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Generation of catecholaminergic polymorphic ventricular tachycardia patient-specific induced pluripotent stem cell line

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ABSTRACT

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) is a genetic disorder characterized by ventricular tachycardia, that can cause the heart to stop beating leading to death. The prevalence is 1/10.000 and in approximately 60% of cases, the syndrome can be due to a mutation of the cardiac ryanodine receptor gene (*RyR2*). We derived an induced pluripotent stem cell (iPSC) line from an 11-year-old patient blood-cells, carrying a heterozygous missense mutation on the 8th exon of the *RyR2* N-terminal part. This reprogrammed CPVT line displayed normal karyotype, expressed pluripotent markers and had a capacity to differentiate in trilineage embryonic layers.

1. Resource table

Unique stem cell line identifier	INSRMi011-A
Alternative name(s) of stem cell line	CPVT_P16_PhyMedExp
Institution	Safe-iPSC Facility INGESTEM, CHU of Montpellier
Contact information of distributor	Albano C. Meli (albano.meli@inserm.fr)
Type of cell line	iPSC
Origin	Human
Additional origin info required for human ESC or iPSC	Age: 11 years
Cell Source	Sex: Male
Clonality	Blood
Method of reprogramming	Mixed
Genetic Modification	Sendai virus kit 2.0 expressing Klf4–Oct3/4–Sox2 (KOS), c-Myc.
Type of Genetic Modification	YES
	Heterozygous missense mutation in the exon 8 of the <i>RyR2</i> gene
	The c-Myc vector contains a temperature sensitivity mutation, cells were incubated

(continued on next column)

(continued)

Evidence of the reprogramming transgene loss (including genomic copy if applicable)	for 5 days at 38–39 °C. Transgene loss is shown by RT-PCR with primers specific to the Sendai virus from RNA of cells at passage 10
Associated disease	Catecholaminergic Polymorphic Ventricular Tachycardia
Gene/locus	Gene: <i>RyR2</i> Locus: Between q42.1 and q43
Date archived/stock date	Not available
Cell line repository/bank	https://hpscreg.eu/user/cellline/edit/INSRMi011-A
Ethical approval	CPP 1003 – HPS2 CHU Montpellier

1.1. Resource utility

CPVT syndrome induces cardiac arrhythmias and can lead to sudden death after acute stress. We generated iPSC cell lines from a CPVT patient experiencing cardiac and neuro-behavioural syndromes. These

Abbreviations: RyR2, Ryanodine receptor type 2; CPVT, Catecholaminergic polymorphic ventricular tachycardia; iPSC, Induced pluripotent stem cells; SR, Sarcoplasmic reticulum; PBMC, Peripheral blood mononuclear cells; EB, Embryoid body.

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Fig. 1. A) CPVT induced pluripotent stem cell lines generated by reprogramming of PBMC from patients with RyR2-R169P single-point mutation. The mutation (in pink) is shown by the white arrows on the 3D structure of RyR2. B) Characterization of CPVT iPSC P16 colonies by alkaline phosphatase staining and morphology (bright field picture). Immunofluorescence staining after 10 passages analysis reveals specific pluripotency marker expression including OCT4, SSEA4, SOX2, and TRA-1-60. Analysis of the expression of pluripotency markers by flow cytometry reveals expression of OCT4, SSEA4, SOX2 and NANOG. C) Heatmap transcriptome analysis of supervised clustering on genes involved in pluripotency. The list of genes involved in pluripotency and upon differentiation is indicated. Gene expression for pluripotency is increased between iPSC cell lines (CPVT iPSC P16, iPSC PBMC, iPSC BJ) while embryoid bodies (EB 4F) exhibit increased gene expression for differentiation. A negative control of pluripotency (Fibroblast BJ) was used. D) Karyotype analyses on RHG-banding indicates that CPVT iPSC P16 line has a normal chromosomal content. E) *In vivo* teratoma derivation revealing structures corresponding to the three embryonic germ layers, including endodermal derivative (epithelium with muciparous cells), neuro-ectodermal derivative (neural tube) and mesodermal derivative (smooth muscular cells and cartilage). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Normal	Fig. 1 panel B
Phenotype	Qualitative analysis (Immunocytochemistry)	Assess staining of pluripotency markers: Oct4, Sox2, SSEA-4, Tra 1-60	Fig. 1 panel B
	Quantitative analysis (Flow cytometry)	NANOG: 99,66%, OCT4: 99,80%, SOX2: 99,85%, SSEA-4: 99,99%	Fig. 1 panel B
Genotype	Karyotype (G-banding) and resolution	46XY, Resolution 450-500	Fig. 1 panel D
Identity	DNA profiling		Not performed
	STR analysis	18 STR sites tested, and matched between the original cell lines and the reprogrammed one	Submitted in archive with journal
Mutation analysis	Sequencing Southern Blot OR WGS	c.506G > Cp.Arg169Pro was confirmed by standard Sanger sequencing Not Performed	Not available
Microbiology and virology	Mycoplasma (MycAlert® Detection Kit Negative)	Negative	
Differentiation potential	Histologic analysis of teratoma formation	Teratoma shows differentiation zones derived from the three layers, endoderm (epithelium with muciparous cells), ectoderm (neural tube) and mesoderm (smooth muscular cell and cartilage)	Fig. 1 panel E
List of recommended germ layer markers	Germ layer markers expression, RT PCR	Ectodermal markers (MAP2, PAX6, SOX2), endodermal markers (SOX17, ITGAV) and mesodermal markers (CDH2, DLL1) expression showed by RT-qPCR	primers listed in Table 2
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not Performed	e.g. not shown but available with author
Genotype additional info (OPTIONAL)	Blood group genotyping	Not Performed	e.g. not shown but available with author
	HLA tissue typing	Not Performed	e.g. not shown but available with author

