Targeted resequencing identifies *TRPM4* as a major gene predisposing to progressive familial heart block type I

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Authors' contributions

XD, MYA, HA, RR and JJS conceived the study, wrote the manuscript and are the guarantors of the project; PL, EC, JB and SLS contributed to the data processing; SB and EB performed the sequencing; BB and SN contributed to the functional and biochemical analyses; SF, AT and FK recruited the patients; HLM, NM, JBG and VP provided expert clinical advice; CD led the statistical analysis. All authors interpreted the data, contributed and commented on drafts of the article, and approved the final version.

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Background: Progressive cardiac conduction disease (PCCD) is one of the most common cardiac conduction disturbances. It has been causally related to rare mutations in several genes including *SCN5A*, *SCN1B*, *TRPM4*, *LMNA* and *GJA5*.

Methods and results: In this study, by applying targeted next-generation sequencing (NGS) in 95 unrelated patients with PCCD, we have identified 13 rare variants in the *TRPM4* gene, two of which are currently absent from public databases. This gene encodes a cardiac calcium-activated cationic channel whose precise role and importance in cardiac conduction and disease is still debated. One novel variant, *TRPM4*-p.I376T, is carried by the proband of a large French 4-generation pedigree. Systematic familial screening showed that a total of 13 family members carry the mutation, including 10 out of the 11 tested affected individuals versus only 1 out of the 21 unaffected ones. Functional and biochemical analyses were performed using HEK293 cells, in whole-cell patch-clamp configuration and Western blotting. *TRPM4*-p.I376T results in an increased current density concomitant to an augmented *TRPM4* channel expression at the cell surface.

Conclusions: This study is the first extensive NGS-based screening of *TRPM4* coding variants in patients with PCCD. It reports the third largest pedigree diagnosed with isolated Progressive Familial Heart Block type I and confirms that this subtype of PCCD is caused by mutation-induced gain-of-expression and function of the *TRPM4* ion channel.

1. Introduction

Progressive cardiac conduction defect (PCCD) was first described in the sixties by Lenègre [1] and Lev [2] as a fibrosis process affecting the conduction system. It is one of the most common cardiac conduction disturbances characterized by a progressive alteration of cardiac conduction through the His-Purkinje system with right or left bundle branch block (RBBB or LBBB) and widening of QRS complexes, leading to complete atrioventricular block (AVB), syncope and sudden death.

Familial cases of PCCD have been reported with an autosomal dominant inheritance and causally related to rare mutations in genes involved in cardiac impulse propagation (*SCN5A* [3,4], *SCN1B* [5] and *TRPM4* [6,7]), in the structure of the nuclear lamina (*LMNA* [8,9]) and in cell-to-cell communication (*GJA5* [10]).

Among these genes, *TRPM4*, encoding a calcium-activated cationic channel that is expressed in Purkinje fibers and nodal tissue [6,7], has first been linked to progressive familial heart block type I (PFHBI) in two large pedigrees, respectively from South Africa [6] and Lebanon [7]. PFHBI is associated with a progressive impairment of the His bundle branches conduction, typically starting with RBBB and then left anterior hemiblock (LAHB) and that may progress to a complete AVB. QRS duration increases with time while PR and QTc intervals remain constant [11–13].

Conduction defects in *TRPM4*-dependent familial cases were shown to be related to gain-of-function mutations proposed to be caused by a reduction of the deSUMOylation of *TRPM4* channels and an impaired endocytosis resulting in stabilization and overexpression of mutant channels at the plasma membrane [6,7]. Since these two seminal reports, eighteen gain- or loss-of-function variants have been identified as causing diverse forms of cardiac conduction defects and/or Brugada syndrome [14–16].

Here, using next generation sequencing (NGS) technologies, we report novel *TRPM4* variants including a *TRPM4* variant (c.T1127C; p.I376T) which is segregating with the third largest reported PCCD family. This missense mutation segregates in 39 relatives of a 4-generation pedigree and was observed to lead to gain-of-expression and function of the mutant channel. These findings strongly support a central role of *TRPM4* in cardiac conduction and cardiac conduction disorders.

2. Methods

2.1. Patient phenotyping

The study was conducted according to the French guidelines for genetic research and approved by the ethic committee of the Nantes University Hospital. A written consent was obtained for each family member who accepted to participate in the study.

The investigation included a physical examination with particular attention to the cardiovascular system and a 12-lead ECG. Heart rate, PR interval, QRS, QTc duration, P and QRS axes were measured automatically at rest (Mac Vu Marquette Inc, Milwaukee, Wisconsin, USA). Conduction defects were defined using the conventional classification [17,18]. Two expert physicians, blinded to the clinical status, independently and systematically reviewed the ECG parameters.

