Research Article

Mutation-Specific Phenotypes in hiPSC-Derived Cardiomyocytes Carrying Either Myosin-Binding Protein C Or α-Tropomyosin Mutation for Hypertrophic Cardiomyopathy

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Received 21 May 2015; Revised 22 July 2015; Accepted 20 September 2015

1. Introduction

Hypertrophic cardiomyopathy (HCM) is one of the most common genetic cardiac diseases with worldwide prevalence of 1:500, as well as the most common cause of sudden cardiac death (SCD) among young competing athletes. HCM is inherited in an autosomal dominant pattern. Nevertheless, a large clinical diversity and age-related penetrance are typical for HCM. On the tissue level, HCM is characterized by the disarray of cardiomyocytes (CMs) and fibrosis of cardiac tissue, as well as thickened interventricular septum or free left ventricular wall. Clinical symptoms include arrhythmias, progressive heart failure, and even SCD, but on the other hand the mutation carrier can be completely asymptomatic.

Altogether more than 1400 mutations in 11 genes encoding for the sarcomeric proteins have been identified and related to HCM. The majority of the mutations are found either in the β-myosin heavy chain (MYH7) or in the myosin-binding protein C (MYBPC3) genes [1]. In Finland, two founder mutations located in MYBPC3 and α-tropomyosin (TPM1) genes and one common mutation in MYH7 gene together account around 24% of all Finnish HCM cases [2, 3].

Although the genetic information related to HCM has been growing in the recent years due to the development of sequencing technologies, exact information of the disease mechanisms remains unclear. Thus, current medication of the disease is directed toward the symptom relief and there is no specific therapy to prevent the onset or progression of the...
Most of the HCM studies have been conducted with model systems, mainly either with transgenic mice or by studying human tissues obtained from surgical myectomy from end-stage HCM patients [4]. However, animal models carry only the mutated gene lacking the rest of the genome and myectomy samples are obtained from patients in the late stage of HCM development. Therefore, the discovery of the human induced pluripotent stem cells (hiPSCs) has offered a new valuable tool to model HCM and other cardiac diseases and to study the underlying disease mechanisms [5]. To date, hiPSCs have already been used to model a variety of cardiac diseases: electrical defects, for example, long-QT syndrome [6–8] and catecholaminergic polymorphic ventricular tachycardia (CPVT) [9, 10] as well as cardiomyopathies including dilated cardiomyopathy (DCM) [11] and HCM [12–14].

Here we have derived hiPSCs from patients carrying two of the Finnish HCM founder mutations either in MYBPC3 (MYBPC3-Gln1061X) or in TPM1 (TPM1-Asp175Asn) gene. We have differentiated the patient-specific hiPSCs into CMs and compared the phenotypes of the diseased and control CMs.

2. Materials and Methods

2.1. Ethical Issues. This study was conducted in accordance with the Ethics Committee of Pirkanmaa Hospital District to establish, culture, and differentiate hiPSC lines (R08070). Skin biopsies for hiPSC establishment were received from the Heart Hospital, Tampere University Hospital, Tampere, Finland. Patients donating skin biopsies signed an informed consent after receiving both oral and written descriptions of the study. The teratoma assay, described in Section 2.3.6, was approved by ELLA-Animal Experiment Board of Regional State Administrative Agency for Southern Finland (ESAVI/6543/04.10.03/2011).

2.2. Generation and Culture of Patient-Specific hiPSC Lines. hiPSC lines were generated from skin fibroblasts either with Sendai reprogramming vectors OCT4, KLF4, c-MYC, and SOX2 using CytoTune-IPS Reprogramming Kit (Life Technologies Ltd., Paisley, UK) according to the manufacturer’s instructions or by using pMX retroviral vectors OCT4, KLF4, c-MYC, and SOX2 with or without Cre-LoxP site as described earlier [6, 15]. UTA.13602.HCM, UTA.02912.HCM, and UTA.04511.WT hiPSC lines were generated by using Sendai vectors and UTA.07801.HCM and UTA.06108.HCM by using pMX retroviral vectors with Cre-LoxP site and UTA.04602.WT was generated by using pMX retroviral vectors without Cre-LoxP site. In the present study, one line of each patient was used. hiPSC lines were derived and cultured on mouse embryonic fibroblast (MEF) feeder cell layers (26000 cells/cm², CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany) in human pluripotent stem cell (hPSC) culture medium consisting of knockout-DMEM (koDMEM, Gibco, Life Technologies Ltd.) supplemented with 20% knockout serum replacement (ko-SR, Gibco, Life Technologies Ltd.), 1% nonessential amino acids (NEAA, Lonza Group Ltd., Basel, Switzerland), 2 mM GlutaMax (Gibco, Life Technologies Ltd.), 50 U/mL penicillin/streptomycin (Lonza Group Ltd.), 0.1 mM 2-mercaptoethanol (Lonza Group Ltd.), and 4 ng/mL basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ, USA).