cells will be further differentiated into cardiomyocytes and neurons, to study the impact of the RyR2 mutation on the cellular properties.

2. Resource details

CPVT is an inherited cardiac syndrome characterized by ventricular arrhythmias under stress or exercise. CPVT is mostly due to mutations on the cardiac ryanodine receptor/calcium release channel (*RyR2*) gene which protein is localized on the sarcoplasmic reticulum (SR). This syndrome is characterized by an abnormal heart rhythm, ventricular tachycardia, which occurs after catecholamine release, in response to stress or physical activity. CPVT-induced arrhythmias can lead to sudden cardiac death. We have reported some pathological links between CPVT-induced ventricular arrhythmias and neurobehavioral disorders (Lacampagne et al., 2017; Liu, 2012). In CPVT mice, epileptic seizure independent of cardiac arrhythmias has been reported along with the *RyR2* mutation-induced SR calcium leak in brain and heart (Lehnart, 2008). While the animal models offer limited access to what is observed in clinic, such pathological mechanism is unclear in CPVT patients. Furthermore, it is difficult to obtain human cardiac and neuronal biopsies for ethical reasons. The iPSC model, via the differentiation of neurons and cardiomyocytes, is an exciting technology to study the CPVT molecular mechanisms using the patient-specific genetic background and cellular environment. To this aim, peripheral blood mononuclear cells (PBMC), obtained under approval of the Montpellier CHU (CPP 1003-HPS2), were isolated from a blood sample of a 11-year-old CPVT patient, carrying a missense mutation on the 8th exon of the *RyR2* gene and experiencing stress-induced cardiac arrhythmias and a neurobehavioral syndrome. This mutation leads to a substitution of an

Arginine by a Proline at position 169, located on the RyR2 N-terminal domain (Fig. 1 panel A) (Peng, et al., 2016).

PBMCs were subjected to transient expression of the four reprogramming factors OCT3/4, SOX2, KLF4 and C-MYC using the Sendai virus gene delivery method without integration. Characterization of the CPVT iPSC line by bright field picture, alkaline phosphatase, immunofluorescence staining, and flow cytometry showed a normal morphology (Fig. 1 panel B). The CPVT iPSC line had pluripotent features indicated by its differentiation into the three germ layers using teratoma formation in immune compromised mouse containing iPSC derived in endoderm (epithelium with muciparous Goblet cells, left), neuro-ectoderm (neural tube, middle left), and mesoderm (smooth muscular cells and cartilage, middle right and right) (Fig. 1 Panel E). The pluripotency is also showed in the transcriptomic analysis of pluripotent genes (Fig. 1 panel C) and RT-PCR. The iPSC CPVT cell line carry CPVT mutation and presented no major genomic aberrations after analysis through karyotype (Fig. 1 Panel D) according the ACLF (*Association des Cytogénéticiens de la Langue Française*) resolution recommendations. Characterization of the CPVT iPSC cell line is summarized in Table 1.

