Next Generation Sequencing and Linkage Analysis for the Molecular Diagnosis of a Novel Overlapping Syndrome Characterized by Hypertrophic Cardiomyopathy and Typical Electrical Instability of Brugada Syndrome

Ruggiero Mango, MD, PhD; Andrea Luchetti, BSc; Raffaele Sanguolo, MD; Valentina Ferradini, BSc; Nicola Briglia, MD; Emiliano Giardina, BSc; Fabrizio Ferrè, BSc; Manuela Helmer Citterich, BSc; Francesco Romeo, MD; Giuseppe Novelli, BSc; Federica Sanguolo, BSc

**Background:** Familial hypertrophic cardiomyopathy (HCM) is an autosomal dominant inherited disorder; mutations in at least 20 genes have been associated. Brugada syndrome (BrS) is an autosomal dominant inherited disorder caused by mutations mainly in the SCN5A gene. A new clinical entity that consists of HCM, typical electrical instability of BrS and sudden death (SD), is described.

**Methods and Results:** The family was constituted by 7 members, 4 of who presented clinical features of HCM and electrical instability of BrS. The clinical presentation of proband was ventricular fibrillation. All members were clinically evaluated by physical examination, 12-lead electrocardiography, 2-dimensional echocardiography, stress test, electrocardiogram Holter, flecainide test, and electrophysiological study. An integrated linkage analysis and next generation sequencing (NGS) approach was used to identify the causative mutation. Linkage with the α-tropomyosin (TPM1) gene on chromosome 15q22 was identified. The NGS study identified a missense mutation within the TPM1 gene (c.574G>A; p.E192K), exactly located in a binding domain with polycystin-2 protein. No other pathogenic mutations were identified.

**Conclusions:** This is the first report of an association between HCM and BrS, and the first to use a combined approach of linkage and NGS to identify a causative mutation in SD. The present study expands the clinical spectrum of disorders associated with the TPM1 gene and may be useful to report novel mechanisms of electrical instability in HCM and BrS. (Circ J 2016; 80: 938–949)

**Key Words:** Brugada syndrome; Hypertrophic cardiomyopathy; Next generation sequencing; Sudden death
The genetic abnormalities that cause BrS have been linked to mutations in the ion channel gene, SCN5A, which encodes for the α-subunit of the cardiac sodium channel. Many others genes have been subsequently identified explaining, however, only 35% of reported cases, and indicating a marked genetic heterogeneity in the pathogenesis of the BrS. As several genes are potentially implicated in the clinical phenotype described above, the combined use of linkage analysis and a large multi-gene disease-targeted panel based on next generation sequencing (NGS) technologies could aid in the identification of causative mutations.
Target Region Panel Design

We designed 2 custom panels whose content was selected to focus on functional portions of the human genome with known involvement in sudden cardiac death.

In the first design, we attempted to target all exonic regions (with 100 base pair exon padding) of 61 genes by an in-solution target enrichment (Ion TargetSeq Custom Panel; Life Technologies). The design comprised 1,299 exons, targeting 418.6 kb (Table 1). In the second design, we utilized ultrahigh multiplex PCR (Ion AmpliSeq Custom Panel, Life Technologies) to analyze those regions not covered in the first panel. The design comprises 334 regions, targeting 55.18 kb. Combining these 2 panels, we managed to achieve 98% region design coverage.

Samples Preparation

DNA was isolated from peripheral blood using a Flexigene Kit (Qiagen). Genomic DNA samples were quality-checked both on a DNA NanoDrop 1000, and with the Qubit2.0 fluorometer using the Quant-IT dsDNA BR Assay (Invitrogen).

For TargetSeq library preparation, 1 µg gDNA was used as input for fragmentation according to the manufacturer’s instructions. DNA fragmentation was assessed by running the taking this approach, we obtained the molecular diagnosis of a new clinical entity characterized by clinical features of HCM and BrS and caused by a mutation in the α-tropomyosin (TPM1) gene.

Methods

Patients

The family was constituted by 7 members, 4 of whom presented with clinical features of HCM and BrS (Figure 1A). The clinical presentation of the proband was ventricular fibrillation (VF). All family members were clinically evaluated by a review of medical history, a complete physical examination, a 12-lead ECG, a 2-dimensional echocardiographic examination, stress test and an ECG Holter. HCM was diagnosed by unexplained thickening of the left ventricular myocardium, according to international guidelines. All individuals underwent a flecainide test (2 mg/kg IV administered as a bolus over 10 min) and an invasive electrophysiological study (EFS), according to standard methods.

Informed written consent was obtained from each family member who agreed to participate to the study.