Because of the prevalence of minor conduction defects in the general population and in order to decrease the risk of misclassification, only the most obviously affected patients were considered as affected. QRS axis was classified as normal when its value was between -30° and $+90^{\circ}$. PR duration shorter than 210 ms was considered as normal. Patients were considered as affected if they have been implanted with a pacemaker (PM) for PCCD or if they have an ECG showing a major conduction defect (complete AVB, complete RBBB,

complete LBBB, parietal block (PB) defined as a QRS wider than 115 ms without morphology of RBBB or LBBB, LAHB or left posterior hemiblock (LPHB)). Given the progressive nature of the disease, only patients older than 45 without any conduction defect were considered as unaffected. All the other patients were considered as undetermined and not included for the evaluation of the ECG parameters. Cardiac morphological diseases were excluded by echocardiography in all patients.

2.2. Targeted sequencing

Genomic DNA was extracted from peripheral blood lymphocytes by standard protocols. The DNA yields were assessed by measurements using Quant-IT™ dsDNA Assay Kit, Broad Range (Life Technologies, Q33130). The purity of the DNA was assessed by spectrophotometry (OD 260:280 and 260:230 ratios) using a Nanodrop instrument (Thermo Scientific). DNA integrity was assessed by separation in a E-Gel® 96 Agarose Gels, 1% (Life Technologies, G700801). For multiplex amplification, we used the HaloPlex™ Target Enrichment System (Agilent Technologies, 1-500 kb, ILMFST, 96 reactions, G9901B), Protocol Version D.2 (November, 2012). We applied a custom HaloPlex™ design enabling high-throughput sequencing of the coding regions of 45 genes previously linked to cardiac arrhythmias or conduction defects and/or sudden cardiac death, including 19 genes known or suspected to be involved in PCCD. In this study we focused solely on the relevant 19 PCCD genes including *SCN5A*, *SCN1B*, *TRPM4*, *GJA5* and *LMNA* that have already been associated with isolated cardiac conduction defects together with *GJA1*, *GJC1*, *SCN10A*, *NKX2-5*, *TBX5*, *SNTA1*, *PRKAG2*, *RYR2*, *EMD*, *BMP2*, *BMPRIA*, *GATA4*, *MSX2* and *TNNI3K* as likely candidate genes. The targeted coding regions (exons) $\pm$ 10 bp correspond to 141 kb of genomic sequence.

Target enrichment and sequencing were performed as previously described [19]. First, 200 ng of gDNA samples were digested in eight different restriction reactions, each containing two restriction enzymes, to create a library of gDNA restriction fragments. These gDNA restriction fragments were hybridized to the HaloPlex probe capture library. Probes are designed to hybridize and circularize targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences were incorporated into the targeted fragments. The circularized target DNA biotinylated HaloPlex probe complexes were captured on magnetic streptavidin beads. We proceeded to a ligation reaction of the circularized complexes followed by an elution reaction before PCR amplification. The amplified target DNA was purified using AMPure XP bead (Beckman Coulter, A63881). To validate enrichment of target DNA in each library sample by microfluidics analysis, we used the 2200 TapeStation (Agilent Technologies, G2964AA), with D1K ScreenTape (Agilent Technologies, 5067-5361), and D1K Reagents (Agilent Technologies, 5067-5362). We ensured that the majority of amplicons range from 175 to 625 bp. Finally we quantified each library by qPCR using KAPA Library Quantification Kit (Clinisciences, KK4854). Libraries were pooled to an equimolar concentration and DNA was then denatured with NaOH. Finally libraries pool was diluted to a 4 pM final concentration before proceeding to 100 bp paired-end Illumina sequencing on HiSeq.

2.3. Detection of rare coding variation in TRPM4

Raw sequence reads were aligned to the human reference genome (GRCh37) using BWAMEM (version 0.7.5a) after removing sequences corresponding to Illumina adapters with Cutadapt v1.2. GATK was used for indel realignment and base recalibration, following GATK DNaseq Best Practices. Variants were called for each sample separately using GATK UnifiedGenotyper (version 2.8) and Samtools mpileup (version 0.1.19), and variants were

considered for further analyses if found by both GATK and Samtools with a minimum quality score of 25.

