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Generation of human induced pluripotent stem cells (NIHTVBi004-A, NIHTVBi005-A, NIHTVBi006-A, NIHTVBi007-A, NIHTVBi008-A) from 5 CADASIL patients with *NOTCH3* mutation

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Abstract

We have successfully generated induced pluripotent stem cell (iPSC) lines derived from peripheral blood mononuclear cells of five patients with Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). These cells carry the genetic *NOTCH3* mutation present in their parental cells. These iPSC cells exhibited normal karyotype and phenotype, which were sustained through propagation. Furthermore, these iPSCs displayed the capacity of differentiating toward the three germ layers *in vitro*.

Resource Utility

Human induced pluripotent stem cells (hiPSCs) from CADASIL patients may provide a powerful tool for studying the effects of NOTCH3 on human development and the pathophysiology of CADASIL disease.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101821.

Resource Details

Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited disease caused by mutations in the *NOTCH3* gene located on chromosome 19. These autosomal dominant mutations are nonsynonymous substitutions, resulting in the addition or deletion of a cysteine residue within the NOTCH3 extracellular domain coding sequences (Tournier–Lasserve et al., 1993; and Joutel et al., 1996). This disease is characterized by migraines, white matter changes, recurrent strokes and early onset dementia through a largely unknown mechanism that is thought to primarily affect the small blood vessels in the brain. (Opherk et al., 2004; Rutten et al., 2016).

The five CADASIL patients were enrolled into one of our NHLBI clinical protocols (16-H-0132). Information regarding clinical onset was obtained using a standard clinical interview format. All 5 patients have been found to have heterozygous missense mutations in the gene *NOTCH3* located on chromosome 19. The mutations result in the addition or deletion of a cysteine residue within epidermal growth factor (EGF)-like repeats of NOTCH3 extracellular domain which is responsible for CADASIL disease. The details of genetic information are shown in Table 1, and Supplement file 1. The patient NIHTVBi004-A (HT405A) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.994C>*T*; p.Arg332Cys) which affects the calcium binding domain in EGF8; the patient NIHTVBi005-A (HT406D) has been found to have heterozygous missense mutations in the gene NOTCH3 (c.505C > *T*; p.Arg169Cys) which affects the calcium binding domain in EGF4; the patient NIHTVBi006-A (HT407D) has been found to have heterozygous missense mutations in the gene *NOTCH3* (c.697T > G; p.Cys233Gly) which affects the protein binding domain of EGF5; the patient NIHTVBi007-A (HT409A) has been found to have heterozygous missense mutations in the gene *NOTCH3* (c.665G > A; p.Cys222Tyr) which affects the protein binding domain of EGF5; and the patient NIHTVBi008-A (HT454D) has been found to have heterozygous missense mutations in the gene *NOTCH3* (c.1364G > *A*; p.Cys455Tyr) which affects the calcium binding domain in EGF11. Blood samples were collected at the NIH Clinical Center. Using a Sendai viral vector delivery system expressing four transcription factors (*OCT4, SOX2, KLF4* and *C-MYC*), we successfully generated hiPSC lines carrying each of the five *NOTCH3* mutations. The hiPSCeCADASIL lines maintained typical morphologies and expressed the common pluripotency markers OCT4, NANOG, TRA-1–60, SSEA4 and SOX2, as shown by immunocytochemistry (Fig. 1A), flow cytometry (Fig. 1B) and/or real-time (RT)-qPCR (Fig. 1D). HiPSCeCADASIL genotyping confirmed NOTCH3 mutations corresponding to each parental cell line (Fig. 1C). To test the differentiation potential of these cell lines, we performed a monolayer differentiation assay to drive cells towards the three germ layers *in vitro*. We determined the marker gene expression for the endoderm (*AFP*), ectoderm (*NES*) and mesoderm (*RUNX1*) by RT-qPCR, which showed up-regulated expression levels in differentiating cells at day 7 compared to undifferentiated hiPSCeCADASIL cells (Fig. 1D). All five iPSC lines demonstrated chromosomal stability and normal karyotype with G-banding (Fig. 1E). Short tandem repeat (STR) profiles indicated that all hiPSCeCADASIL lines matched their parental blood cells completely in 15 amplified STR loci (Supplementary

file 2). All cell cultures were routinely tested for *Mycoplasma* contamination and were found to be *Mycoplasma* free (Supplementary file 3). The iPSCs were free of Sendai virus after the 15th passage as shown by PCR (Supplementary file 4). In conclusion, hiPSCeCADSASIL lines exhibited pluripotent potential for self-renewal, proliferation and differentiation. To the best of our knowledge, this is the first published study in which hiPSC lines were generated from individuals with the CADASIL carrying *NOTCH3* mutations (Table 2).