Table 1. Targetseq and Ampliseq Analysed Genes

| Gene name | Chr. | Exons | NM_number | Location/function               |
|-----------|------|-------|-----------|---------------------------------|
| ABCC9     | 12p12.1 | 36    | NM_020297 | ATP-sensitive potassium channel |
| ACTC1     | 15q14 | 6     | NM_005159 | Sarcomere                       |
| ACTN2     | 1q43  | 21    | NM_001103 | Z disk                          |
| AKAP9     | 7q21.2 | 51    | NM_005751 | Bind ion channels on sarcolemma |
| ANK2      | 4q25-q26 | 46    | NM_001148 | Bind ion channels on sarcolemma |
| BAG3      | 10q25.2-q26.2 | 4    | NM_004281 | Anti-apoptotic activity         |
| CACNA1C   | 12p13.33 | 47   | NM_001129844 | Calcium ion channel           |
| CACNB2    | 10q12.32 | 13    | NM_201596 | Calcium ion channel            |
| CALR3     | 19p13.11 | 9     | NM_145046 | Calcium-binding protein        |
| CASQ2     | 1p13.1  | 11    | NM_001232 | Calcium storage and transport   |
| CAV3      | 3p25  | 3     | NM_033337 | Sarcolemma                     |
| CSRPR3    | 11p15.1 | 5     | NM_003476 | Z disk                         |
| DES       | 2q35  | 9     | NM_001927 | Intermediate filament          |
| DSC2      | 18q12.1 | 16    | NM_024422 | Desmosome complex              |
| DSG2      | 18q12.1 | 15    | NM_001943 | Desmosome complex              |
| DSP       | 6p24  | 24    | NM_004415 | Desmosome complex              |
| EYA4      | 6q23  | 19    | NM_172105 | Transcription factor           |
| FKTN      | 9q31.2 | 10    | NM_006731 | Golgi-resident proteins        |
| GPD1L     | 3p22.3 | 8     | NM_015141 | Regulation cardiac Na+ current  |
| HCN4      | 15q24.1 | 8     | NM_005477 | Na+ and K+ transporter         |
| JPH2      | 20q13.12 | 6     | NM_020433 | Junctional membrane complexes   |
| JUP       | 17q21.2 | 15    | NM_002230 | Desmosome complex              |
| KCNE1     | 21q22.2 | 3     | NM_001127670 | Potassium ion channel        |
| KCNE2     | 21q22.11 | 2     | NM_172201 | Potassium ion channel          |
| KCNE3     | 11q13.4 | 3     | NM_005472 | Potassium ion channel          |
| KCNH2     | 7q36.1 | 15    | NM_000238 | Potassium ion channel          |
| KCNJ2     | 17q24.3 | 2     | NM_000891 | Potassium ion channel          |
| KCNH1     | 11p15.5 | 16    | NM_000218 | Potassium ion channel          |
| LDB3      | 10q23.2 | 13    | NM_007078 | Z disk                         |
| LMNA      | 1q22  | 12    | NM_170707 | Nuclear lamina                 |
| MYBPC3    | 11p11.2 | 34    | NM_000256 | Sarcomere                      |
| MYH6      | 14q11.2-q12 | 39 | NM_002471 | Sarcomere                      |

(Table 1 continued the next page.)
TPM1 Mutation by NGS Causing a Novel Syndrome

Sanger Sequencing

TPM1 mutation was confirmed by direct sequencing, using primers designed by Primer Express software. Analysis was performed according to the ABI BigDye Terminator Cycle Sequencing protocol using a 3130×L automated sequencer (Applied Biosystems).

Results

Scene of the Crime: Clinical and Instrumental Characterization of the Family

The proband (II-4), a 22-year-old woman, was admitted to our hospital after successful resuscitation from VF cardiac arrest (Figure 1A). Results of examination and laboratory tests were normal. However, the 12-lead-ECG was highly suggestive for LVH; PQ and QT corrected (QTc) intervals were within normal limits (180 ms and 431 ms, respectively) (Figure 1B).

Transthoracic echocardiography revealed severe left ventricular thickening of the interventricular septum (23 mm) without left ventricular outflow tract obstruction and with a normal systolic left ventricular function (Figure 1C). The patient underwent an EFS that revealed inducibility of polymorphic ventricular tachycardia (VT) with degeneration into VF (data not shown). Interestingly, intravenous flecainide caused ST-segment elevation in the inferior leads and coved-type ST-segment elevation in the right precordial leads, suggesting a link with a typical electrical instability of BrS (Figure 1D).

According to the guidelines,11 the patient underwent the samples on an Agilent High Sensitivity DNA chip on a Bioanalyzer 2100 (Agilent). Each sample was barcoded by ligation of IonXpress adapters (Life Technologies) according to protocol. Libraries were size-selected with an E-Gel system (Invitrogen) to an average length of ~330 bp, purified and amplified. Before hybridization with target probes, 250 ng of DNA from 2 samples were combined. Hybridization, post-hybridization washes and elution of the enriched samples was performed according to the manufacturer’s instructions. The enriched library was diluted, and clonally amplified by emulsion polymerase chain reaction; beads with amplicons were enriched, loaded onto an Ion-318 chip (Life Technologies) and sequenced on the Ion-PGM (Life Technologies). Average base pair coverage was >500× for each sample.

For the Ampliseq Library, 20 ng of gDNA were used to generate the amplicons libraries. Libraries were indexed using the IonXpress Barcode Adapter Kit (Life Technologies) and quantitated using the High Sensitivity DNA Chip on the Agilent BioAnalyzer (Agilent). Appropriate dilutions were performed based on amplicon concentration at the 130–210 bp range. Twenty pmol/L of individual indexed amplicon libraries were pooled for emulsion PCR and all 4 samples were sequenced on the Ion Torrent PGM platform using the Ion-316v2 chip (Life Technologies). Average base pair coverage was >400× for each sample.