Variants were considered of interest if: 1- They present a potential pathogenicity as predicted by Variant Effect Predictor (Ensembl). Variants were considered as having a potential functional consequence if they were annotated with one or more of the following SO terms for at least one RefSeq transcript: “transcript_ablation” (SO:0001893), “splice_donor_variant” (SO:0001575), “splice_acceptor_variant” (SO:0001574), “stop_gained” (SO:0001587), “frameshift_variant” (SO:0001589), “stop_lost” (SO:0001578), “initiator_codon_variant” (SO:0001582), “inframe_insertion” (SO:0001821), “inframe_deletion” (SO:0001822), “missense_variant” (SO:0001583), “transcript_amplification” (SO:0001889); 2- They were rare that is if the minor allele frequency (MAF) was < 1% compared to the 1000 genomes phase 1 data (379 individuals of European origin, integrated release v3, downloaded from <ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20110521>), to the NHLBI GO Exome Sequencing Project (ESP) data – Exome Variant Server (EVS) (4,300 individuals of European origin, ESP6500SI-V2 release, downloaded from <http://evs.gs.washington.edu/EVS>), and to the Exome Aggregation Consortium (ExAC) data (60,706 unrelated individuals including more than 33,300 non-Finnish European individuals, release v1, downloaded from ftp://ftp.broadinstitute.org/pub/ExAC_release).

In case of missense variants SIFT [20] and PolyPhen-2 (PPH-2) [21] were used to predict the impact of the amino acid substitutions. Filtering was performed using Knime4Bio [22].

2.4. Segregation analysis

Familial segregation analyses were carried out by bidirectional direct sequencing of amplified genomic DNA amplicons with variant-specific primers (forward:

CCTCCATCCCTTTGGACAG; reverse: CAGGCCAGGAAAGGTGTCTA) using the “Big Dye Terminator v3.1 Cycle Sequencing Kit” (Applied Biosystems - Life Technologies) following the manufacturer’s recommendations. The capillary sequencing was performed on Applied Biosystems 3730 DNA Analyzer using standard procedures provided by Applied Biosystems (Life Technologies).

The RefSeq NM_017636.3 transcript has been used to compare our sequencing data.

Linkage was assessed between the variant I376K and the disease using standard method comparing likelihood under a recombination fraction of 50% (no linkage) and 0% (full linkage). LOD score [23] calculation was performed with Superlink-Online SNP version 1.1 (<http://cbl-hap.cs.technion.ac.il/superlink-snp/main.php>) [24].

We postulated a rare causal variant (frequency set at 1/10000) and dominant model with high penetrance (80%). The prevalence is 5% and therefore, the phenocopy rate is 0.0499.

A LOD score higher than 3 is considered as significant for linkage.

2.5. Cell culture and transfection

Human embryonic kidney (HEK293) cells were cultured with DMEM medium supplemented with 4 mM Glutamine, 10% FBS and a cocktail of streptomycin-penicillin antibiotics. For the electrophysiological studies, the cells were transiently transfected with 80 ng of HA-TRPM4 WT or HA-TRPM4 p.I376T in a 35 mm dish mixed with 4 μ l of JetPEI (Polyplus transfection, Illkirch, France) and 46 μ l of 150 mM NaCl. The cells were incubated for 24 hours at 37°C with 5% CO₂. All transfections included 400 ng of cDNA encoding CD8 antigen as a reporter gene. Anti-CD8 beads (Dyna[®], Oslo, Norway) were used to identify transfected cells, and only CD8-displaying cells were analyzed. Cells were used 24 hours after transfection.

For the biochemical studies, HEK 293-cells were transiently transfected with 960 ng of either HA-TRPM4 WT, HA-TRPM4 p.I376T variants or empty vector (pcDNA4TO) in a P100 dish (BD Falcon, Durham, North Carolina, USA) mixed with 30 μ l of JetPEI (Polyplus transfection, Illkirch, France) and 250 μ l of 150 mM NaCl. The cells were incubated for 48 hours at 37°C with 5% CO₂.

2.6. Cell surface biotinylation assay

Following 48 hours of incubation, transiently transfected HEK293 cells were treated with EZlinkTM Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, Illinois, USA) 0.5 mg/ml in cold 1X PBS for 15 minutes at 4°C. Subsequently, the cells were washed twice with 200 mM glycine in cold 1X PBS and twice with cold 1X PBS to inactivate and remove the excess biotin, respectively. The cells were then lysed with 1X lysis buffer (50 mM HEPES pH 7.4; 150 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA pH 8.0; 10% Glycerol; 1% Triton X-100; 1X Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany)) for 1 hour at 4°C. Cell lysates were centrifuged at 16,000 g; 4°C for 15 minutes. Two milligram of the supernatant was incubated with 50 μ l Streptavidin Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden) for 2 hours at 4°C, and the remaining supernatant was kept as the input. The beads were subsequently washed five times with 1X lysis buffer before elution with 50 μ l of 2X NuPAGE sample buffer (Invitrogen, Carlsbad, California, USA) plus 100 mM DTT at 37°C for 30 minutes. These biotinylated fractions were analyzed as TRPM4 expressed at the cell surface. The input fractions, analyzed as total expression of TRPM4, were resuspended with 4X NuPAGE Sample Buffer plus 100 mM DTT to give a concentration of 1 mg/ml and incubated at 37°C for 30 minutes.