2.3. Characterization of hiPSC Lines

2.3.1. Mutation Analysis by Genotyping. DNA samples from the hiPSC lines were prepared with TaqMan Sample-to-SNP Kit (Applied Biosystems, Life Technologies Ltd.) and the presence of MYBPC3-Gln1061X and TPM1-Asp175Asn mutation in the patient-specific hiPSC lines was confirmed by custom TaqMan SNP Genotyping Assays (Applied Biosystems, Life Technologies Ltd.) according to the manufacturer’s instructions. In the genotyping assays, MYBPC3-gene as well as TPM1-gene was amplified with specific primers. Furthermore, the presence of the mutations was assessed with mutation-specific FAM labeled probes. VIC labeled probes were used to assess the presence of the wild type allele. Sequences for the primers and probes used in the assay are listed in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/1684792).

2.3.2. The Expression of Mutant and Wild Type Alleles in hiPSC-Derived CMs. RNA samples were collected and extracted from hiPSC-derived CMs (UTA.04511.WT, UTA.02912.HCM, UTA.07801.HCM, and UTA.06108.HCM) with Norgen’s Total RNA Purification Plus Kit (Norgen Biotek Corp., Ontario, Canada) according to manufacturer’s instructions. 50–100 ng of RNA was transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Ltd.). The expression of TPM1-Asp175Asn or MYBPC3-Gln1061X mutation on mRNA level in the hiPSC-derived CMs was studied by Custom TaqMan SNP Genotyping Assays (Applied Biosystems, Life Technologies Ltd.) similarly as that for genotyping described above. Sequences for the primers and probes used in the assay are listed in Supplementary Table 1.

2.3.3. Immunocytochemistry. Undifferentiated hiPSC colonies were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA), stained with primary antibodies for Nanog (R&D systems Inc., Minneapolis, MN, USA), OCT4 (R&D systems Inc.), SOX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRA-1-60 (Millipore, MA, USA), and TRA-1-81 (Millipore), and visualized with secondary antibodies as described before [16]. Finally, the cells were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) containing 40,6-diamidino-2-phenylindole (DAPI) for the nuclei staining and imaged with an Olympus IX51 phase contrast microscope equipped with fluorescence optics and Olympus DP30BW camera (Olympus Corporation, Hamburg, Germany).

2.3.4. RT-PCR. The RNA was extracted from the hiPSC lines by NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co., Düren, Germany) and 500–1000 ng of RNA was transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit
The presence of pluripotency genes Nanog, SOX2, OCT4, and c-MYC and the absence of virally imported exogenes (OCT4, SOX2, c-MYC, and KLF4) were confirmed by RT-PCR. GAPDH was used as an endogenous control. The primer sequences used for detection of Sendai transgenes are described in CytoTune-iPS Reprogramming Kit’s manual (Life Technologies Ltd.).

2.3.5. Karyotype Analysis. The karyotypes of hiPSC lines were studied by G-banding (Medix Laboratories, Espoo, Finland) or by KaryoLite assay [17] (Turku Centre for Biotechnology, University of Turku, Turku, Finland).

2.3.6. Pluripotency Analysis. The pluripotency of hiPSC lines was confirmed in vivo by embryoid body (EB) formation and in vitro by teratoma assay. hiPSCs were removed from feeder cell layer and cultured in suspension to form EBs. The EBs were cultured in EB medium consisting of ko-DMEM supplemented with 20% fetal bovine serum (FBS, Biosera, Boussens, France), 1% NEAA (Lonza Group Ltd.), 2 mM Glutamax (Invitrogen, Life Technologies Ltd.), and 50 U/mL penicillin/streptomycin (Lonza Group Ltd) for 4–6 weeks before RNA extraction. 200 ng of RNA was transcribed to cDNA for the RT-PCR analysis. The presence of all three germ layers, endoderm (APF, SOX17), ectoderm (SOXI, NESTIN, and Musashi), and mesoderm (KDR, alpha cardiac actin), was studied with RT-PCR.

For in vivo pluripotency assay, hiPSCs were injected under the testis capsule of nude mice and the formed teratomas were collected and fixed with 4% PFA 8 weeks after the injection. Teratomas were embedded in paraffin, cut in sections, and stained with haematoxylin and eosin.

2.4. Differentiation of Cardiomyocytes. hiPSCs were differentiated into CMs by coculturing with mouse visceral endodermal-like cells (END-2) (Hubrecht Institute, Utrecht, Netherlands) as described before [18]. After 15–30 days beating areas were cut from cocultures and dissociated into single cells in EB medium by Collagenase A (Roche Diagnostics, Mannheim, Germany) as described earlier [18] and plated to 0.1% gelatin-coated cover slips or well plates for further analysis.

2.5. Characterization of hiPSC-Derived Cardiomyocytes

2.5.1. Immunocytochemistry and Image Analysis. Dissociated CMs were fixed with 4% PFA and stained with Troponin T (cTnT, 1:2000, Abcam, Cambridge, MA, USA), MYBPC (1:400, sc-166081, Santa Cruz Biotechnology), and TPM1 (1:200, sc-73225, Santa Cruz Biotechnology) primary antibodies, followed by labeling with secondary antibodies.