To analyze the data, 2 strategies were proposed: the use of proprietary bioinformatic tools and the use of a custom pipeline.

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\begin{array}{|c|c|c|c|c|}
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\text{Gene name} & \text{Chr.} & \text{Exons} & \text{NM_number} & \text{Location/function} \\
\hline
\text{MYH7} & 14q11.2 & 37 & \text{NM}_000257 & \text{Sarcomere} \\
\text{MYL2} & 12q24.11 & 7 & \text{NM}_000432 & \text{Sarcomere} \\
\text{MYL3} & 3p21.31 & 7 & \text{NM}_000258 & \text{Sarcomere} \\
\text{MYOZ2} & 4q26-q27 & 6 & \text{NM}_016599 & \text{Z disk} \\
\text{NEXN} & 1p31.1 & 13 & \text{NM}_0144573 & \text{Z disk} \\
\text{PKP2} & 12p11 & 14 & \text{NM}_001005242 & \text{Desmosome complex} \\
\text{PLN} & 6q22.31 & 1 & \text{NM}_002667 & \text{Cardiac Ca++ ATPase} \\
\text{PRKAG2} & 7q36.1 & 16 & \text{NM}_016203 & \text{AMP activated protein kinase} \\
\text{PSEN1} & 14q24.3 & 12 & \text{NM}_000201 & \text{Cardiac development} \\
\text{PSEN2} & 1q42.12 & 11 & \text{NM}_000447 & \text{Cardiac development} \\
\text{RBMM20} & 10q25.2 & 14 & \text{NM}_01134963 & \text{Regulator of mRNA splicing} \\
\text{RyR2} & 1q42.1-q43 & 105 & \text{NM}_001035 & \text{Sarcoplasmic reticulum} \\
\text{SCN1B} & 19q13.12 & 6 & \text{NM}_199037 & \text{Sodium ion channel} \\
\text{SCN3B} & 11q24.1 & 7 & \text{NM}_018400 & \text{Sodium ion channel} \\
\text{SCN4B} & 11q23.3 & 5 & \text{NM}_174934 & \text{Sodium ion channel} \\
\text{SCN5A} & 3p22.2 & 28 & \text{NM}_01160161 & \text{Sodium ion channel} \\
\text{SDHA} & 5p15.33 & 15 & \text{NM}_004168 & \text{Oxidative phosphorylation} \\
\text{SGCD} & 5q33.2 & 8 & \text{NM}_01128209 & \text{Dystrophin associated protein} \\
\text{SNTA1} & 20q11.2 & 8 & \text{NM}_003098 & \text{Membrane scaffold proteins} \\
\text{TCAP} & 17q12 & 2 & \text{NM}_003673 & \text{Z disk} \\
\text{TGFBI3} & 14q24.3 & 7 & \text{NM}_003293 & \text{Embryogenesis and cell differentiation} \\
\text{TREM43} & 3p25.1 & 12 & \text{NM}_024334 & \text{Nuclear envelope structure} \\
\text{TMPO} & 12q22 & 4 & \text{NM}_003276 & \text{Nuclear envelope structure} \\
\text{TNNG1} & 3p21.1 & 6 & \text{NM}_003280 & \text{Sarcomere} \\
\text{TNNT3} & 19q13.42 & 8 & \text{NM}_000363 & \text{Sarcomere} \\
\text{TNNT2} & 1q32.1 & 15 & \text{NM}_000364 & \text{Sarcomere} \\
\text{TPM1} & 15q22.2 & 9 & \text{NM}_001018020 & \text{Sarcomere} \\
\text{TTN} & 2q31 & 311 & \text{NM}_133378 & \text{Sarcomere} \\
\text{VCL} & 10q22.2 & 21 & \text{NM}_014000 & \text{Z disk} \\
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Table 2. Clinical Characteristics of the Patients Carrying Mutations in the TPM1 Gene

| Individual | Gender | Age (years) | Clinical presentation | NYHA | Cardiac phenotype | LVH (mm) | EF | OTO | QTc (ms) | Flecainide infusion | VT/VF induction at EPS | 8 years FU events | Mutation |
|------------|--------|-------------|-----------------------|------|-------------------|----------|----|-----|---------|---------------------|-------------------------|-------------------|----------|
| I:2        | F      | 51          | Asymptomatic          | I    | HCM               | 16       | Normal | No  | 431     | Unmasking           | Positive                | No                 | E192K    |
|            |        |             |                       |      |                   |          |        |     |         | Type I Brugada ECG |                        |                   |          |
| II:2       | M      | 28          | Exercise dyspnea and palpitations | II   | HCM               | 24       | Normal | No  | 484     | Unmasking           | Positive                | No                 | E192K    |
|            |        |             |                       |      |                   |          |        |     |         | Type I Brugada ECG |                        |                   |          |
| II:3       | M      | 25          | Asymptomatic          | I    | HCM               | 20       | Normal | No  | 414     | Unmasking           | Positive                | No                 | E192K    |
|            |        |             |                       |      |                   |          |        |     |         | Type I Brugada ECG |                        |                   |          |
| II:4       | F      | 22          | Aborted sudden death  | I    | HCM               | 20       | Normal | No  | 431     | Unmasking           | Positive                | VT                 | E192K    |
|            |        |             |                       |      |                   |          |        |     |         | Type I Brugada ECG |                        |                   |          |

