shared an area on chromosome 1p13-21. Subsequently, homozygous mutations in the cardiac calsequestrin gene (CASQ2) located in that region were found to underlie this recessive form of CPVT. Strikingly, the heterozygous carriers of the mutations were devoid of any clinical symptoms or ECG anomalies. The phenotypes of the recessive CASQ2 CPVT patients seem more severe compared to RYR2 CPVT patients. The CASQ2 gene consists of 11 exons and encodes a protein that serves as the major Ca\textsuperscript{2+} reservoir within the SR lumen of cardiac myocytes. However, although CASQ2 mutations are often autosomal recessive, some reports indicate that autosomal dominant inheritance with reduced penetrance can be part of the clinical spectrum of CASQ2 CPVT.\textsuperscript{3}

**Overlap diseases: Andersen and CPVT, the KCNJ2 gene**

Mutations of the KCNJ2 gene are known to be causative in the Andersen syndrome (AS), a disease characterized by potassium-sensitive periodic paralysis, dysmorphic features, and PVT.\textsuperscript{11} However, mutations in KCNJ2 result in a pleiotropy of the phenotypic AS variations including all, some or just a single characteristic of the AS. Moreover, there is evidence that KCNJ2 only produces CPVT characteristics with a very low penetrance of prominent dysmorphic features, suggesting that some Andersen syndrome patients may present as CPVT patients.\textsuperscript{12-13} These “overlap” patients have syncope, exercise induced polymorphic ventricular tachycardia, and a normal QTc interval. However, in contrast to CPVT, they can also exhibit polymorphic ventricular beats at the resting ECG. The KCNJ2 gene is a background ion channel involved in maintaining the membrane potential of cardiomyocytes during diastole. It is located on chromosome 17q23.1-q24.2 and consists of only one coding exon.
Molecular diagnostics of CPVT

It is clear that there is a strong genetic component in CPVT, and currently in about 60% of the patients underlying mutations are found. Depending on the clinical presentation of the disease and the family history, the appropriate genes can be screened in the patients. The challenge of doing diagnostics for CPVT is the fact that the causative genes differ so much in size. The main gene involved in CPVT, RYR2, has 105 exons, CASQ2 has 11 and KCNJ2 has 1 exon. The strategies for the genetic screening of patients with CPVT thus depend on the size of the genes involved, the equipment available and the cost-effectiveness. Our lab currently uses Denaturing High-Performance liquid chromatography (DHPLC) analysis for the RYR2 and the CASQ2 genes, and sequence analysis for the KCNJ2 gene. Below we will outline the various techniques we employ to screen CPVT patients for the genes of interest. In our lab patients with a CPVT diagnosis are screened first for RYR2, subsequently for CASQ2 and finally for Kir2.1, this order is based upon the prevalence of mutations.

Materials

PCR
1. 10X PCR buffer (Roche)
2. 5X PCR buffer; 1mL: 250 μl 1M Tris-HCl pH 9.0, 140μl 0.5M (NH4)SO4, 100μl DMSO, 75μl Tween-20, 435μl ddH2O
3. MgCl2 25 mM (Applied Biosystems)
4. dNTPs, 1.25mM for each NTP (Amersham Pharmacia)
5. primers (20pmol/μl) (see Note 1)
6. Super Taq (HT Biotechnology)
7. 5mM Betaine (Sigma)
8. Strip tubes/caps (VWR)
9. 96-wells plate (Biozym)

Sequencing
1. BigDye Terminator v3.0 (Applied Biosystems)
2. 5x sequencing buffer (supplied with BigDye Terminator v3.0) (Applied Biosystems)
3. 5mM Betaine (Sigma)
4. Strip tubes/caps (VWR)

Gel electrophoresis analysis
1. Agarose
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2. 1X Tris-acetate-EDTA (TAE): 0.04M Tris-acetate, 0.001M EDTA, pH 8.0
3. Ethidium Bromide (10mg/mL)
4. 10X Loading Buffer; 25ml: 6.25g Ficoll 400, 20ml ddH₂O, 0.5 ml EDTA (0.5mM), 0.25 mk SDS (10%), 0.0625g Orange G (Merck), add ddH₂O till 25 ml.