1. Materials and methods

1.1. Subjects and study approval

iPSCs were generated from blood samples obtained from five CADASIL patients carrying a *NOTCH3* mutation. This study was approved by the NHLBI's Institutional Review Board, and samples were collected after obtaining informed written consent.

1.2. Generation and culture of human iPSCs from peripheral blood mononuclear cells (PBMCs)

The PBMCs were isolated from blood samples through density gradient centrifugation via Ficoll-Paque following the manufacturer's protocol. Approximately 3 to 5×10^5 PBMCs were plated into one well of a 6-well plate within Erythroid Expansion Medium (Stemcell Technologies) for expansion of erythroid progenitor cells. Half of the medium was replaced with fresh medium every other day. At day 9~10, the cells were transduced with CytoTune™ 2.0 Sendai reprogramming vectors (Invitrogen). After three days in culture, the cells were collected and seeded into Matrigel (Corning) pre-coated plates supplemented with TesR-E7 medium (Stemcell Technologies) for 10~15 days. After which the medium was replaced with TesR-E8 medium (Stemcell Technologies) until day 21. Once iPSC colonies emerged in culture, they were picked up and transferred onto fresh Matrigel pre-coated plate for further expansion (Jin et al., 2016).

1.3. Immunofluorescent staining and flow cytometry analysis

iPSCs were fixed with 4% paraformaldehyde and stained for immunohistochemistry. Briefly, cells were incubated with primary antibodies against SSEA4 (Table 3) at 4 C overnight. After washes in PBS, they were incubated with fluorophore-tagged secondary antibodies at room temperature for 1 h After PBS washes, the nuclei were then stained with DAPI and images were captured using a fluorescence microscope (Zeiss).

For FACS analysis, iPSCs were digested to a single cell suspension by incubation with Trypsin-EDTA (Invitrogen). Following by fixation and permeabilization, the cells were stained with antibodies. Analysis was performed on a MACSQuant Flow Cytometer (Miltenyi) and the results were analyzed using FlowJo software (FlowJo, LLC).

1.4. Monolayer differentiation assay

To assess hiPSCeCADASIL ability to differentiate *in vitro*, cell cultures were dissociated into small clumps with 0.5 μM EDTA and cultured on Matrigel Precoated Plates (Corning) with differentiation medium consisting of 90% KnockOut DMEM, 10% FBS, 2 mM $_L$-gluta-

mine, 0.1 mM non-essential amino acids and 0.1 mM 2-mercaptoethanol (Invitrogen). After seven days, cells were harvested for further analysis.

1.5. Gene expression analysis

The total RNA was isolated by using RNeasy Mini Kits (Qiagen). Endogenous mRNA expression levels of *NANOG, SOX2, AFP, NES*, and *RUNX1* were determined in iPSCs and in differentiating cells at day 7 (Fig. 1D). RT-qPCR was performed by using SYBR Green Premix on a Real-Time PCR Detection System (Bio-Rad). Assays were run in triplicate and the results were normalized to 18S ribosomal RNA expression. Primers used for RT-qPCR are shown in Table 3. After 15 passages, iPSC lines were tested for Sendai virus (SeV) residues by using RT-PCR with the primers indicated in Table 2.

1.6. Karyotyping assay

The karyotype of hiPSCeCADASIL was evaluated by the WiCell Research Institute using G-banding metaphase karyotype analysis.

1.7. DNA sequencing and STR analysis

Genomic DNA was extracted by using DNeasy Blood & Tissue Kit (Qiagen). To amplify the corresponding mutation position in *NOTCH3*, PCR was performed with specific primers (Table 3). Following purification, the PCR products were sent to Eurofins Scientific for sequencing.

STR analysis was performed by WiCell Research Institute, which generated a STR profile via the Promega Powerplex® 16 System to verify STR polymorphisms for 15 loci plus amelogenin in genomic DNA extracted from iPSCs and their parental fibroblasts.