2.5.2. Ca2+ Imaging. The clusters of CMs were cut, dissociated, plated on 0.1% gelatin-coated coverslips, and cultured for 1, 3, and 6 weeks. To study the Ca2+ handling properties of hiPSC-derived CMs, cells were loaded with 4 μM Fura-2 AM (Molecular Probes, Life Technologies Ltd.) for 30 minutes in perfusate medium. The perfusate medium consisted of (in mM) 137 NaCl, 5 KCl, 0.44 KH2PO4, 20 HEPES, 4.2 NaHCO3, 5 D-glucose, 2 CaCl2, 1.2 MgCl2, and 1 Na-pyruvate dissolved in H2O. pH of the perfusate medium was adjusted to 7.4 with NaOH. The coverslip, containing the dissociated hiPSC-derived CMs, was mounted to an RC-25 recording chamber and continuously perfused with perfusate medium preheated to 35-36°C by an SH-27B inline-heater controlled by a TC-324B unit (all from Warner Instruments Inc., Hamden, USA). The perfusion was controlled by a gravity driven VC8 application system (ALA Scientific Instruments Inc., NY, USA). Coverslip was perfused for 15 minutes for Fura-2 AM deesterification before experimental recordings.

For Ca2+ imaging analysis, single beating CMs were selected as regions of interests and background noise, recorded from a cell-free area in the same coverslip, was subtracted before further processing. Data is presented as ratios of 340/380 nm (F340/F380). The spontaneously beating CMs were divided into five different rhythm categories based on the abnormalities observed in their Ca2+ signals: normal beating with regular peaks (normal); more than three peaks which do not return to the baseline (oscillation); signals with small or medium sized amplitude events in the beginning, in the end, or in between two Ca2+ spikes (low/middle peaks); two or three peaks which do not return to the baseline (double peaks); Ca2+ spikes with prolonged rise or decay time (plateau abnormality). In the low/middle peaks category, the small amplitude was at least 10% from the preceding Ca2+ spike amplitude. Full-length, 10–30 seconds long recordings were analyzed, while most of the analyzed recordings were 12 seconds long. The distribution of CMs in different categories is presented for each cell lines separately.

2.5.3. Electrophysiological Measurements: Recording and Analysis of Action Potentials. The action potentials (APs) were recorded by conventional patch clamp [19] in perforated patch configuration using Amphotericin B [20] in final concentration of 0.24 mg/mL. Data acquisition was
Table 1: TaqMan assays used in qRT-PCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description/alias</th>
<th>Function</th>
<th>TaqMan assay ID</th>
</tr>
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<tbody>
<tr>
<td>EF1A1</td>
<td>Eukaryotic translation elongation factor 1 alpha 1</td>
<td>Housekeeping gene</td>
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<td>Sarcomeric gene</td>
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<td>Sarcomeric gene</td>
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<td>α-actinin 2</td>
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<td>α-tropomyosin</td>
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<td>Sarcomeric gene</td>
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<td>Titin</td>
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<td>Ca2+ binding protein in SR</td>
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<tr>
<td>RYR2</td>
<td>Ryanodine receptor 2 (cardiac)</td>
<td>Ryanodine receptor</td>
<td>Hs00892883_m1</td>
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Conducted using Axon Series 200B patch-clamp amplifier connected to Digidata 1440a AD/DA converter driven by pCLAMP 10.2 software (all from Molecular devices LLC). On the day of use, the coverslips containing dissociated hiPSC-derived CMs were transferred to RC-24N recording chamber (Warner Instruments Inc.) and mounted on an inverted Olympus IX71 microscope (Olympus Corporation). The patch electrodes had tip resistance of 3.0–3.5 MΩ and contained the following intracellular solution (in mM): 132 KMeSO4, 20 KCl, 1 MgCl2, and 1 CaCl2 (pH was adjusted to 7.2 with KOH). The extracellular solution contained (in mM) 143 NaCl, 4.8 KCl, 1.8 CaCl2, 1.2 MgCl2, 5 glucose, and 10 HEPES (pH was adjusted to 7.4 with NaOH). The preheated extracellular solution was continuously perfused with similar setup compared to what is presented in Section 2.5.2. Patch pipettes (Harvard Apparatus Ltd., Holliston, MA, USA) were freshly prepared using PC-10 micropipette puller and then flame-polished with MF-830 microforge (both from Narishige Int., Tokyo, Japan).

APs were recorded in the gap-free mode in the current clamp from the spontaneously beating hiPSC-derived CMs. Current-clamp recordings were digitally sampled at 20 kHz and filtered at 2 kHz using low pass Bessel filter on recording amplifier. Beats per minute (BPM), AP duration (APD90 and APD50), AP amplitude (APA), and maximum diastolic potential (MDP) were analyzed from the recorded APs by using Origin 9.1 (OriginLab Corp., Northampton, MA, USA). Only ventricular-like waveforms are presented here to avoid any biasness among different hiPSC lines. The ventricular-like CMs were characterized by APD90/APD50 < 1.3 and APA > 90 mV.

2.5.4. Real-Time qRT-PCR Analysis. After one week of culture, dissociated CMs were collected into a lysis solution buffer of CellsDirect One-Step qRT-PCR Kit (Life Technologies Ltd.) according to the manufacturer’s instructions. Two replicate samples were collected and stored at -70°C until the DNase I digestion and reverse transcription-specific target amplification (RT-STA) by using CellsDirect One-Step qRT-PCR Kit. Real-Time qPCR was performed with Biomark HD system (Fluidigm Corp., San Francisco, USA) according to the manufacturer’s instructions. The TaqMan assays (Life Technologies Ltd.) used in the qRT-PCR are collected in Table 1. All samples were analyzed in duplicate and the fold changes were calculated by the 2-ΔΔCT method [21]. EF1A1 and GAPDH genes were used as endogenous control genes and UTA.045II.WT cell line was used as a calibrator.