**Denaturing High-Performance liquid chromatography (DHPLC)**
1. 0.1 M triethylamine acetate (pH 7.0) (Transgenomic)
2. Acetonitrile (Transgenomic) (see Note 2)

**Equipment**
1. Thermocycler
2. Gel electrophoresis device
3. Gel image capture device, Eagle-eye II (Stratagene)
4. ABI Prism DNA Analyser (Applied Biosystems)
5. Macintosh or PC with sequence analysis software (Applied Biosystems) and WAVE Maker softwareTM (Transgenomic)
6. DHPLC-WAVETM system (Transgenomic)

**Methods**

**Direct Sequence PCR Reaction**
1. Amplify genomic DNA for sequence analysis in 20μl reaction mixtures containing 3.0μl genomic DNA (25 ng/μl), 4.0μl 5X PCR buffer, 1.2μl MgCl₂ 25 mM, 2.25μl dNTPs (1.25mM), 0.4μl of each primer (20pmol/μl), 0.1μl Super Taq (HT Biotechnology), 4.0μl Betaine 5mM, and 4.65μl ddH₂O (see Note 3).

2. Perform PCR using an initial denaturating step of 94°C for 3 minutes, followed by 35 cycles of the following touch-down PCR protocol: denaturation at 94°C for 45 seconds, annealing at 66-52°C (see Note 4) for 45 seconds, and the extension at 72°C for 1 minute.

3. Store the product mixture at 40°C until further use.

**Gel electrophoresis analysis**
1. The PCR products are tested on an agarose gel to determine the outcome and specificity of the reaction. Single, crisp bands indicate a successful PCR product that can be used for
subsequent sequence analysis.

2. Prepare a 1.5% (w/v) agarose gel with 1X TAE, with 5μl of 10mg/mL ethidium bromide added to each 100mL of gel solution. Run the electrophoresis in a 1X TAE buffer.

3. Add 1μl of 10x loading buffer to a sample of 5μl of each PCR product, subsequently load the reaction mixture onto the agarose gel. Use a stock ladder (100bp ladder) to determine size of products.

4. Perform electrophoresis at 100V/100mA; running time depends on product size and gel length.

5. Capture the gel image (photo/computer) under long wave UV to determine the result of the PCR. If the PCR was successful, the sample is sequenced.

**Sequencing Reaction**

1. Assemble the sequencing reaction (10μl total volume) by adding 3.0μl of 1/5 diluted PCR product to a strip tube containing 1.0μl BigDye Terminator v3.0, 1.0μl of sequencing primer (2pmol/μl), 2.0μl 5x sequencing buffer, 2.0μl 5mM Betaine and 1.0μl ddH2O (see Note 3 and 5).

2. Denature the DNA initially by incubating the reaction mixture at 94°C for 2 minutes.

3. Perform cycle sequencing with 24 cycles of denaturation at 96°C for 10s, primer annealing at 50°C for 5s, and primer extension at 60°C for 4 minutes, and a single, final extension step at 60°C for 10 minutes (see Note 6).

4. Store the product mixture at 40°C until loading onto the capillary DNA sequencer.

**Denaturing High-Performance liquid chromatography (DHPLC)**

**PCR Reaction**

1. Amplify genomic DNA for DHPLC analysis in 50μl reaction mixtures containing 2.0μl genomic DNA (25 ng/μl), 5.0μl 10X (DHPLC)-PCR buffer (Roche), 3.0μl MgCl2 25 mM, 8.0μl dNTPs 1.25mM, 1.0μl of each primer (20pmol/μl), 0.1μl Super Taq (HT Biotechnology) and 30μl ddH2O (see Note 7).

2. Perform PCR using an initial denaturing step of 94°C for 3 minutes, followed by 35
cycles of the following touch-down PCR protocol: denaturation at 94°C for 45 seconds, an-
ealing at 66-52°C for 45 seconds, and the extension at 72°C for 1 minute (see Note 4)

3. Store the product mixture at 40°C until further use

**Gel analysis of PCR products**

1. PCR products for subsequent DHPLC analysis are checked using agarose gels in the
same fashion as described under subheading 3.1.3. Only PCR products with single, crisp
bands are to be used in the DHPLC analysis (see Note 8).

**DHPLC analysis**

1. For each amplicon, the PCR product of wildtype DNA (healthy control) has to be mixed
with the patient’s product at equimolar ratios (see Note 9); subsequently the resulting mix
is subjected to a 5 min 95°C denaturating step in a PCR machine, followed by a gradual
re-annealing from 95°C to room temperature over 1 hour. This ensures the formation of
equimolar ratios of homo- and heteroduplex species. The mixed products can be stored at
40°C for several weeks, or used immediately.