ECG, electrocardiogram; EF, ejection fraction; EPS, electrophysiologic study; F, female; FU, follow up; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; mm, maximum wall thickness; M, male; NYHA, New York Heart Association; OTO, outflow tract obstruction; QTc, corrected QT interval; VT, ventricular tachycardia; VF, ventricular fibrillation.

Figure 2. Microsatellite analysis demonstrating linkage of the TPM1 locus on chromosome 15 and the flecainide test in affected subjects. Family members are identified by generations and numbers. Square, male family member; circle, female member; symbols with a slash, deceased members; closed symbols, affected members; open symbols, unaffected members; red arrow, proband; +, carrier of the heterozygous missense mutation; -, non carrier. During the administration of flecainide, the diagnostic type I Brugada ECG pattern appeared in the right precordial leads (V1–V3) of all affected subjects. Interestingly, the coved ST elevation also appeared in the inferior leads (DII, DIII, aVF).
implantation of an automatic cardioverter defibrillator (AICD) as secondary prevention. The instrumental screening of the entire family displayed a severe left ventricular thickening of the interventricular septum in the mother (Table 2; II-3) and 2 brothers (Table 2; II-2 and II-3) of the proband. Very intriguingly, all these subjects presented a positive response to flecainide tests; accordingly, all these subjects presented a positive response to flecainide; the flecainide tests in unaffected subjects were negative, as expected (data not shown). The flecainide tests showed a mean coverage of 500×, with a mean uniformity of coverage 90% for the target regions. A total of 313 variants were identified by the single nucleotide polymorphism (SNP) calling procedure in the II-2 sample, 270 of which were previously reported (in dbSNP v136) and 43 were novel variants. Moreover, 297 variants (282 known, 15 novel) were identified in the II-3 sample, and 386 (346 known, 40 novel) in the II-4 sample (Figure 3).

### Table 3. Markers for Linkage Analysis

| Gene | Markers | Chr. |
|------|---------|------|
| Titin (TTN) | D2S118, D2S335, D2S364, D2S2188 | 2q31 |
| Troponin T type 2 (TNNT2) | D1S412, D1S413, D1S249, D1S2692 | 1q32.1 |
| AMP-activated protein kinase γ 2 subunit (PRKAG2) | D7S661, D7S636, D7S483, D7S798 | 7q36.1 |
| Frataxin (FXN) | D6S81817, D9S1874, D9S273, D9S175 | 9q21.11 |
| Myosin binding protein C (MYBPC3) | D1S51416, D1S902, D1S4190, D1S915 | 11p11.2 |
| Myosin light chain 2 (MYL2) | D1S2613, D1S2583, D1S2614, D1S2579 | 12q24.11 |
| Myosin heavy chain 6–7 (MYH6 and MYH7) | D14S283, D14S990, D14S972, D14S275 | 14q12 |
| α-cardiac actin (ACTC1) | D15S1007, D15S1040, D15S118, D15S1012 | 15q14 |
| Tropomyosin α (TPM1) | D15S1033, D15S1036, D15S1011, D15S974, D15S997, D15S987, D15S1009, D15S1020, D15S3651, D15S213, D15S153, D15S988 | 15q22.1 |
| Myotonic dystrophy protein kinase (DMPK) | D19S420, D19S903, D19S902, D19S904 | 19q13.3 |
| Myosin light chain kinase 2 (MYLK2) | D20S912, D20S871, D20S195, D20S107 | 20q11.21 |
| Troponin I type 3 (TNNT3) | D19S572, D19S418, D19S210 | 19q13.4 |
| Cysteine and glycine-rich protein 3 (CSRP3) | D15S4116, D1S902, D1S4190, D1S915 | 11p15.1 |
| Myosin light chain 3 (MYL3) | D3S5321, D3S3685, D3S1851, D3S1289 | 3p21.31 |
| Sodium channel protein type 5 subunit α (SCN5A) | D3S1277, D3S3521, D3S3685, D3S1581 | 3p21.2 |
| Galactosidase α (GLA) | DXS8077, DXS8020, DXS1106, DXS1059 | Xq22 |

Chr., chromosome.

On the Hill of the Assassin: Molecular Analysis

Genetic counselling of the family showed a strong familiarity for SD of maternal origin, with no history of SD or syncpe present from a paternal origin. Because of the overlap between HCM and BrS, we carried out segregation analysis of microsatellite markers in order to confirm or exclude putative linkage to several but more frequently candidate chromosomal regions linked to both diseases. Four microsatellite markers, 2 centromeric and 2 telomeric, were used to test the presence of linkage for each candidate locus, as reported in Table 3. Segregation analysis excluded evidence of linkage for all loci except for the TPM1 locus on chromosome 15 (Figure 2). Successively, the linkage analysis of the TPM1 locus was deepened using further 8 microsatellite markers (Table 3, Figure 2). All together, these markers, mapped on the TPM1 locus, generated a positive logarithm of odds score value (value = 0.426).