2.5.5. Western Blot. hiPSC-derived CMs were lysed in M-PER protein extraction reagent (Thermo Scientific, Life Technologies Ltd.), supplemented with complete protease inhibitor cocktail (Roche Diagnostics). The protein concentration was quantified with BCA protein assay kit (Thermo Scientific, Life Technologies Ltd.). 10 μg of protein was run to 4–15% mini-PROTEAN TGX precast polyacrylamide gel
(Bio-Rad, Hercules, CA, USA) and transferred to PVDF membrane (Amersham Hybond-P, GE Healthcare, Little Chalfont, UK). Membranes were blocked with 5% milk for 1 h at RT and proteins were stained with MYBPC (1:1500, sc-166081, Santa Cruz Biotechnology), cTnT (1:2000, ab64623, Abcam), TPM1 (1:200, sc-73225, Santa Cruz Biotechnology), or β-actin (1:1000, sc-47778, Santa Cruz Biotechnology) primary antibodies overnight at +4°C. Horseradish peroxidase-(HRP-) conjugated polyclonal rabbit anti-mouse (DAKO, P0260) and rabbit anti-goat IgG (Santa Cruz Biotechnology, sc-2922) were used as secondary antibodies. Stained proteins were detected by using Clarity ECL substrate (Bio-Rad) and visualized by Molecular Imager ChemiDoc XRS+ (Bio-Rad). ImageJ software (National Institutes of Health, USA) was used to compare the expression of MYBPC, cTnT, and TPM1 with the β-actin expression from the same cell line.

2.6. Statistical Analysis. For statistical analysis, control cell lines and cell lines in each mutation were combined in groups: UTA.04602.WT and UTA.04511.WT hiPSC lines in WT-CM group, UTA.02912.HCMT and UTA.13602.HCMT in HCMT-CM group; and UTA.07801.HCMM and UTA.06108.HCMM in HCMM-CM group. Mann-Whitney U test with Bonferroni’s correction was used to analyze the differences between WT-, HCMT-, and HCMM-CMs in cell size analysis, proportion of multinucleated CMs, and Ca^{2+} imaging experiments as well as in gene expression analysis. For the statistical comparison between the three groups, one-way ANOVA followed by Tukey test was used for the patch-clamp result analysis. p < 0.05 was considered statistically significant. All error bars are presented as standard error of the mean (SEM).

3. Results

3.1. hiPSCs Were Derived from HCM Patients with Different Backgrounds. We derived hiPSCs from four patients carrying a HCM causing mutation either in TPM1 (TPM1-Asp175Asn) or in MYBPC3 (MYBPC3-Gln1061X). UTA.13602.HCMT and UTA.02912.HCMTs carry TPM1-Asp175Asn and UTA.07801.HCMM and UTA.06108.HCMM in HCMM-CM group. Mann-Whitney U test with Bonferroni’s correction was used to analyze the differences between WT-, HCMT-, and HCMM-CMs in cell size analysis, proportion of multinucleated CMs, and Ca^{2+} imaging experiments as well as in gene expression analysis. For the statistical comparison between the three groups, one-way ANOVA followed by Tukey test was used for the patch-clamp result analysis. p < 0.05 was considered statistically significant. All error bars are presented as standard error of the mean (SEM).

3.2. Mutation-Specific HCM Phenotypes Were Observed in hiPSC-Derived Cardiomyocytes. All the cell lines used in the present study differentiated into cardiomyocytes similarly. Beating aggregates were formed 14–20 days after the initiation of coculturing with END-2 cells and there was no difference of the beating areas. After cardiac differentiation, beating clusters were dissociated into single cells and cultured for 1, 3, and 6 weeks. The differences in the cell sizes and Ca^{2+} handling properties between different mutants and control cells were analyzed at each time point. The size of the hiPSC-derived CMs in all groups increased when the cells cultured for three weeks. Within each group, there were no differences in cell sizes between three and six weeks, except with HCMT-CMs, which seemed to be smaller in size 6-week time point. However, during 6 weeks of culture, cell types other than CMs had the tendency to overgrow the CM culture, which might have affected the cellular enlargement. This phenomenon was

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell line</th>
<th>Mutation</th>
<th>Name in Figure 2(f)</th>
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<tbody>
<tr>
<td>WT</td>
<td>UTA.04602.WT</td>
<td>—</td>
<td>WTI</td>
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<td></td>
<td>UTA.04511.WT</td>
<td>—</td>
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<td>UTA.06108.HCMM</td>
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Figure 1: Characterization of UTA.13602.HCMT cell line. (a) The hiPSCs formed colonies expressing Nanog, OCT4, SOX2, TRA-1-60, and TRA-1-81. Scale bars: 200 μm. (b) The virally transferred Sendai exogenes, exo-OCT4 (483 bp), exo-KLF4 (410 bp), exo-SOX2 (451 bp), and exo-c-MYC (532 bp), were silenced in the hiPSCs. + indicates positive controls, for which RNA was extracted from cells 1 week after transduction. hiPSCs expressed endogenous Nanog (287 bp), OCT4 (144 bp), REX1 (306 bp), SOX2 (151 bp), and c-MYC (328 bp). GAPDH (302 bp) was used as a housekeeping control. (c) The hiPSC line was karyotypically normal, 46 XX. (d) The pluripotency of hiPSCs was confirmed by in vivo teratoma assay, in which hiPSCs formed all three germ layers (mesoderm, endoderm, and ectoderm).
Figure 2: Continued.
observed especially with UTA.13602.HCMT cell line (data not shown). When all time points in each group (WT-CM, HCMT-CM, and HCMM-CM) were combined, the number of multinucleated CMs was significantly higher in HCMT-CMs than in WT-CMs and HCMM-CMs (n = 6 in statistical analysis, p < 0.05, Figure 2(c)).