2. The temperatures needed for successful resolution of heteroduplex molecules are de-
termined by running amplicon-specific melting curves and by using the DHPLC melting
algorithm WAVE™ Maker of the WAVE™ instrument. Melting curves are determined as
follows: the elution time of a specific fragment under standard conditions is determined.
This specific gradient is then tested for the same PCR product for temperatures ranging
from 48°C to 70°C, and retention time versus temperature are graphed to yield a fragment
specific melting curve. The optimal analysis temperature is defined by a maximal decrease
of retention time in a minimal temperature range. However, the complexity of the melting
curve may need between one and four different temperatures for fragment analysis. On
average, 3 different temperatures to cover the entire DNA fragment of interest are necessary
(see Note 10). The chosen temperatures are stored by the software and can be re-used for
subsequent samples for the same amplicon.

3. The (mixed) samples are loaded into the DHPLC machine and covered with a (96 well)
rubber mat to avoid evaporation of the products. It is advisable to also use a negative con-
trol (water-control of the PCR) and a positive control (a known aberration in the amplicon
examined) during each DHPLC run. Enter into the DHPLC software whether you are using
strips or plates and use an injection volume of 8μl per (mixed) DNA sample per tempera-
ture.
4. The (mixed) samples are separated at a flow rate of 0.9ml/min by means of a linear acetonitrile gradient. Generally, analysis takes approximately 4-min/sample including column regeneration and re-equilibration to starting conditions. The column mobile phase consisted of a mixture of 0.1 M triethylamine acetate (pH 7.0) (buffer B) with 25% acetonitrile (buffer A).

5. Subsequently the DHPLC is run with the optimal temperatures and gradients, and the results are stored for analysis.

**Sequence variation identification by DNA analysis**

Whether you want to determine sequence variations of aberrant DHPLC samples or directly identify variation in a patient’s sequence, the methods for finding sequence variations remain the same. Always sequence at least two different individuals, i.e. a (confirmed) healthy control DNA sample together with DNA samples of the patient(s) of interest. After the sequencing run on the ABI Prism 310 DNA Analyzer (or similar machines), trim and re-analyse the sequence traces of all the sequences with the ABI Sequence Analysis software. In this way, poor quality bases can be removed and the sequencing signal can be boosted. Subsequently the ABI Factura program is used to mark and annotate sequence variations and areas of low signal quality. The final step is to align the cleaned up sequences. For this, we use the ABI Sequence Navigator program. After importing 8 sequences into the program (including a healthy control), “Clustal alignment” with the default settings will provide the best alignment. If necessary, sequences done with a reverse primer can also be imported and converted to inversed complementary sequences to complete the alignment (see Note 11). After the alignment, we let the program mark all the differences in a given position with the option “create shadow/compute ambiguity sequences”. This marks all the places in the alignment with possible differences, including homozygous mutations/polymorphisms (Figure 1). Similar options for marking ambiguity in sequences are available in the ABI Auto Assembler program. We subsequently display the alignments at the points of interest (places with ambiguity) and determine the source of the ambiguity (Figure 1). In case of a possible mutation, polymorphism, or disruption of the reading frame, screenshots are taken for archiving, and the sequence of interest can be printed. It is important to understand that sequence variations can always arise from the PCR technique itself and therefore aberrant sequences need to be validated by amplifying control DNA and patient DNA in a additional subsequent PCR reaction. Moreover, it is important to check whether the aberrant sequence is really a mutation, or a (rare) polymorphism. In general, an aberration is labelled as a mutation if it is absent from more than 200 control alleles. However, the causality of any
Figure 1: Example of a clustal alignment of sequences in which any ambiguity between sequences is marked with an asterisk; in the lower panel, a close up of the ambiguity is displayed. Note that up to eight sequences can be aligned and analyzed at the same time.
given mutation depends on: DNA analysis in family members coupled to the clinical history and expression analysis. Good quality sequences and the above-described approach provide a balance between speed of analysis and accuracy. We employ the sequencing techniques for various exons of CASQ2 (see Note 12) and all the Kir2.1 samples. Upon identifying a possible mutation, it is imperative to finish screening the remaining exons as well.

**Sequence variation identification by DHPLC analysis**

The main reason to perform DHPLC analysis is that it is a fast and accurate method for mutations screening. Samples resulting in aberrant patterns always have to be sequenced to determine the nature of the sequence variation. Even if the DHPLC pattern resembles a pattern of a known polymorphism for the amplicon examined, sequencing is still necessary, as this doesn’t exclude the possibility that a mutation close to the polymorphism is masked, or that an unknown mutation causes a similar pattern. DHPLC is used to filter out the wildtype sequences in order to decrease the amount of patient samples to be sequenced. It is important to analyse the patient’s samples at each temperature indicated by the WAVE maker™ software, as some sequence aberrations can only be seen at one specific temperature. In order to obtain a reliable analysis, a second researcher should always check the DHPLC results. DHPLC samples are compared at each temperature and any aberrant patterns are marked and printed for archiving purposes (Figure 2). Subsequently, the sequence analysis on the aberrant samples can be done directly from the PCR product loaded in the DHPLC machine using the above-described methods. It is important to understand that amplicons with numerous sequence variations will severely hamper a reliable DHPLC analysis. Therefore DHPLC analysis is only performed on amplicons that do not contain frequent polymorphisms.