1.8. Mycoplasma detection

To validate that the hiPSCeCADASIL cultures were *Mycoplasma* free, mycoplasma analysis was performed using the MycoAlert™ Mycoplasma Detection Kit (Lonza, LT27–224). Briefly, the culture media was collected after culturing for 48 h while the confluency of cells was at last 80%. As according to manufacturer's protocol, 100 μl of clearer supernatant was used as the test sample to screen for mycoplasma contamination. The absence of mycoplasma contamination was confirmed in all hiPSCeCADASIL culture tested (Supplement file 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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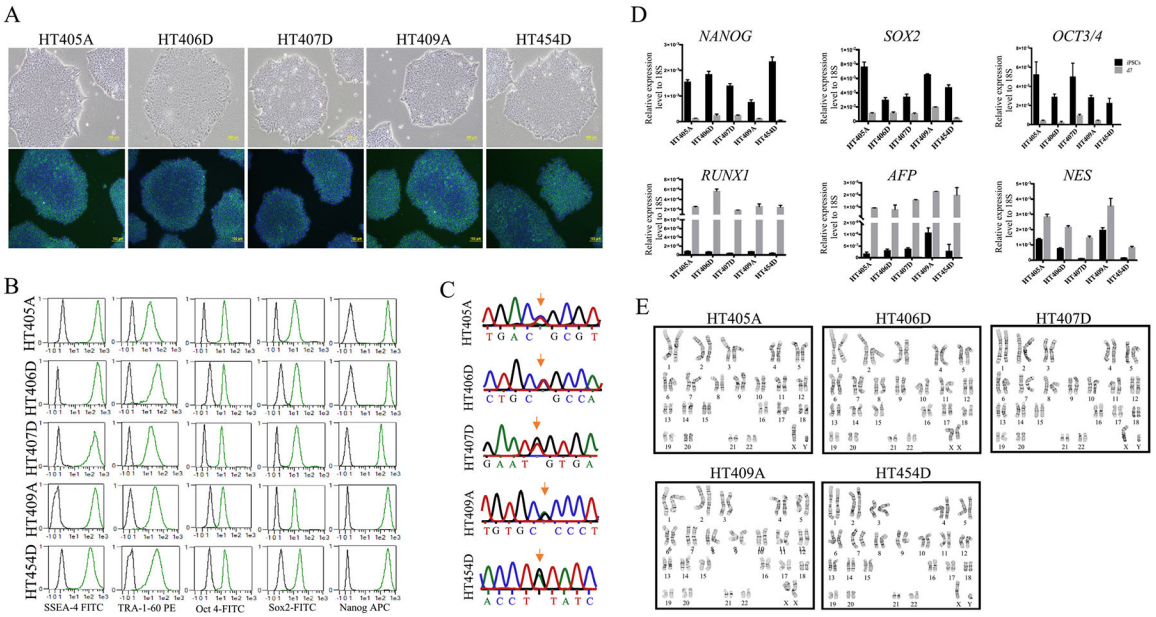

Fig. 1.

Characterization of human iPSC lines derived from five CADASIL patients with a *NOTCH3* mutation. (A) iPSCs were cultured to passage 15 on a feeder-coated plate. Phase contrast images of iPSC lines derived from five CADASIL patients with a *NOTCH3* mutation (HT405A, HT406D, HT407D, HT409A and HT454D) (top column). Expression of pluripotent markers SSEA4 was analyzed by immunofluorescence; DAPI staining of cell nuclei in blue (bottom columns) (all scale bars: 100 μm). (B) The expression level of pluripotent markers (SSEA4, TRA-1–60, Oct4, Sox2, and Nanog) was quantitative analysis by Flow Cytometry Analysis. (C) PCR and DNA sequencing identified mutations in *NOTCH3* in the iPSC lines from the five patients (red arrows). (D) Expression of pluripotent genes (*NANOG, SOX2*, and *OCT3/4*) was confirmed in all hiPSC lines derived from CADASIL patients as assessed by RT-qPCR (black bar). All five hiPSC lines from CADASIL patients were able to differentiate into three germ layers using monolayer differentiation in vitroat day 7, as shown by gene expression of *AFP, NESTIN* and *RUNX1* (gray bar). Data are represented as means ± SEM relative to mRNA levels. (E) All five hiPSC lines from CADASIL patients showed a normal karyotype with G-band analysis.

Table 1

Summary of patients with a CADASIL disease.

iPSC line names	Abbreviation in figures	Gender	Age (years)	Ethnicity	Genotype of locus	Disease
NIHTVBi004-A	HT405A	M	43	White/non-hispanic	Notch 3, C.994C > *T;* p.Arg332Cys	CADASIL
NIHTVBi005-A	HT406D	F	61	White/non-hispanic	Notch 3, C.505C > *T;* p.Argl69Cys	CADASIL
NIHTVBi006-A	HT407D	M	43	White/non-hispanic	Notch 3, C.697T > *G;* p.Cys233Gly	CADASIL
NIHTVBi007-A	HT409A	F	62	White/non-hispanic	Notch 3, C.665G > *A;* p.Cys222Tyr	CADASIL
NIHTVBi008-A	HT454D	M	50	White/non-hispanic	Notch 3, C.1364G > *A;* p.Cys455Tyr	CADASIL

Table 2

Characterization and validation.