In order to characterize the genetic defect in the TPM1 gene, to clarify the pro-arythmic intrafamilial variability and because of the overlapping phenotype (HCM and BrS), we expanded genetic analysis to all known genes causing cardiomyopathies and channelopathies. For this purpose, DNA was analysed using 2 multi gene panels in which the coding regions of 61 genes were screened (Table 1). Each sample showed a mean coverage of 500×, with a mean uniformity of coverage 90% for the target regions. A total of 313 variants were identified by the single nucleotide polymorphism (SNP) calling procedure in the II-2 sample, 270 of which were previously reported (in dbSNP v136) and 43 were novel variants. Moreover, 297 variants (282 known, 15 novel) were identified in the II-3 sample, and 386 (346 known, 40 novel) in the II-4 sample (Figure 3).

Twenty-six variants are shared in the 3 samples, 25 of which fall into 15 genes, and 1 intergenic. Seven distinct variants were mapped into the gene CALR3, 2 into CACNB2, SCN3B, TTN and VCL, and 1 each into ACTC1, DES, KCNH2, KCNJ2, MYH7, PKP2, RYR2, SCN5A, SDHA, and TPM1. The transcript context of each of the 26 variants was estimated on all the alternative splicing variants that the involved genes can encode. Hence, the same variant can be classified multiple times and in different manners depending on the splicing isoform. We obtained 44 variants classified as intronic, 19 as synonymous, 10 as non-synonymous, and 1 falling in the 3’UTR. Only one variant in TTN (S21716L) and 1 found in TPM1 (E192K) were missense.

The missense mutation (c.574G>A) (Table 4), which segregates with disease in the family, was identified within the coding sequence of the TPM1 gene with either the built-in or the in-house pipeline analysis (Figure 4A). All the individuals carrying the mutation developed a pathological phenotype, suggesting complete penetrance of the genetic defect. The mutation was confirmed by direct Sanger sequencing (Figure 4B) and resulted to be absent in healthy family individuals (Figure 4C; II-1 and II-5).

### SNPs as Partners in Crime

Although the mutation showed a complete penetrance, only the proband displayed arrhythmias. As previously described,12 SNPs can interact with mutations modulating cellular and clinical manifestation of cardiomyopathies and channelopathies.
acts with polycystin-2 protein (PKD2), an intracellular calcium channel expressed in renal epithelial cells and in cardiomyocytes, acting as modulator of intracellular calcium and cardiac function. Because potentially causative variants of BrS are also found in the calcium channel genes and PKD2 interacts with different sarcomeric proteins regulating calcium signalling, we investigated in silico the functional effect of E192K mutation on TPM1-PKD2 interaction. The modelling and docking procedures are described in the Supplementary Section. We first modelled the 3-dimensional structure of the wild-type tropomyosin protein using the Sus scrofa tropomyosin to model the human tropomyosin-coiled coil homodimer. The E192 residue of each monomer is involved in electrostatic interactions with residues K189 and E192 of the opposite chain. We then verified the possibility of reconstructing the coiled coil using docking procedures. The best docking solution overlaps quite well with the modelled coiled coil, having a root mean squared deviation of 1.17Å. The residue E192 in the best docking solution is involved in electrostatic interactions with K189, E192 and K198 of the opposite monomer. We then assessed the impact of the E192K mutation on the formation of the tropomyosin coiled coil, predicting a slight destabilizing effect (∆∆G+0.7 kJ/mol) and the loss of the 2 electrostatic interactions involving residue 192, while showing 4 new interactions (with residues E187, E194, E195, and E196). None of the top 20 ranked docking solutions can be

With this background and in order to explain the variable expression of the disease in terms of arrhythmic events, we isolated all identified rare variants exclusively present in the proband analysed genes. Five of these were non synonymous (3 in the TTN gene, 2 in TMEM43 gene), and between them, only the TMEM43 variations (rs4685076 and rs2340917) showed a possible functional effect following in silico analysis (see Supplementary Section). TMEM43 is a nuclear inner membrane protein; the 2 analyzed variants occur in 2 distinct consecutive exons that are part of a large protein region in the perinuclear space. The rs4685076 variant, by abolishing an exonic splicing enhancer, could impair the splicing of the exon containing it, leading to the retention of the 24 residues encoded by the domain. Similarly, the rs2340917, by creating an exonic splicing silencer, could lead to the inclusion of the mature mRNA of the exon containing it. Further speculations are difficult, because no known protein coding alternative splicing variants are currently reported in the Ensembl database.