![Figure 2: Examples of aberrant DHPLC profiles due to the presence of (A) no, one or two polymorphisms in exon 43 of RYR2 and (B) a known mutation, E4076K, in exon 90 of RYR2.](image-url)
phisms (see Note 12). We employ DHPLC analysis for the majority of the CASQ2 samples and all the RYR2 samples. Upon identifying a possible mutation, it is imperative to finish screening the remaining exons as well.

**Notes**

1. Primers were designed with a target melting temperature of 600°C. It is important to design the forward and reverse primer in introns as far upstream or downstream as necessary, as this will ensure that any sequence variations around the splice- and branch sites are included in the analysis. Make sure that the chosen primers do not overlap with SNPs. Moreover, it is important to keep in mind that accurate DHPLC analysis is only possible ±50bp down or upstream from any given primer. Primers for RYR2 are available from Tiso et al.;5 primers for CASQ2 and Kir2.1 are available from the authors upon request.

2. Acetonitrile is a hazardous chemical and can penetrate the skin, therefore caution is warranted when replacing the containers, always use gloves. Moreover, the waste product of the DHPLC machine also contains acetonitrile and it is therefore recommend to place a fumehood above the waste container to capture any hazardous fumes.

3. Betaine is used in the PCR and sequencing steps to reduce self-coiling of GC-rich areas and to lower melting temperatures; in our experience betaine has substantial beneficial effects on all PCR/sequence reactions.

4. In a PCR touchdown protocol, the annealing temperature is lowered (proportionally) each successive cycle; the first cycle is performed at the first temperature indicated in the protocol and is subsequently lowered each cycle until the last temperature mentioned is reached at the end of the final cycle.

5. Sequence reactions can be purified using spin-columns or gel-filtration, however in our experience, a dilution of 1/5 of the PCR product works equally well, and saves time and costs. If the dilution of a PCR product produces unusable results during sequencing, one can always revert to other methods of purification.

6. If the product is GC rich, it might help if the temperature of the annealing step of the sequencing reaction is raised, for instance from 50°C to 55°C or even 58°C.

7. When using the DHPLC in patient analysis, it is crucial to use PCR reaction buffers that do not contain solvent or detergents, such as DMSO, glycerol, TWEEN or betaine (often found in most PCR buffers). These products can damage the columns of the DHPLC
machine; therefore PCR buffers without such products should be used. However, if these products are necessary for a specific PCR, the PCR products can be filtered by column purification and subsequently used in the DHPLC.

8. PCR products for subsequent DHPLC analysis need to be specific; any double bands or smears on gel analysis points to a PCR with side products. These side products will interfere with the DHPLC analysis by producing incorrect patterns and might even mask real polymorphisms or mutations.

9. Homozygous mutations are difficult to detect in a DHPLC. This is because aberrant homozygous patterns might differ only slightly, or even not at all, compared to (homozygous) normal alleles. Mixing of wildtype DNA with patient DNA results in heteroduplexes that are easier to detect. However, in case of a clear family history with autosomal dominant inheritance (most of the CPVT cases) one could take the risk to analyse patient DNA without mixing with wildtype DNA to speed up analysis.

10. A set of random samples, plus a positive control, is to be used for the initial optimisation of the PCR amplicons of interest. The DHPLC conditions are adjusted so that the elution profile of the positive control is different from the wildtype sample. When no positive control is available, conditions are considered to be optimal at the temperature and corresponding gradient immediately before a significant decrease in the retention time of the amplicon and/or an excessive broadening of the peak, indicating excess denaturation. Useful temperatures are typically located in the range of 50 to 75% helical fraction for the amplicons analysed.

11. Initially unidirectional sequencing per patient is used (i.e. only a forward primer). If aberrant sequences are found, a new PCR is done and in the subsequent sequence reaction, both the forward and reverse primers are used.

12. Direct sequencing is warranted for CASQ2 exons 1, 7 (contains a poly T tail in the 5’ intron; therefore a reverse primer is recommended), 8, 9 and 10 as they contain numerous polymorphisms.
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References