3. Materials and methods

3.1. Reprogramming PBMC to iPSC

From blood samples, PBMC were isolated and cultured in SFEM II (StemSpan™ SFEM II Stemcell, cat# 09605) medium containing cytokines (StemSpan™ Erythroid Expansion Supplement (100X) Stemcell, Catalog cat#02692) as previously described (Gatinois, Aug. 2019). The

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers Flow cytometry	Nanog XP® Rabbit mAb (Clone D73G4) IgG	1:200	Cell Signaling Technology Cat# 4903	RRID: AB_10559205
Pluripotency Markers Flow cytometry	Oct-4A Rabbit mAb (Clone C30A3) IgG	1:200	Cell Signaling Technology Cat# 2840	RRID:AB_2167691
Pluripotency Markers Flow cytometry	Sox2 XP® Rabbit mAb (Clone D6D9) IgG	1:200	Cell Signaling Technology Cat# 3579	RRID:AB_2195767
Pluripotency Markers Flow cytometry	SSEA4 Mouse mAb (Clone MC813) IgG3	1:200	Cell Signaling Technology Cat# 4755	RRID:AB_1264259
Pluripotency Markers Flow cytometry	TRA-1-60(S) Mouse mAb (Clone TRA-1-60(S)) IgM	1:200	Cell Signaling Technology Cat# 4746	RRID:AB_2119059
Pluripotency Markers Immunostaining	PE Mouse anti-human Nanog (Clone: N31-355)	1:5	BD Biosciences Cat# 560791	RRID:AB_1937305
Pluripotency Markers Immunostaining	PerCP-CyTM 5.5 Mouse anti-Oct3/4 (Clone: 40/Oct-3)	1:5	BD Biosciences Cat# 560794	RRID:AB_1937313
Pluripotency Markers Immunostaining	Alexa FluorR 647 Mouse anti-Sox2 (Clone: 245610)	1:5	BD Biosciences Cat# 560301	RRID:AB_1645308
Pluripotency Markers Immunostaining	Alexa FluorR 647 Mouse anti-SSEA-4 (Clone: MC813-70)	1:5	BD Biosciences Cat# 560796	RRID:AB_2033991
Pluripotency Markers Immunostaining	PE Mouse IgG1, κ_Isotype Control (Clone MOPC-21)	1:5	BD Biosciences Cat# 554121	RRID:AB_395252
Pluripotency Markers Immunostaining	PerCP-Cy5.5 Mouse IgG1, κ_Isotype Control (Clone: X40)	1:5	BD Biosciences Cat# 347202	RRID:AB_400265
Pluripotency Markers Immunostaining	Alexa Fluor®_647 Mouse IgG2a, κ_Isotype Control (Clone: MOPC-173)	1:5	BD Biosciences Cat# 558020	RRID:AB_396989
Pluripotency Markers Immunostaining	Secondary Antibody Alexa Fluor®_488 conjugate Goat anti-Rabbit IgG	1:400	Invitrogen-Thermo Fisher Scientific Cat# A-11034	RRID: AB_2576217
Pluripotency Markers Immunostaining	Secondary Antibody Alexa Fluor®_555 conjugate Goat anti-Rabbit IgG	1:400	Invitrogen-Thermo Fisher Scientific Cat# A-21424	RRID:AB_141780
Primers				
Target	Size of band	Forward/Reverse primer (5'-3')		
Virus RNA sendai.	181bp	GGATCACTAGGTGATAT/ACCAGACAAGAGTTTAAGAGATATGTATC		
Homo sapiens cadherin 2 (CDH2)	136bp	AGCCAAACCTTAACTGAGGAGT/GGCAAGTTGATTGGAGGGATG		
Homo sapiens delta-like 1 (DLL1)	168bp	GATTCTCTGATGACCTCGCA/TCCGTAGTAGTGTTCGTGACA		
Homo sapiens integrin, alpha V (ITGAV)	183bp	GGCTGCATATTTCCGATTTTCTG/CCATTAGCTTTGTCGCTGCTGG		
Homo sapiens microtubule-associated protein 2 (MAP2)	91bp	TGGTGCCGAGTGAGAAGAAG/AGTGGTTGGTTAATAAGCCGAAG		
Homo sapiens paired box 6 (PAX6)	120bp	AACGATAACATACCAAGCGTGT/GGTCTGCCCGTTCAACATC		
Homo sapiens SRY (sex determining region Y)-box 2 (SOX2)	215bp	TGGACAGTTACGCGCACAT/CGAGTAGGACATGCTGTAGGT		
Homo sapiens SRY (sex determining region Y)-box 17 (SOX17)	94bp	CTCCGGTGTGAATCTCCCC/CACGTCAGGATAGTTGCAGTAAT		

cells were transduced by Sendai Virus using the CytoTune® iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific, cat#A34546) containing the Klf4, Oct3/4, Sox2 and c-Myc vectors, as previously described (Gatinois, 2019).