2.7. Western Blot experiments

Protein samples were analyzed on 9% polyacrylamide gels, transferred with the TurboBlot dry blot system (Biorad, Hercules, CA, USA) and detected with anti-TRPM4 (generated by Pineda, Berlin, Germany), anti α -actin A2066 (Sigma-Aldrich, St. Louis, Missouri, USA) antibodies using SNAP i.d. (Millipore, Billerica, MA, USA). The anti-TRPM4 antibody was generated by Pineda (Berlin, Germany) using the following peptide sequence: NH₂-CRDKRESDSERLKRTSQKV-CONH₂. A fraction of the antisera, which was subsequently used in this study, was then affinity purified.

2.8. Cellular Electrophysiology

For patch-clamp experiments in whole-cell configuration, glass pipettes (tip resistance, 1.5–3 M Ω) were filled with an intracellular solution containing (in mM): 100 CsAsp, 20 CsCl, 4 Na₂ATP, 1 MgCl₂, 10 EGTA, and 10 HEPES. The pH was adjusted to 7.20 with CsOH, and the free Ca²⁺ concentration at 100 μ M with CaCl₂ using WEBMAXCLITE program (<http://www.stanford.edu/~cpatton/downloads.htm>). Access resistance ranges was from 3 to 5 M Ω . Extracellular solution contained (in mM): 156 NaCl, 1.5 CaCl₂, 1 MgCl₂, 6 CsCl, 10 glucose and 10 HEPES. The pH was adjusted to 7.40 with NaOH. Patch-clamp recordings were carried-out in the whole-cell configuration at room temperature (23–25°C). TRPM4 currents were investigated using a ramp protocol. The holding potential was -60 mV. The 400 ms increasing ramp from -100 to +100 mV ends with a 300 ms step at +100 mV then 300 ms at -100 mV. A new ramp was performed every 2 seconds. Before seal formation, liquid junction potential was compensated to keep the baseline at 0 mV. Using a Digidata 1440A analog-digital interface (Axon Instruments, Inc.), currents were filtered at 5 kHz and the sampling frequency was at 50 kHz. Current densities were obtained by dividing the peak current recorded at -100 mV by the cell capacitance (17 $\pm$ 2 pF and 16 $\pm$ 1 pF, respectively

transfected with WT and I376T-TRPM4 channels). Of note, capacitances and series resistances were not compensated.

2.9. Data analysis and statistical methods

Currents were analyzed with Clampfit software (Axon Instruments, Inc). Data were analyzed using a combination of pClamp10, Excel (Microsoft) and Prism (Graphpad).

Comparisons between groups were performed with unpaired two-tailed Student's t test. Data are expressed as mean + SEM. A p-value <0.05 was considered significant.

3. Results

3.1. Mutational Screening

Ninety-five patients with PCCD were recruited through the French National Referral Center for Sudden Cardiac Death as previously described [25]. Nineteen genes known or suspected to be involved in conduction defects were sequenced in these patients using the HaloPlex™ System, resulting in a mean coverage depth of 578x per sample: *SCN5A*, *SCN1B*, *TRPM4*, *GJA5* and *LMNA* that have already been associated with isolated cardiac conduction defects together with *GJA1*, *GJC1*, *SCN10A*, *NKX2-5*, *TBX5*, *SNTA1*, *PRKAG2*, *RYR2*, *EMD*, *BMP2*, *BMPR1A*, *GATA4*, *MSX2* and *TNNI3K*. A graphical representation of the mean coverage obtained for the 5 major genes is provided in supplemental figure 1.

When selecting only genetic variants with a potential pathogenicity as predicted by Variant Effect Predictor (see methods) and an MAF below 1% in public databases, we identified a total of 45 variants in 43 patients: 11 novel variants and 34 rare variants (see supplemental table 1). Among these variants, 13 have already been associated with cardiac pathologies such

as the Brugada syndrome and cardiac conduction defects (and one with Small Fibre Neuropathy; see supplemental table 1).

3.2. TRPM4 is the most frequently affected gene

The most frequently affected gene is *TRPM4*, with a total of 13 rare variants identified and then validated by capillary sequencing (Table 1). Seven of these variants (54%) are located in the intracellular N-terminal region (Fig. 1a). Two of them – p.I376T and p.R892H – are absent from public databases and thus considered as novel.

The *TRPM4*-p.I376T missense variant, which resides in the intracellular N-terminal domain (Fig. 1a), was identified in the male patient 9 (Fig. 1b). No other rare variant altering any other known PCCD-susceptibility genes could be identified in this patient. The affected amino acid is located in a highly conserved region across vertebrates as indicated by its *Genomic Evolutionary Rate Profiling* score [26] of 4.24 (supplemental figure 2). It is predicted as deleterious (0.02) by SIFT [20] but benign (0.323) by PolyPhen-2 (PPH-2) [21].