The \(Ca^{2+}\) handling properties of hiPSC-derived CMs were studied by \(Ca^{2+}\) imaging. HCMT-CMs had significantly more abnormalities than WT-CMs and HCMM-CMs, when all time points in each group were combined (Figure 2(d), n = 6 in statistical analysis, p < 0.05). Spontaneously beating CMs were divided into five different rhythm categories (normal, oscillation, low/middle peaks, double peaks, and plateau abnormality) based on the abnormalities observed in their \(Ca^{2+}\) signals (Figures 2(e) and 2(f)). HCMT-CMs had significantly higher number of double peaks than HCMM-CMs (p < 0.05, n = 6 in statistical analysis) when both cell lines and all time points were combined for each group.

**3.3. Action Potential Characteristics of WT and HCM hiPSC-Derived Cardiomyocytes.** The spontaneous action potentials were recorded from the beating hiPSC-derived CMs to establish the electrophysiological baselines. Most of the cells (>80%) were ventricular-like CMs in all the hiPSC lines studied. For this reason, only ventricular-like waveforms are presented here. We first analyzed the percentage of the arrhythmias in each cell line (Figures 3(a)–3(f)) and found similar percentage in both cell lines within the groups (UTA.04602.WT (13%) versus UTA.04511.WT (15%), UTA.02912.HCMT (42%) versus UTA.13602.HCMT (47%), and UTA.07801.HCMM (50%) versus UTA.06108.HCMM (50%). Based on the percentage of the arrhythmias, we combined hiPSC-derived CMs into groups (WT-CM, HCMT-CM, and HCMM-CM) for further analysis.

Both HCMT-CMs and HCMM-CMs had more arrhythmic events including delayed after depolarizations (DADs) and early after depolarizations (EADs) when compared to the WT-CMs (WT-CM (14%), HCMT-CM (45%), and HCMM-CM (50%)). We quantified the occurrence of DADs in hiPSC-derived CMs as a rate (DADs/min) calculated as total number of DADs/total number of APs. We found that the DAD rate in HCMM-CMs was significantly higher than in WT-CMs (Figure 3(g), p < 0.005).
Figure 3: Arrhythmogenic events (DADs and EADs) were observed in HCM-CMs. (a)–(e) Representative recordings of control hiPSC-derived CMs (WT) and hiPSC-derived CMs carrying TPM1-Asp175Asn (HCMT) or MYBPC3-Gln1061X (HCMM) mutations. Typical DADs (arrows) are presented in (b) and (c) and EADs (arrows) in (d) and (e) for HCMT-CMs and HCMM-CMs, respectively. Scale bars represent 40 mv and 5 seconds, respectively. Scale bars in (a) are representative for (b) and (c), and scale bars in (d) are representative for (e). (f) Distribution of CMs exhibiting arrhythmogenic events in each cell line. (g) DAD rate was significantly higher in HCMM-CMs than in WT-CMs (**p < 0.005).
Table 3: AP properties of ventricular-like CMs derived from control hiPSC lines (WT) and from hiPSC lines carrying TPM1-Asp175Asn (HCMT) or MYBPC3-Gln1061X (HCMM) mutations. In the results, the data of each group is comprised from two separate cell lines.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Beating rate (BPM)</th>
<th>APD&lt;sub&gt;90&lt;/sub&gt; (ms)</th>
<th>APD&lt;sub&gt;90&lt;/sub&gt; (ms)</th>
<th>APA (mV)</th>
<th>MDP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>43</td>
<td>58.1 ± 2.3</td>
<td>277.3 ± 13.0</td>
<td>323.6 ± 13.9</td>
<td>119.5 ± 1.1</td>
<td>-76.8 ± 0.8</td>
</tr>
<tr>
<td>HCMT</td>
<td>71</td>
<td>48.4 ± 1.5**</td>
<td>372.3 ± 13.2**</td>
<td>433.1 ± 14.0**</td>
<td>121.2 ± 1.1</td>
<td>-75.8 ± 0.7</td>
</tr>
<tr>
<td>HCMM</td>
<td>54</td>
<td>47.1 ± 1.8**</td>
<td>319.5 ± 13.7&lt;sup&gt;§&lt;/sup&gt;</td>
<td>377.6 ± 15.0&lt;sup&gt;*&lt;sup&gt;§&lt;/sup&gt;</td>
<td>124.3 ± 1.4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-77.9 ± 0.8</td>
</tr>
</tbody>
</table>

<sup>1</sup>HCM or HCMM versus WT.  
<sup>§</sup>HCMM versus HCMT.  
<sup.§</sup> or *p < 0.05 and **p < 0.005.

