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An induced pluripotent stem cell line (NCATS-CL9075) from a patient carrying compound heterozygote mutations, p.R390P and p.L318P, in the *NGLY1* gene

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Abstract

NGLY1 deficiency is a rare disorder caused by mutations in the *NGLY1* gene which codes for the highly conserved N-glycanase1 (NGLY1). This enzyme functions in cytosolic deglycosylation of N-linked glycoproteins. An induced pluripotent stem cell (iPSC) line was generated from the dermal fibroblasts of a 2-year-old patient carrying compound heterozygous mutations, p.R390P and p.L318P in the *NGLY1* gene. This cell-based iPSC disease model provides a resource to study disease pathophysiology and to develop a cell-based disease model for drug development for NGLY1 patients.

1. Resource utility

Currently, there are no effective treatments for NGLY1 deficiency. This patient-specific hiPSC line provides a unique approach for cell-based disease modeling to further investigate the pathogenesis and pathophysiology of the NGLY1 disease.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102400.

2. Resource details

N-glycanase 1 (NGLY1) deficiency is a rare autosomal recessive congenital disorder of deglycosylation (CDDG) caused by mutations in the *NGLY1* gene. This conserved deglycosylation enzyme is responsible for the cleavage of N-linked glycans in glycoproteins including misfolded glycoproteins in the ERAD pathway [1,2]. Mutations in this gene that cause a loss of NGLY1 function leads to inactivation of Nrf1 and proteasomal dysfunction [1,3]. In this particular compound heterozygote, the missense mutations (L318P and R390P) most likely lead to a misfolding and degradation of NGLY1 as little to no protein can be detected in these cells (data not shown). Patients with this disorder have severe clinical symptoms such as developmental delay, movement disorder, hypotonia and alacrimia. New disease models can aid in the development of novel therapeutics for patients [3].

In this study, a patient-specific iPS cell line (NCATS-CL9075) was derived from dermal fibroblasts of a 2-year-old male patient (GM26602, Coriell Institute) carrying heterozygous mutations of p.L318P and p. R390P in the *NGLY1* gene. Sendai virus (SeV) vector containing four essential transcription factors OCT3/4, KLF4, SOX2 and c-MYC were used to reprogram the fibroblasts. The iPSC's were expanded and then validated using cell morphology and pluripotential markers SOX2, OCT4, NANOG, SSEA4 and TRA-1–60 (Fig. 1A). Similarly, the quantitative analysis revealed expression levels of 99.44% and 97.60% for TRA-1–60 and Nanog, respectively (Fig. 1B). G-banded karyotype showed diploid 46, XY without any unusual abnormalities (Fig. 1C). Furthermore, the presence of two mutations, p.L318P and p.R390P, on the NGLY1 gene was confirmed using Sanger sequencing, consistent with the original description on the Coriell Cell Repository website (Fig. 1D). After passage 15, both the reprograming factors and the vector were cleared from NCATS-CL9075 iPSCs (Fig. 1E). Routine mycoplasma checks showed no presence of contamination (Supplemental Fig. S1). The experiment of *in vivo* teratoma formation validated the pluripotency of the iPSC line through differentiation into three germ layers of ectoderm, mesoderm and endoderm (Fig. 1F). Last, the 18 loci STR DNA profiling analysis of NCATS-CL9075 revealed a match with the parental fibroblasts (data not shown) (see Table 1).

3. Materials and methods

3.1. Cell culture and reprogramming

The original patient dermal fibroblasts (GM22602) were acquired from Coriell Cell Repositories. The cells were cultured in DMEM medium (Thermo Fisher) supplemented with 10% fetal bovine serum, 100 ug/ml streptomycin, 100 units/ml penicillin,and incubated at 37 °C with 5% CO_2 and 5% O_2. After a few passages, fibroblasts were reprogrammed into iPSCs using the integration-free CytoTune Sendai viral vector kit (A16517, Thermo Fisher Scientific) following a previous method [4]. The generated iPSCs were cultured on Matrigel (Corning)-coated six-well plate in Stemflex medium (Stem cell technologies) at 5% CO_2 and 5% O_2. The iPSCs were cultured at 70% confluency using EZ-Lift (Sigma-Aldrich) and then frozen using CryoStor® CS10 (StemCell Technologies) freezing medium.