Crime Weapon: Molecular Docking Showed That E192K in TPM1 Could Alter Interactions With a Patch of Positively Charged Residues of PKD2
In order to explain the molecular basis of the Brugada phenomenon secondary to flecainide administration in TPM1-mutated patients, we explored literature data on TPM1 function and protein interaction. As previously described, TPM1 interacts with polycystin-2 protein (PKD2), an intracellular calcium channel expressed in renal epithelial cells and in cardiomyocytes, acting as modulator of intracellular calcium and cardiac function. Because potentially causative variants of BrS are also found in the calcium channel genes and PKD2 interacts with different sarcomeric proteins regulating calcium signalling, we investigated in silico the functional effect of E192K mutation on TPM1-PKD2 interaction. The modelling and docking procedures are described in the Supplementary Section. We first modelled the 3-dimensional structure of the wild-type tropomyosin protein using the Sus scrofa tropomyosin to model the human tropomyosin-coiled coil homodimer. The E192 residue of each monomer is involved in electrostatic interactions with residues K189 and E192 of the opposite chain. We then verified the possibility of reconstructing the coiled coil using docking procedures. The best docking solution overlaps quite well with the modelled coiled coil, having a root mean squared deviation of 1.17Å. The residue E192 in the best docking solution is involved in electrostatic interactions with K189, E192 and K198 of the opposite monomer.

We then assessed the impact of the E192K mutation on the formation of the tropomyosin coiled coil, predicting a slight destabilizing effect (∆∆G+0.7 kJ/mol) and the loss of the 2 electrostatic interactions involving residue 192, while showing 4 new interactions (with residues E187, E194, E195, and E196). None of the top 20 ranked docking solutions can be
### Table 4. In Silico Analysis of the TPM1 Mutation

| Chr | chr15 |
|-----|-------|
| Start | 63353922 |
| End | 63353922 |
| Ref | G |
| Alt | A |
| Func | Exonic |
| Gene | TPM1 |

| ExonicFunc | Nonsynonymous SNV |
|-----------|------------------|
| AAChange | TPM1:NM_001018008:exon5:c.G466A:p.E156K |
| ESP6500 si ALL | – |
| ESP6500 si EA | – |
| ESP6500 si AA | – |
| ESP6500 si AS | – |
| ESP6500 si EUR | – |
| ESP6500 si SAS | – |
| ExAC Freq | – |
| ExAC AFR | – |
| ExAC AMR | – |
| ExAC EOS | – |
| ExAC FIN | – |
| ExAC NFE | – |
| ExAC OTH | – |
| ExAC SAS | – |
| 1000G ALL | – |
| 1000G AFR | – |
| 1000G AMR | – |
| 1000G EAS | – |
| 1000G EUR | – |
| 1000G SAS | – |
| ExAC Freq | – |
| ExAC AFR | – |
| ExAC AMR | – |
| ExAC EOS | – |
| ExAC FIN | – |
| ExAC NFE | – |
| ExAC OTH | – |
| ExAC SAS | – |
| 1000G ALL | – |
| 1000G AFR | – |
| 1000G AMR | – |
| 1000G EAS | – |
| 1000G EUR | – |
| 1000G SAS | – |
| ExAC Freq | – |
| ExAC AFR | – |
| ExAC AMR | – |
| ExAC EOS | – |
| ExAC FIN | – |
| ExAC NFE | – |
| ExAC OTH | – |
| ExAC SAS | – |
| 1000G ALL | – |
| 1000G AFR | – |
| 1000G AMR | – |
| 1000G EAS | – |
| 1000G EUR | – |
| 1000G SAS | – |
| ExAC Freq | – |
| ExAC AFR | – |
| ExAC AMR | – |
| ExAC EOS | – |
| ExAC FIN | – |
| ExAC NFE | – |
| ExAC OTH | – |
| ExAC SAS | – |
| ESP6500 si ALL | – |
| ESP6500 si EA | – |
| CSIC DIS | – |
| dbSNP | rs199476315 |
| COSMIC ID | COSM9636661 |
| ClinVar ID | RCV000024578.1 |
| ClinVar ID | RCV000036627.3 |
| ClinVar ID | RCV000054795.23 |
| ClinVar ID | RCV000159372.1 |
| ClinVar ID | RCV000074435.1 |
| ClinVar ID | RCV000159372.1 |
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|ClinVar ID | RCV000054795.23 |
|ClinVar ID | RCV000159372.1 |
|GWAS DIS | – |
|GWAS OR | – |
|GWAS BETA | – |
|GWAS PUBMED | – |
|GWAS SNP | – |
|GWAS P | – |
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*(Table 4 continued the next column.)*
Figure 4. Next generation sequencing (NGS), Sanger sequencing and molecular docking. (A) c.574G>A mutation in the TPM1 gene identified by NGS sequencing in the affected sample. (B) Mutation confirmation of the variant by Sanger sequencing on the same DNA. (C) No nucleotide variation was detected in the control DNA. (D) Molecular docking of the PKD2-TPM1 interaction. (Left) Ribbon representation of the modelled coiled coil interaction between PKD2 (red) and the wild-type TPM1 (green). Highlighted are the E192 TPM1 residue, and the arginine residues of PKD2 with which it can establish electrostatic interactions. (Right) Ribbon representation of the modelled coiled coil interaction between PKD2 (red) and the E192K TPM1 mutant (yellow). Highlighted are the K192 and the 3 PKD2 arginine residues that can form destabilizing interactions.
well superimposed to the native coiled coil structure; all have a (usually large) positive energy and the pattern of electrostatic interactions is disrupted in all examined dockings. These results suggest that the E192K mutation can have a profound impact on the establishment of the correct tropomyosin homodimeric coiled coil quaternary structure.