The average APD at 50% repolarization (APD<sub>90</sub>) and 90% repolarization (APD<sub>90</sub>) of HCMT-CMs was significantly longer than those of the WT-CMs (APD<sub>90</sub> (p < 0.005) and APD<sub>90</sub> (p < 0.005)) and HCMM-CMs (APD<sub>90</sub> (p < 0.05) and APD<sub>90</sub> (p < 0.05)) (Table 3). APD<sub>90</sub> of HCMM-CMs was significantly longer than that of the WT-CMs (p < 0.05) (Table 3). Furthermore, the beating rates of both HCMT-CMs and HCMM-CMs were significantly lower than in WT-CMs (WT-CM versus HCMT-CM (p < 0.005) and WT-CM versus HCMM-CM (p < 0.005)). In addition, the APA of HCMM-CMs was significantly higher than in the WT-CMs (p < 0.05). However, no significant differences were found for the MDP between any groups (Table 3).

3.4. Differences in the Gene Expression Profiles of hiPSC-Derived Cardiomyocytes. Dissociated hiPSC-derived CMs were cultured for one week before qRT-PCR analysis was performed. The results are presented in Figure 4. The expression of sarcomeric genes MYBPC3, TNNT2, ACTN2, TTN, MYL7, and MYL9 was significantly higher in both HCMT-CMs and HCMM-CMs than in the WT-CMs (p < 0.005 in all cases). The expression of TPM1 and TNNT2 was significantly increased only in the HCMM-CMs when compared to WT-CMs (p < 0.005 in both cases). On the other hand, the expression of MYH6 was on the same level in all hiPSC-derived CMs. Moreover, the expression of some sarcomeric genes (TNNT2, ACTN2, TNNT2, TTN, MYL7, and MYL9) was significantly higher in the HCMM-CMs than in the HCMT-CMs (p < 0.005 in other than TNNT2 and MYL9 p < 0.05). The expression of natriuretic peptide A (NPPA) was similar in all hiPSC-derived CMs while the expression of natriuretic peptide B (NPPB) was increased in HCMT-CMs and HCMM-CMs when compared to WT-CMs (p < 0.005 in both cases). Nodal marker HCN4 was also significantly increased in both HCMT-CMs when compared to WT-CMs (p < 0.05 for HCMT-CMs versus WT-CMs and p < 0.005 for HCMM-CMs versus WT-CMs). Further, the highest expression of potassium channel KCNQ1 and sodium channel SCN5A and SCN8A was observed in HCMM-CMs (p < 0.05 when compared to WT-CMs in all cases).

We found differences also in the expression of genes related to the Ca<sup>2+</sup> handling. The expression of CACNA1C and PLN was increased in both HCMT-CMs (p < 0.05 for CACNA1C and p < 0.005 for PLN) and HCMM-CMs (p < 0.005 in both cases) when compared to WT-CMs while the expression of ATP2A2 and ITPR2 was on the same level in all hiPSC-derived CMs. However, the expression of CASQ2 and RYR2 was significantly higher in HCMT-CMs and HCMM-CMs when compared to WT-CMs (p < 0.005 in all cases). Moreover, the expression of RYR2 was almost six times higher in the HCMM-CMs than in the WT-CMs (p < 0.005) and around three times higher than in HCMM-CMs (p < 0.005).

3.5. Truncated MYBPC Protein Was Not Detected in hiPSC-Derived Cardiomyocytes Carrying MYBPC3-Gln1061X Mutation. Both wild type and the mutant TPM1 mRNA were present in HCM-CMs carrying the TPM1-Asp175Asn mutation while hiPSC-derived control CMs expressed only wild type mRNA. However, in hiPSC-CMs carrying the MYBPC3-Gln1061X mutation the mutant mRNA was not clearly detected (Supplementary Figure 7). At a protein level all hiPSC-derived CMs expressed MYBPC, cTnT, and TPM1 (Figures 5(a) and 5(b)). The truncated MYBPC protein (predicted size: 116 kDa) was not detected in HCMM-CMs with western blot analysis (Figure 5(b)). However, the expression level of total MYBPC was slightly reduced in HCMM-CMs when compared to WT-CMs (Figure 5(c)). In addition, the expression of cTnT and TPM1 was elevated in both HCM-CMs (Figure 5(c)).

4. Discussion

Here we have analyzed characteristics of hiPSC-derived HCM-CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X gene mutation. The size of the MYBPC3-Gln1061X CMs was significantly larger than that of TPM1-Asp175Asn CMs, while the CMs carrying the latter mutation had significantly more abnormal Ca<sup>2+</sup> transients. Additionally, CMs with TPM1-Asp175Asn mutation had significantly more prolonged action potentials. However, both types of HCM-CMs had increased amount of arrhythmogenic events (DADs and EADs) in electrophysiological recordings when compared to control CMs. In addition to morphological and functional differences, also gene expression profiles were different between CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X gene mutation.