The *TRPM4*-p.R892H variant has been identified in the patient 10, who presents with a complete AVB. We found that the same patient also carries a rare missense variant in *SCN5A* (p.A572D), suggesting that the *TRPM4*-p.R892H variant alone may not be responsible for the observed cardiac conduction defects. Another substitution affecting the same amino acid - *TRPM4*-p.R892C - was detected in a second patient (patient 3), but was also reported at an MAF below 1% in public databases (Table 1).

3.3. Familial recruitment

Patient nine carrying the *TRPM4*-p.I376T variant (patient IV-5 in the pedigree) was diagnosed with complete RBBB and LAHB (Fig. 2) and was implanted with a PM for

conduction disorders at the age of 32. Familial investigation has been undertaken for this patient, indicated as the proband IV-5 on Fig. 1c.

A total of 96 family members could be identified, among which 57 have been recruited (Fig. 1c). Twelve patients were diagnosed with conduction defects, of which six (50%) were implanted with a PM (Table 2). Ten of the 12 patients presented with RBBB, among which 8 showed LAHB. The eleventh patient (V-18) exhibited an isolated LBBB; the last one (III-2) PB (Table 2).

Two patients (V-16 and VI-1) exhibiting at birth 2:1 AVB with RBBB and LAHB QRS morphology alternant with complete AVB and were classified as patients with a congenital AVB, as well as the patient V-18 who exhibited a permanent complete AVB with a 30 bpm ventricular escape rhythm with a complete LBBB QRS morphology. This patient also met the magnetic resonance image diagnostic criteria for a left ventricular non compaction while echocardiography had failed to identify this phenotype (Fig. 1c). Note that the patient IV-6 presented with minor conduction defects (QRS duration of 118ms) and a slight left axis deviation (-14°), but was not considered as affected following our criteria and thus was classified as ‘unknown’.

Age at last clinical evaluation (34 ± 25 vs 22 ± 16 , ns), PR (169 ± 24 ms vs 138 ± 20 ms, $p < 0.001$), QRS (138 ± 26 ms vs 88 ± 13 ms, $p < 10^{-10}$) and QTc (438 ± 42 ms vs 417 ± 22 ms, $p < 0.05$) durations were higher in the affected members compared to non-affected members while the heart rate was lower in the affected group (61 ± 16 bpm vs 77 ± 16 bpm, $p < 0.01$) (Table 3).

The novel *TRPM4*-c.T1127C variant (*TRPM4*-p.I376T) was systematically assessed among family members (Fig. 1c). We were able to test 39 family members: 10 out of the 11 affected patients that we tested (90.9%) carried the *TRPM4* variant versus only 1 out of 21 unaffected family members (4.8%). The twelfth patient suffering from cardiac conduction disease

(patient VI-1) was born in 2012 and thus was not genotyped given his young age. The two-point logarithm of the odds ratio (LOD) score was estimated at 4.1182 for this locus - assuming a disease allele frequency of 0.01%, a disease penetrance of 80 % and a recombination fraction of 0 %. These findings indicate a genotype-phenotype co-segregation in an autosomal dominant manner in this large French family affected by PFHBI. The patient III-2, while appearing as a phenocopy, may show conduction defects caused by a previous anterior myocardial infarct while the patient V-17 (born in 1994) was still young at recruitment time (17 years old), which may explain the absence of conductance disturbance for this variant carrier. This patient will be subjected to regular clinical follow-up since carrying the putative causal variant may confer higher risk to develop PCCD with ageing.

3.4. The p.I376T variant induces a gain-of-function of TRPM4 channel

To investigate the effect of the p.I376T variant on TRPM4 expression levels, we performed western blot and cell surface biotinylation experiments. As previously published [27], we observed that the TRPM4 channel is expressed in fully and core glycosylated forms (Fig. 3). In the presence of the p.I376T variant, we observed an increased expression of these two forms at the cell membrane (Fig. 3). The functional consequences of the p.I376T variant were investigated using the whole-cell configuration of the patch-clamp technique. As reported by our group [28], TRPM4 currents recorded over time show two distinct phases (Fig. 4a). After the membrane rupture, a fast transient phase is observed; it is followed by a plateau phase in which the current amplitude is stable (Fig. 4a). The functional characterization of the p.I376T variant shows in this condition an increase of TRPM4 current densities in both transient and plateau phases, (Table 4, Fig. 4b, c and d).