We tested whether the E192K tropomyosin mutant can establish a successful interaction with the polycystin-2 (PKD2) gene product. Interaction between TPM1 and PKD2 was experimentally verified\(^1\) and ascribed to the 821–878 region of PKD2 and to the 152–196 region of TPM1. The E192 residue therefore falls within this region. PKD2 is a large transmembrane protein in which the region 838–926 is reported as coiled coil in the UniProt entry (UniProt AC Q13563). This suggests the formation of a heterodimeric coiled coil interaction between PKD2 and the TPM1 interaction domain. PKD2 was modelled using the C-terminal coiled coil domain of the human transient receptor potential channel subfamily P member 2 (TRP2), a member of the polycystin family. The model covers the PKD2 residues from 833 to 895, corresponding to a large portion of the TPM1 interacting region. The best docking solution between PKD2 and the wild type TPM1 has the lowest energy (−73.57 kJ/mol) and the largest number of electrostatic interactions (n=39), two of which involve residue E192 (with R845 and R848 of PKD2) (Figure 4D Left). When the modelled structure of the TPM1 E192K mutant was subjected to docking with the PKD2 model, the best solution has an estimated energy of −30.73 kJ/mol and a larger number of electrostatic interactions when compared with the best solution obtained using the native TPM1 model (57 vs. 39), but also a larger number of unfavourable electrostatic interactions (53 vs. 31). In particular, residue 192 is involved in 3 unfavourable interactions, with a patch of positively charged residues of PKD2 (R844, R845 and R848) that were instead favourable interactions, with a patch of positively charged residues on the modelled structure of the TPM1 E192K mutant weresubsequently submitted with a patch of positively charged residues of PKD2. The lack of TPM1 interaction with PKD2 protein, as described for other sarcomeric proteins, could result in perturbation of calcium ion flux.

**Discussion**

From a genetic point of view, this is the first report combining linkage analysis and NGS for the identification of a genetic defect causing SD. The identification of causative genes or those that act synergistically to contribute to an increase in the risk of a disease is one of the aims of medical genetics. Genetic testing in patients with HCM provides prognostic and diagnostic benefits and can substantially assist the clinical management of index cases and family members. Detection of a mutation in an index case increases the probability of diagnosis of uncertain cases in the same family without diagnostic clinical features. Genetic analysis has prognostic value in certain index cases, but generally, there are significant obstacles to accurate genetic prognostication. These obstacles include: (1) the existence of many ‘private’ mutations with unknown functional characteristics; (2) the variable disease severity, seen even among patients carrying identical mutations; and (3) the genetic and allelic heterogeneity.

**Role of NGS in Detecting Mutations Causing Overlapping Phenotypes**

Both diseases, HCM and BrS, are a clinically and genetically heterogeneous group of pathologies; over 450 mutations in 20 sarcomere-related and myofilament-related genes have been identified in HCM.\(^{1,16}\) Mutations in the genes for myosin-heavy chain (MYH7), myosin-binding protein C (MYBPC3), and cardiac troponin I (TNNT3) and troponin T (TNNT2) account for the majority of reported genotyped cases.\(^{17–23}\) Regarding BrS mutations, the first was described in 1998 in the SCN5A gene. Since then, more than 350 pathogenic mutations have been identified in 16 genes encoding mainly subunits of cardiac sodium, potassium, and calcium channels, or genes involved in the trafficking/regulation of these channels.\(^{24–26}\) To complicate the genetic diagnosis of HCM and BrS is the allelic heterogeneity. Mutations in a variety of sarcomeric genes lead to pleiotropic cardiac phenotypes ranging from HCM to dilated cardiomyopathy (DCM); each TNNT3 mutation can be associated with diverse morphologies, and with both hypertrophic and restrictive cardiomyopathy, within the same family.\(^{27,28}\)

Pathogenic mutations in the SCN5A gene have been associated with several diseases, such as Long QT Syndrome (LQTS), Atrial Fibrillation (AF), Sick Sinus Syndrome (SSS), Progressive Conduction Cardiac Disease (PCCD) and Sudden Infant Death Syndrome (SIDS).\(^{29,30}\) Despite most of these diseases belonging to the group of channelopathies without structural heart alterations, SCN5A mutations have also been reported in association with DCM.\(^{31}\) In this scenario, novel technical approaches, such as NGS, promise to transform clinical practice. The major current application of NGS in diagnostics is through disease-targeted tests using single genes or multi-gene disease-targeted panels. The technology is convenient when applied to disorders for which both allelic and locus heterogeneity are substantial, because it dramatically increases clinical sensitivity allowing an analysis that would have been impossible to do using the traditional Sanger sequencing method. By this genotyping method, it is possible to analyse a very large number of genes simultaneously and even the entire genome quickly, and at a reasonable cost. However, the amount of data generated by this method is considerable and this makes the process of interpretation very difficult. To overcome these difficulties, our approach was to combine a classical linkage analysis with NGS technologies to guide the analysis and interpretation of NGS-identified variations. This approach was recently described to be a successful strategy for the identification of causative mutations.\(^{32}\) Here, it allowed us to rapidly obtain the molecular diagnosis of the present overlapping syndrome characterized by clinical features of HCM and BrS caused by mutation in the α-tropomyosin (TPM1) gene.