Genetic HCM is primarily due to mutations in sarcomeric genes, while changes at the cellular level include disturbed Ca<sup>2+</sup> metabolism and decreased contraction force generation in addition to enlarged cell size [23]. To our knowledge,
Figure 4: Continued.
total of three reports studying the characteristics as well as the pathophysiological mechanisms of the HCM by using the patient-specific hiPSCs have been published [12–14]. In two of these publications, the mutation is located in the MYH7 (MYH7-R663H or MYH7-R442G) [12, 13], whereas, in the most recent publication, the hiPSCs were derived from three HCM patients, from whom one carried the MYBPC3-999-1004del2, while the other mutations were unknown [14]. In addition to these three publications, hiPSC-derived CMs carrying MYBPC3 mutations have been used in one study where the effects of serum on the phenotype of neonatal rat CMs as well as hPSC-derived CMs have been explored [24]. hiPSC-derived CMs carrying MYBPC3 mutation were used only when studying the effects of serum on the cellular enlargement [24]. In this current study, we obtained hiPSCs from HCM patients carrying either the MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. We believe that this is the first report where hiPSC-derived CMs carrying different gene mutations have been compared in the same study with similar experimental settings.

Our HCM-CMs demonstrated cellular enlargement similarly to previous HCM studies with hiPSCs [12–14]. However, we observed a significant difference in the cellular enlargement between the two HCM mutations. CMs carrying the MYBPC3-Gln1061X mutation presented more pronounced and earlier cellular enlargement than CMs carrying the TPM1-Asp175Asn mutation. In a previous study, serum has been shown to mask hypertrophic phenotype of the CMs with mutations in the MYBPC3 [24]. The CMs from the HCM patients were larger in serum-free conditions without any external stimuli, while the serum seemed to increase the cellular enlargement in WT-CMs but not in CMs with HCM mutations [24]. We used 20% serum in our CM culture medium that did not seem to mask the cellular enlargement with our CMs. Already after one week of culture, CMs carrying MYBPC3-Gln1061X mutation were significantly larger than the WT-CMs. The enlargement of CMs carrying TPM1-Asp175Asn mutation was detectable only after three weeks of culture. In the previous clinical studies, as well as in the patient data analyzed in this current study, the hypertrophy has been in the same range in patients carrying either of these two mutations [2]. Therefore, these differences in CM size between the two mutations do not correlate with the extent of clinical hypertrophy.

The higher Ca$^{2+}$ sensitivity, observed in animal models and myectomy samples, has been suggested to be a common feature for all HCM mutations [25, 26]. In the previous studies with hiPSC-derived CMs, irregularities in Ca$^{2+}$ transients have been observed in the MYH7-R663H and the MYH7-R442G mutations [12, 13]. The higher Ca$^{2+}$ sensitivity has been related to lower phosphorylation levels of the MYBPC and the Troponin I proteins and the difference could at least partly be explained by hypophosphorylation of the sarcromeric proteins compared to the actual mutations [26]. In our study, the amount of abnormalities in Ca$^{2+}$ transients was significantly increased only in the hiPSC-derived CMs with the TPM1-Asp175Asn mutation. Indeed, the amount of irregularities in Ca$^{2+}$ handling properties was similar in the MYBPC3-Gln1061X compared to that in the WT-CMs. The phosphorylation of the proteins was not analyzed in this current study. However, we analyzed the expression of genes related to Ca$^{2+}$ handling and they were at the highest level in hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation. This might be at least partly due to the larger cell size of these CMs. Han and coworkers found decreased level of RYR2 expression in HCM-CMs carrying MYH7-R442G mutation [13], while in our study the expression of RYR2 was significantly higher in both mutations and almost six times higher in the hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation than in the WT-CMs. These observations suggest that abnormal Ca$^{2+}$ transients in HCM-CMs carrying different mutations may be caused by distinct mechanisms.

One of the fundamental features of the HCM is its association with ventricular arrhythmias responsible for severe cardiac malfunctions including sudden cardiac death [27, 28]. We found increased amount of arrhythmogenic events (DADs and EADs) in both HCM-CMs. Furthermore, decreased beating rate was observed in both types of HCM-CMs, which could be due to higher occurrence of DADs between the two consecutive APs. In addition, the APD$_{90}$ of hiPSC-derived CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X mutation was longer than in WT-CMs, which is in line with previous findings with different mutations [12, 13, 29]. The mechanism of arrhythmias in HCM is not yet fully understood; however, imbalances in Ca$^{2+}$ homeostasis are considered as a main cause of arrhythmias shown in the previous studies [12, 13]. Clinically, patients carrying TPM1-Asp175Asn mutation have been reported to
Figure 5: Cardiac-specific protein expression in hiPSC-derived CMs. (a) Representative images of hiPSC-derived CMs carrying TPM1-Asp175Asn mutation (HCMT) or MYBPC3-Gln1061X mutation (HCMM) and hiPSC-derived control CMs (WT) stained with cTnT, MYBPC, and TPM1. These images are not to quantify the protein expression but to demonstrate the presence of cTnT, MYBPC, and TPM1 proteins in the hiPSC-derived CMs. (b) hiPSC-derived CMs from all cell lines expressed MYBPC, cTnT, and TPM1 proteins. The truncated MYBPC was not detected in HCMM cells (size of the wild type protein 141 kDa and the predicted size of the truncated protein 117 kDa). (c) The expression of MYBPC, cTnT, and TPM1 in WT-CMs, HCMT-CMs, and HCMM-CMs normalized to the expression of β-actin. Protein expressions were quantified from western blots using ImageJ software. Quantitation data show the averages of MYBPC/β-actin, cTnT/β-actin, and TPM1/β-actin relations from hiPSC-derived CMs from two different hiPSC lines in each group. Because of the lack of replicates, statistical analysis was not performed.
be more prone to arrhythmias than those carrying MYBPC3-Gln1061X mutation [30]. Our data with hiPSC-derived CMs support this finding by demonstrating more abnormal Ca\(^{2+}\) transients and longer APD\(_{90}\) in TPM1-Asp175Asn CMs than in HCM-CMs carrying the MYBPC3-Gln1061X mutation.