4. Discussion

In the present study, thirteen variants in the *TRPM4* gene were identified using NGS technologies upon screening of a cohort of 95 patients with PCCD. Eleven of these variants were previously listed in at least one of the used public databases. Two of them (p.A432T and p.G844D) were previously reported in familial autosomal conduction block and were shown as deleterious [7]. Five other variants (p.R252H, p.D561A, p.G582S, p.Q854R and p.P1204L) have been reported in sporadic cases presenting with conduction disorders and/or Brugada syndrome [15,16]. Of interest p.R252H was identified in 3 unrelated patients all of which exhibiting a type 2 second-degree AVB (patients 35, 36 and 37). The four other variants (three missense variants p.R442C, p.G737R and p.R892C and one synonymous variant predicted to affect splicing of the seventh intron p.T286T) have not been causally related to conduction disorders and/or arrhythmia so far.

Another variant, *TRPM4*-p.R892H, is novel since absent from public databases. However, as the patient carrying this variant also carries an *SCN5A*-p.A572D variant, no conclusion could be drawn on the relative pathogenicity of each of these two variants.

The last variant identified in *TRPM4* (p.I376T) is also novel. Familial investigations led to the identification of 96 members including 12 patients with conduction disorders. This is the third largest pedigree diagnosed with PFHBI in which a *TRPM4* mutation significantly segregates in an autosomal dominant manner with the pathology. Thus, this study represents the first NGS-based detection of a *TRPM4* variant that has led to the recruitment of a large 4-generation pedigree from the proband (patient IV-5 on Fig. 1c) carrying the mutation p.I376T. Of interest, the novel variant p.I376T is located in the same intracellular N-terminal domain as the 2 causal variants previously identified in large pedigrees (Fig. 1a) [11, 18]. Noteworthy, 6 out of the 11 low-frequency variants identified in this study also reside in the same

intracellular N-terminal domain, thus suggesting that this domain could be a preferential site for PFHBI causing mutations.

In the present family, a large majority of affected members present with RBBB and anterior hemiblocks, without any LBBB. This pattern is similar to the clinical descriptions of the families previously linked to mutations in *TRPM4* [6,7], which corresponds to the PFHB type IB definition. Our study, in combination with previously published works [6,7,16] strongly support the prominent role of this cardiac TRP channel in this subtype of conduction disease. The clinical onset of conduction disturbances tends to occur at an early age among affected patients. In particular, the presence of three cases of congenital AVB implanted with a PM during the first year of life also suggests an important role of heritability in disease severity. Furthermore, the observation that these three congenital AVB patients are first- or second-degree relatives suggests that additional genetic factors are strengthening the disease susceptibility in these patients.

Expression and functional analyses were performed using HEK293 cells, in whole-cell patch-clamp configuration and Western blotting. The *TRPM4* p.I376T results in an increased current density that may be caused by an augmented TRPM4 channel expression at the cell surface as previously described [6,7]. The underlying mechanisms leading to conduction block caused by TRPM4 dysfunction are not yet understood. It has been proposed [15,29] that gain-of-function mutations may depolarize the cells of the conduction system, reduce the availability of the cardiac sodium channels and current and thereby alter the normal impulse propagation in Purkinje fibers. This model is consistent with the large QRS complexes observed in PFHBI patients. Conversely, loss-of-function mutations of TRPM4 may lead to a hyperpolarization of the membrane potential, and so reduce cellular excitability and conduction. A detailed analysis of the molecular mechanisms leading to the mutation-induced gain of expression and

function was out of the scope of the present work. These findings, however, strongly support the role of TRPM4 gain-of-function in slowed cardiac impulse propagation.

5. Limitations

Next generation-based targeted resequencing such as HaloPlex™ System allows high-throughput genetic screening in a large number of individuals but some target sequences may be uncovered due to biases in DNA digestion by restriction enzymes. Thus some relevant variations may be missed in small subsets of coding regions: this problem is inherent to sequencing strategies based on DNA enrichment.

Furthermore this high-throughput candidate-gene approach was used to screen 19 candidate genes in 95 unrelated patients. Except for the patient carrying the *TRPM4*-p.I376T variant (patient 9) for which a familial recruitment, segregation tests and a LOD score calculation strongly suggest an association between this variant and the phenotype, the implication of variants in other unknown involved genes cannot be excluded in isolated PCCD cases.

6. Conclusion

In this study we identified one large family with 10 members diagnosed with PFHBI and carrying a *TRPM4* gain-of-expression and function mutation. This represents the first NGS-based detection of a *TRPM4* variant that has led to the recruitment of a large PCCD pedigree. This work confirms that gain-of-function mutations in the intracellular N-terminal region of TRPM4 are responsible for PFHBI and further underline the crucial role of TRPM4 channel in cardiac conduction disorders.