TPM1 is a member of the tropomyosin family of highly conserved, widely distributed actin-binding proteins. Defects in the TPM1 gene are the cause of familial HCM type \(^{3,38}\) (CMH3 [MIM:115196]), DCM type 1Y (CMD1Y [MIM: 611878]) and left ventricular non-compaction 9 (LVNC9 [MIM:611878]).\(^{34}\) Several mutations in TPM1 have been reported as causative of HCM.

The described E192K mutation occurs in exon 6 and segregates in all affected members. It consists in a missense mutation inducing a non-conservative change (Glu to Lys) within a highly conserved region ranging from amino acid 166 to 207, and considered essential for the binding to actin,\(^{13,38}\) in addition to lying near the calcium-dependent troponin-T binding domain. Mutations occurring within the tropomyosin-binding domain of cardiac troponin T and altering the charge of the residue have been already reported as causative of HCM, because critical for cooperative actin binding and regulatory function.\(^{36}\)

The E192K mutation was first described by Probst et al\(^{34}\) in a family with LVNC, but has never been associated with the
Brugada phenomenon. Our data confirm that the mutation, seen as associated with the 2 clinically distinct entities, HCM and LVNC, confers a malignant phenotype with complete penetrance and variable expressivity.

Regarding the variable expressivity of the disease in terms of arrhythmic events, only the proband manifested episodes of VT. In our study, we failed to find a genetic explanation of this variability using the identified SNPs as partner in crime. However, this variability cannot be explained solely by the genetic factors, polymorphisms and modifier genes, and environmental factors such as lifestyle, degree of physical exercise and blood pressure.

From a clinical point of view, the present study describes, for the first time, an association between HCM, SD and flecainide-induced ST-segment elevation in the right precordial and inferior leads. This association was observed in all affected members carrying the c.574G>A TPM1 mutation. Our data support and expand the previous evidence that the Brugada phenomenon can be demonstrated under sodium channel blocker provocation in patients with LQT3 syndrome, ARVD/C with structural epi-endocardial heterogeneity, and in typical BrS without structural heart disease. In the present study, the genetic screening excluded mutations or SNPs in genes most frequently associated with BrS. However, even if several pathogenic mutations in 16 genes have been associated with BrS, all these genes account only for 35% of total cases. It is noteworthy that the Brugada phenomenon observed in the proband could be due to mutations/SNPs present inside genes that are still unknown to be associated with BrS. Nevertheless, it is likely that Brugada phenomenon could be generated by alterations in the action potentials not directly connected to the genetic defect responsible for the syndrome. Thus, the lack of interaction between sarcomeric proteins and proteins regulating ion channel activity will likely trigger the Brugada phenomenon.

In the present study, the altered interaction between TPM1 and PKD2 proteins, demonstrated by molecular docking, might influence the calcium ion flux thus altering the transmembrane electric potential. This effect becomes more evident on ECG when a sodium channel blocking drug is used. In this scenario, the flecainide/ajmaline-induced Brugada phenomenon might represent a marker of electrical instability that might be useful to unmask and stratify subjects carrying mutations at high risk to develop arrhythmia and SD. Moreover, our study confirms the previous evidence that sodium channel blocker drug-induced ST-segment elevation in peripheral leads is an independent predictor for a malignant arrhythmic event.

Recently, Haruki et al. reported that oral flecainide administration may be considered a useful therapy to improve left ventricular pressure gradient and symptoms in patients with obstructive HCM, particularly for those with disopyramide-induced vagolytic side effects. Although the authors did not observe any flecainide-induced Brugada phenomenon or proarrhythmic events in treated patients, their data must be viewed with caution given the small number of patients enrolled and in light of our data. Therefore, further studies are needed to assess the safety of flecainide therapy in patients with symptomatic obstructive HCM, and the role of the flecainide test in unmasking and stratifying HCM subjects at high risk to develop SD.

Conclusions

There are 3 main features of our study: (1) the discovery of the disease-causing variant of a novel overlapping syndrome characterized by HCM, typical electrical instability of BrS and SD; (2) these results were obtained combining together linkage and target resequencing analysis; and (3) the ajmaline or flecainide test, if confirmed in larger cohort of patients with cardiomiopathies, might be useful to unmask and stratify subjects at high risk of developing arrhythmia.

Finally, the multidisciplinary approach used in the study is an example of molecular medicine. Combining the expertise of a cardiologist, geneticist, molecular biologist and a specialist in bioinformatics has been shown to be fundamental in obtaining a rapid characterization of the genetic defect, and a molecular explanation of the clinical evidence with possible important future clinical implications.

Used appropriately, genetic analysis can have an important role in the prognosis, genetic counselling and clinical management of patients with HCM and their families.

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Disclosures

No conflicts of interest are declared.

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