Classification	Test	Result	Data
Morphology Phenotype	Phase-contrast microscope Qualitative analysis (immunofluorescence staining) Quantitative analysis (flow cytometry analysis, RT-qPCR)	Normal Expression of pluripotency markers: Oct4, Nanog, SSEA4 and Tra-1–60 Expression of pluripotency markers: *S0X2* and *NANOG*	Fig. 1A Fig. 1A Fig. 1B, 1D
Genotype	Karyotype (G-banding) and resolution	46.XX or 46.XY; resolution 450–500 bands	Fig. 1E
Identity	Microsatellite PCR OR STR analysis	Not performed 15 sites tested, 100% match	N/A Supplementary file 2
Mutation analysis (IF APPLICABLE)	DNA sequencing Southern blot OR WGS	Notch 3, Chromosome 19, GRch38 Not performed	Fig. 1C N/A
Microbiology and virology	*Mycoplasma* testing by luminescence	Negative	Supplementary file 3
Differentiation potential	Monolayer differentiation assay	Differentiating cells are expression of *RUNX1, AFP,* and *NES;* iPSCs were able to differentiate into three germ layers	Fig. 1D
Donor screening (OPTIONAL)	HIV1 + HIV2, hepatitis B virus, hepatitis C virus	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	Not performed Not performed	N/A N/A

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Table 3

Reagents.

Antibodies used for immunocytochemistry and *Flow Cytometry Analysis*					
	Antibody	**Dilution**	**Company**	**Cat#**	**RRID**
Primary antibodies	Mouse anti-SSEA4	1:100	MilliporeSigma	MAB4304	AB_177,629
	Alexa Fluor 488 anti-SSEA4 Antibody	1:10	BioLegend	330,412	AB_l,089,198
	PE anti-TRA-1–60 Antibody	1:10	BioLegend	330,610	AB_2,119,065
	Alexa Fluor 488 anti-SOX2 Antibody	1:10	BioLegend	656,110	AB_2,563,957
	Alexa Fluor 488 anti-OCT4 Antibody	1:10	BioLegend	653,708	AB_2,563,184
	Alexa Fluor 647 anti-NANOG Antibody	1:10	BioLegend	674,210	AB_2,650,619
Secondary antibodies	Alexa Fluor 488 Donkey anti-mouse	1:300	Life Technologies	A21202	AB_141,607
Primers used for RT-qPCR and PCR					
Target	Forward/reverse primer (5’ – 3’)				
NANOG	AGG GAA ACA ACC CAC TTC T/CCT TCT GCG TCA CAC CAT T				
S0X2	CCC AGC AGA CTT CAC ATG T/CCT CCC ATT TCC CTC GTT TT				
AFP	AGC TTG GTG GAT GAA AC/CCC TCT TCA GCA AAG CAG AC				
NESTIN	GCG TTG GAA CAG AGG TTG GA/TGG GAG CAA AGA TCC AAG AC				
RUNX1	CTG CCC ATC GCT TTC AAG GT/GCC GAG TAG TTT TCA TTG CC				
Sev	GGA TCA CTA GGT GAT ATC GAG C/ACC AC JA CAA GAG TTT AAG AGA TAT GTA TC				
Sev c-MYC	TAA CTG ACT AGC AGG CTT GTC G/TCC AC A TAC AGT CCT GGA TGA TG				
N0TCH3 HT405A/407D	ACG ACT GTG CCT GTC TTC CT/TGC CCA GCC TAG CAT AAT CT				
N0TCH3	GGG GTG TGG TCA GTC CTA AA/CTG ACC CTC AAA CCC TAG CA				
HT406D/409A	GGA GCT CCA TCG TCT GTG A/ACC TCC TTC CAG GCT TCA GT				
N0TCH3					
HT454D					
β-ACTIN	GAG AAG ATG ACC CAG ATC ATG TTT/GGC AGC TCG TAG CTC TTC TCC A				

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Resource Table

Unique stem cell lines identifier	NIHTVBi004-A NIHTVBi005-A NIHTVBi006-A NIHTVBi007-A NIHTVBi008-A
Alternative names of stem cell lines	HT405A HT406D HT407D HT409A HT454D
Institution	National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, Maryland, USA
Contact information of distributor	Manfred Boehm; HYPERLINK “mailto: boehmm@nhlbi.-nih.gov
Type of cell lines	iPSC
Origin	Human
Cell Source	Peripheral blood mononuclear cells
Clonality	Clonal cell lines
Method of reprogramming	Sendai vectors containing the transcription factors Oct-4, Klf4, Sox2 and c-MYC
Multiline rationale	Lines derived from the five individuals
Gene modification	Yes
Type of modification	Hereditary
Associated disease	None
Gene/locus	N0TCH3, Chromosome 19, GRch38.pl2
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	December 2013
Cell line repository/bank	N/A
Ethical approval	National Institutes of Health Ethics Committee (Approval Number: 16-H-0132)