Like most of the HCM mutations located in the MYBPC3, also MYBPC3-Gln1061X is a nonsense mutation that leads to premature stop-codon [31]. Nonsense mutations are suggested to act through haploinsufficiency in which the mutated protein is either degraded or not produced at all. The truncated form of MYBPC has not been found in human cardiac samples while the total expression level of MYBPC has been reported to vary from being decreased to even increased [32–36]. Interestingly, when studying myectomy samples from HCM hearts with increased myocardial fibrosis, Helms et al. observed that the total amount of MYBPC mRNA was increased, while the total amount of MYBPC protein was on the same level compared to that in the control hearts. They hypothesized that the upregulation of the MYBPC compensates the degraded truncated MYBPC protein [35].

We could not detect mutant allele on mRNA expression level or truncated MYBPC protein in hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation. Similar absence of truncated protein has been reported earlier with a different MYBPC mutation in hiPSC-derived CMs [14]. These data suggest that the mutant mRNA might be degraded. However, further research is still needed to confirm the results and to discover the actual degradation mechanism.

The two HCM mutations analyzed in this study are the most frequent mutations in Finland accounting for about 18% of all Finnish HCM patients [3]. In our study, we found differences in the morphological and biochemical properties, as well as in Ca\(^{2+}\) cycling and electrophysiological properties between the CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X mutation. However, we have not analyzed the possible effects of other gene mutations and epigenetic factors on the phenotype differences observed in our study. Additionally, we used only two hiPSC lines from two different patients in each mutation in our experiments. In the future, we need to extend our studies including further patients and studying the effects of additional gene mutations and epigenetic factors. The immature nature of hiPSC-derived CMs is a general limitation when using these cells in disease modeling. Further development in the differentiation and maturation protocols will increase the reliability of studies conducted with hiPSC-derived CMs. Finally, the exact pathophysiology in abnormal Ca\(^{2+}\) transients or electrical abnormalities is not known and further research with combined patch clamp and Ca\(^{2+}\) imaging is required in the future to reveal the significance of the cellular findings for clinical phenotypes as well as for treatment options.

5. Conclusions

In conclusion, both HCM hiPSC-derived CMs either carrying the TPM1-Asp175Asn or MYBPC3-Gln1061X mutation exhibited pathological changes related to HCM. However, significant differences between the two mutations were observed. The hiPSC-derived cell models, established in this study, can be exploited to study further the pathophysiological mechanisms of HCM as well as to screen drugs and potentially optimize treatments in mutation-specific way.

Abbreviations

- AP: Action potential
- APA: Action potential amplitude
- APD: Action potential duration
- bFGF: Basic fibroblast growth factor
- BPM: Beats per minute
- CM: Cardiomyocyte
- CPVT: Catecholaminergic polymorphic ventricular tachycardia
- cTnT: Troponin T
- DAD: Delayed after depolarization
- DAPI: 40,6-Diamidino-2-phenylindole
- DCM: Dilated cardiomyopathy
- EAD: Early after depolarization
- EB: Embryoid body
- END-2: Mouse endodermal-like cells
- FBS: Fetal bovine serum
- HCM: Hypertrophic cardiomyopathy
- HCM-CM: Cardiomyocytes derived from hiPSCs carrying HCM mutation
- HCM-M-CM: Cardiomyocytes derived from hiPSCs carrying MYBPC3-Gln1061X mutation
- HCM-T-CM: Cardiomyocytes derived from hiPSCs carrying TPM1-Asp175Asn mutation
- hiPSC: Human induced pluripotent stem cell
- hPSC: Human pluripotent stem cell
- HRP: Horseradish peroxidase
- ICD: Implantable cardioverter defibrillator
- ko: Knockout
- ko-SR: Knockout serum replacement
- MDP: Maximum diastolic potential
- MEF: Mouse embryonic fibroblast
- MYBPC: Myosin-binding protein C
- MYH7: ß-myosin heavy chain
- NEAA: Nonessential amino acids
- RT-STA: Reverse transcription-specific target amplification
- SCD: Sudden cardiac death
- SEM: Standard error of the mean
- TPM1: ß-tropomyosin
- WT-CM: Cardiomyocytes derived from control hiPSCs

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study was funded by the Finnish Foundation for Cardiovascular Research, the Finnish Funding Agency for
Technology and Innovation, Competitive Research Funding of the Pirkanmaa Hospital District, Finnish Cultural Foundation, Aarne and Aili Turunen Foundation, Maud Kuistila Memorial Foundation, and Aarne Koskelo Foundation. The authors would like to acknowledge Markus Haponen, Merja Lehtinen, Kirsi Penttinen, Julia Silvennoinen, and Henna Venäläinen for the general technical support, Turkka Mannila for the help with data analysis, Reija Autio for the help with statistical analysis, and Christine Mummery (Leiden University Medical Center, Leiden, Netherlands) for providing the END-2 cells.

References