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Table 1 Characteristics of identified Amino Acid Variants in *TRPM4*

No	Patient	Exon	Nucleotide	Amino Acid	Effect	Genotype	Other variant(s) in susceptibility genes	SIFT PPH-2	dbSNP141 database id	EUR AF (1000 Genomes) (%)	EVS UA MAF (%)	AllelicFreq NFE (ExAC) (%)	ECG Morphology	Phenotype
1.1	35	6	c.755 G>A	R252H [16]	missense_variant	Heterozygous	0	deleterious(0.01) possibly_damaging(0.772)	rs146564314	0	0,63	0,818	RBBB	type 2 AVB 2°
1.2	36	6	c.755 G>A	R252H [16]	missense_variant	Heterozygous	0	deleterious(0.01) possibly_damaging(0.772)	rs146564314	0	0,63	0,818	LBBB	type 2 AVB 2°
1.3	37	6	c.755 G>A	R252H [16]	missense_variant	Heterozygous	0	deleterious(0.01) possibly_damaging(0.772)	rs146564314	0	0,63	0,818	Normal	type 2 AVB 2°
2	13	7	c.858 G>A	T286T	splice_region_variant & synonymous_variant	Heterozygous	1 (<i>GJA5</i>)			0	0,00	0,001	Normal	AVB 3°
3	9	9	c.1127 T>C	I376T	missense_variant	Heterozygous	0	deleterious(0.02) benign(0.323)		0	0,00	0	RBBB + LAHB	Normal
4	4	11	c.1294 G>A	A432T [7,15,16]	missense_variant	Heterozygous	2 (<i>TRPM4</i> and <i>RYR2</i>)	deleterious(0) probably_damaging(0.97)	rs201907325	0,13	0,10	0,056	LBBB	AVB 3°
5	24	11	c.1324 C>T	R442C	missense_variant	Heterozygous	1 (<i>SCN5A</i>)	deleterious(0) probably_damaging(0.996)	rs148867331	0	0,02	0,018	RBBB + LAHB	Normal
6.1	21	12	c.1682 A>C	D561A [16]	missense_variant	Heterozygous	1 (<i>SCN1B</i>)	tolerated(0.22) benign(0.086)	rs56355369	0,13	0,55	0,618	RBBB	AVB 3°
6.2	28	12	c.1682 A>C	D561A [16]	missense_variant	Heterozygous	1 (<i>SCN5A</i>)	tolerated(0.22) benign(0.086)	rs56355369	0,13	0,55	0,618	RBBB	AVB 3°
7	4	13	c.1744 G>A	G582S [15,16]	missense_variant & splice_region_variant	Heterozygous	2 (<i>TRPM4</i> and <i>RYR2</i>)	tolerated(0.34) benign(0.037)	rs172149856	0,13	0,10	0,060	LBBB	AVB 3°
8.1	38	16	c.2209 G>A	G737R [15]	missense_variant &	Heterozygous	0	tolerated(0.59)	rs145847114	0,4	0,17	0,180	LBBB	AVB 1° +

splice_region_variant														benign(0.007)		type 2 AVB 2°	
8.2	39	16	c.2209 G>A	G737R [15]	missense_variant & splice_region_variant	Heterozygous	0	tolerated(0.59) benign(0.007)	rs145847114	0,4	0,17	0,180	RBBB + LPHB	AVB 1°			
9	40	17	c.2531 G>A	G844D* [7,16]	missense_variant	Homozygous	0	tolerated(0.2) probably_damaging(0.945)	rs200038418	0,13	0,16	0,431	RBBB + LAHB	AVB 2/1, 3/1			
10	41	17	c.2561 A>G	Q854R [15,16]	missense_variant	Heterozygous	0	tolerated(0.29) benign(0.029)	rs172155862	0,26	0,12	0,289	LAD	type 2 AVB 2°			
11	3	18	c.2674 C>T	R892C	missense_variant	Heterozygous	3 (TNNI3K, SCN1B and RYR2)	deleterious(0) probably_damaging(0.985)	rs147854826	0	0,10	0,081	Normal	AVB 3°			
12	10	18	c.2675 G>A	R892H	missense_variant	Heterozygous	1 (SCN5A)	deleterious(0.02) benign(0.252)		0	0,00	0	RBBB + LAHB	AVB 3°			
13.1	42	24	c.3611 C>T	P1204L [15,16]	missense_variant	Heterozygous	0	tolerated(0.21) unknown(0)	rs150391806	0,13	0,33	0,505	Normal	AVB 3°			
13.2	43	24	c.3611 C>T	P1204L [15,16]	missense_variant	Heterozygous	0	tolerated(0.21) unknown(0)	rs150391806	0,13	0,33	0,505	RBBB + LAHB	AVB 1°			

RBBB: Right Bundle Branch Block; LBBB: Left Bundle Branch Block; LAHB: Left Anterior HemiBlock; LPHB: Left Posterior HemiBlock; LAD: Left Axis Deviation; AVB: AtrioVentricular Block