3.2. iPSC culture and genomic DNA extraction

iPSC were maintained on extracellular matrix Matrigel hES qualified (Fisher Scientific, cat#354277, lot number: 5194015, with a dilution factor 255 µl as recommended by the manufacturer) and cultured in Essential 8™ culture media (Thermo Fisher Scientific, cat#A15169-01) according to the manufacturer's instruction at 37 °C in 5% O₂ and 5% CO₂. Cells were dissociated using Versene solution (Thermo Fisher Scientific; cat#15040066) for manual dissociation. iPSC were differentiated via EB formation using AggreWell™ EB Formation Medium (Stem Cell, no. 05893) and AggreWell 800 plates (Stem cell, no. 34811). Once formed, EB were transferred in non-adherent 6 well plates Corning (Stem Cell, no. 3471). EB were kept for 15 days in culture. For DNA extraction and isolation, we employed the QIAamp DNA Mini Kit (Qiagen) and followed the described protocol as performed before (Gatinois, 2019).

3.3. Flow cytometry analysis

To perform flow cytometry analysis, single cell (P12) suspension was fixed in 4% paraformaldehyde in PBS and labelled after permeabilization according to standard protocols of BD Stemflow Human and Mouse Pluripotent Stem Cell Analysis Kit (no.560477) and BD Stemflow Human

Pluripotent Stem Cell Transcription Factor Analysis Kit (no.560589). Specific pluripotent markers were stained using specific antibodies as it is described in the Table 2. Cells were analyzed on a CANTO II Becton Dickinson and analysis was made with Flow-JO.

3.4. Immunofluorescence

Cells (P10) grown on coverslips were fixed in 4% paraformaldehyde in PBS and labelled overnight at room temperature, after 0,1% Saponin permeabilization in the blocking buffer (5% goat serum) for 60 min according to the standard protocol of StemLight™ Pluripotency Antibody Kit (cat#9656). Fluorochrome-conjugated anti primary antibodies with Alexa Fluor® 488 and Alexa Fluor® 555 dyes were applied 60 min. DNA was stained with DAPI (ImmunoChemistry, cat#6244) (Fig. 1 Panel B). Antibodies are listed in Table 2.

3.5. Transcriptomic analysis

Total RNA isolation was performed using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA was hybridized on a Affymetrix GeneChip Human Genome U133 Plus 2.0 array (Fig. 1 Panel C).

3.6. RT-PCR

RNA was extracted with the Qiagen RNA easy kit following the kit instructions (Cat # 74104). The fold increase was calculated using the

algorithm, also known as delta-delta-Ct. The level of gene expression from the EBs after 15 days of differentiation was compared to the original iPSC (P20) The primer sequences listed in Table 2.

3.7. Karyotype analysis

Karyotype analysis was performed on actively dividing cells (P20) on RHG-banding using standard procedures at SAFE iPSC facility (Gatinois, 2019). A minimum of 10 to 15 metaphases were counted and scored, up to 50 when a mosaicism was suspected. Results are shown in Fig. 1 panel D.

3.8. Teratoma induction and histological analysis

The differentiation potency was evaluated by *in vivo* teratoma derivation. Clusters corresponding to approximately 3×10^6 iPSC (P20) cells were injected into anesthetized NOD SCID gamma (NOD.CgPrkdcscidIl2rg tm1Wjl/SzJ). After 4–8 weeks of latency, a 100% derivation efficiency was observed and teratomas were fixed, embedded in paraffin blocks, stained with Hematoxylin-Eosin-Saffron and analysed by a pathologist for the presence of structures corresponding to the three embryonic germ layers (Fig. 1 panel E).

3.9. Short tandem repeat analysis (STR)

STR analysis was performed with ATCC kit on 18 STR.

3.10. Mycoplasma detection

To detect mycoplasma, we used MycoAlert® Detection Kit (Lonza)

according to manufacturer's instructions at passage 20

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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