Variants already described in some articles are noted : [7] Liu *et al.* 2010, [16] Stallmeyer *et al.* 2011, [15] Liu *et al.* 2013

* : the patient 40 has been identified as homozygous for this variant (G844D)

Table 2 Clinical data of the affected family members

Patient N°	Age at last clinical examination	Heart rate (bpm)	PR (ms)	QRS (ms)	QTc (ms)	ECG Morphology	Conduction	PM (Age)
IV-5 (proband)	32	69	160	170	474	RBBB + LAHB	Normal	32 y.o.
II-1	85	63	138	126	439	RBBB	Normal	
III-2	60	81	170	128	439	PB	Normal	
III-3	50	35	200	120	336	RBBB + LAHB	type 2 AVB 2°	50 y.o.
III-4	51	55	188	158	439	RBBB + LAHB	Normal	
IV-8	33	59	180	168	436	RBBB + LAHB	Normal	31 y.o.
IV-9	25	76	170	166	504	RBBB + LAHB	Normal	
IV-25	40	74	200	130	434	RBBB + LAHB	AVB 1°	
V-16	8	45		148	398	RBBB + LAHB	type 2 AVB 2° and AVB 3°	at birth
V-18*	12	35				LBBB	AVB 3°	at birth
V-21	11	73	136	123	441	RBBB	Normal	

VI-1

0

62

144

90

439

RBBB + LAHB

type 2 AVB 2°

8-month-old

RBBB: Right Bundle Branch Block; LAHB: Left Anterior HemiBlock; PB: Parietal Block; LBBB: Left Bundle Branch Block; AVB:

AtrioVentricular Block

***This patient presented with a left ventricular non-compaction phenotype**

Table 3 Comparison of age at last clinical evaluation, heart rate, PR, QRS and QTc durations between affected and unaffected members of the family

	Affected	Unaffected	p value (affected vs unaffected)
Patients (N)	12	45	
Age (years)	34 ± 25	24 ± 16	NS
Heart rate (bpm)	61 ± 16	75 ± 17	< 0.01
PR (ms)	169 ± 24	140 ± 21	< 0.001
QRS (ms)	138 ± 26	90 ± 13	< 10 ⁻¹⁰
QTc (ms)	438 ± 42	415 ± 24	< 0.05

Table 4 The functional characterization of the I376T variant

	TRPM4 WT	TRPM4 p.I376T
Current density of	-161 ± 31	-678 ± 113
Transient Phase (pA/pF)	n=12	n=9
Current density of Plateau	-772 ± 138	-1390 ± 134
Phase (pA/pF)	n=8	n=7

Figure captions

Fig. 1 The *TRPM4*-p.I376T variant is responsible for PFHBI. (A) Distribution of rare coding variation detected among 95 patients with PCCD in the *TRPM4* channel. Novel variants are shown in red, low-frequency ones in blue. The two rare variants previously reported as causing PFHBI [6,7] are indicated in green. (B) Capillary sequencing of the exon 9 of *TRPM4* for the patient 9 confirms the presence of a novel variant resulting in the p.I376T substitution. (C) Family tree of patient 9 (the proband, IV-5). Plus symbols (+) denotes p.I376T mutation carriers and minus symbol (-) non-carriers. 'PM' indicates patients implanted with a pacemaker, 'LVNC' stands for Left Ventricular Non-Compaction and 'C' indicates congenital forms of conduction defects.

Fig. 2 The ECG profile of the proband IV-5. This patient presented with a heart rate of 69 bpm, a complete right bundle branch block and a left anterior hemiblock enlarging the QRS complex to 170 ms. ECG was recorded at a 25 mm/s paper speed and 0,1 mV/mm signal amplitude. A Premature ventricular beat can also be observed in the first QRS complex of the precordial lead.

Fig. 3 Expression of the WT and p.I376T *TRPM4* channels. (A) Western blots showing the expression of *TRPM4* at the total (left panel) and surface levels (right panel) with white and black arrowheads representing fully glycosylated and core-glycosylated forms of *TRPM4*, respectively. (B) Quantification of the Western blots is shown as relative intensity of protein bands for both fully- and core-glycosylated forms of *TRPM4* in each fraction. *P<0.05, **P<0.01

Fig. 4 Whole cell patch clamp recording for the WT and p.I376T TRPM4 channels. (A) Time course recording of the TRPM4 current. (B) Individual current traces of the WT and p.I376T TRPM4 channels recorded as transient and plateau phases. (C) Quantification of current density of the WT and p.I376T TRPM4 channels for both phases. The current densities are measured at the pic current at -100 mV. (D) Current-Voltage relationships of the WT and p.I376T TRPM4 channels. *P<0.05, **P<0.01, ***P<0.001

Figure1

Figure2

Patient 5 (IV-5 on the pedigree)

Figure3

Figure 4