4. NGLY1 mutation analysis

The genome analysis for the *NGLY1* mutations was conducted by Genecopoeia (Rockville, MD, USA). Genomic DNA was extracted from the iPSCs. Primers (Table 2) were designed specifically for the amplification of the two target sites. Sanger sequencing analysis was used to confirm the p.L318p and p.R390P mutations in the patient iPSC line.

5. Immunocytochemistry

The iPSCs were fixed in 96-well plates with 4% paraformaldehyde for 30 mins at room temperature (RT), permeabilized with 0.3% Triton X-100 in Dulbecco's phosphate-buffered saline (DPBS) for 15 mins, and washed with DPBS. Next, the cells were blocked using Image-iT™ FX signal enhancer (Thermo Fisher Scientific) for 30 mins at RT. After the removal of the blocking buffer, cells were incubated overnight at 4 °C with primary antibodies (Table 2) diluted in the blocking buffer. Following, the cells were washed with DPBS and incubated with corresponding secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Table 2) for 1 h at RT. Finally, the nuclei were stained with Hoechst 33,342 for 15 mins and imaged using an INCell Analyzer 2500 imaging system (Cytiva, Marlborough, MA) with 20x objective lens and Texas Red, FITC and DAPI filter sets.

6. G-banding karyotyping and short tandem repeat (STR) analysis

The G-banding karyotype analysis was performed at WiCell Research Institute (Madison, WI, USA) using standard cytogenetic protocols. A total of 20 cells at metaphase were examined and analyzed to check for potential clonal abnormalities.

The STR analysis for both the fibroblasts and iPSCs were conducted at the Johns Hopkins University Genetic Resources Core Facility using the Promega PowerPlex 18D kit. The PCR product was electrophoresed on an ABI Prism® 3730xl Genetic Analyzer and analyzed using GeneMapper® v 4.0 software (Applied Biosystems).

7. Flow cytometry analysis

The iPSCs were detached using TrypLE Express (Thermo Fisher Scientific) and fixed with 4% paraformaldehyde for 10 min at RT. Following, the cells were washed with DPBS and permeabilized with 0.2% Tween-20 in DPBS for 10 min at RT. They were then stained with fluorophore-conjugated antibodies (Table 2) for 1 h at 4 °C on a shaker. After incubation, cells were analyzed on a BD Accuri™ C6 Flow Cytometry system (BD Biosciences).

8. Sendai virus detection

RNA extraction, cDNA synthesis and amplification were performed on the iPSCs following a previously published protocol [5]. Human fibroblasts (GM05659, Coriell Institute) transfected with Sendai virus served as the positive control. The final PCR products were loaded on E-Gel® 1.2% with SYBR Safe™ gel, and imaged by G: Box Chemi-XX6 gel doc system (Syngene).

9. Teratoma formation assay

Patient iPSCs cultured in 6-well plates were dissociated using EZ-Lift (Sigma-Aldrich). Approximately 1×10^7 dissociated cells were resuspended in 400 µl culture medium supplemented with 25 mM HEPES (pH 7.4) and chilled on ice. Next, 200 µl of cold Matrigel (Corning, 354277) was added to the cells. This mixture was injected subcutaneously into NSG mice (JAX No. 005557) at 150 µl per injection site. Visible tumors were removed 6–8 weeks post injection, immediately fixed in 10% neutral buffered formalin and embedded in paraffin. Finally, the tissue was stained with hematoxylin and eosin for visualization of teratoma formation.

10. Mycoplasma detection

Mycoplasma levels were detected and analyzed using the Lonza MycoAlert Kit following manufacturer's instruction (Ratio B/A >1.2 mycoplasma positive; 0.9–1.2 ambiguous result; <0.9 mycoplasma negative).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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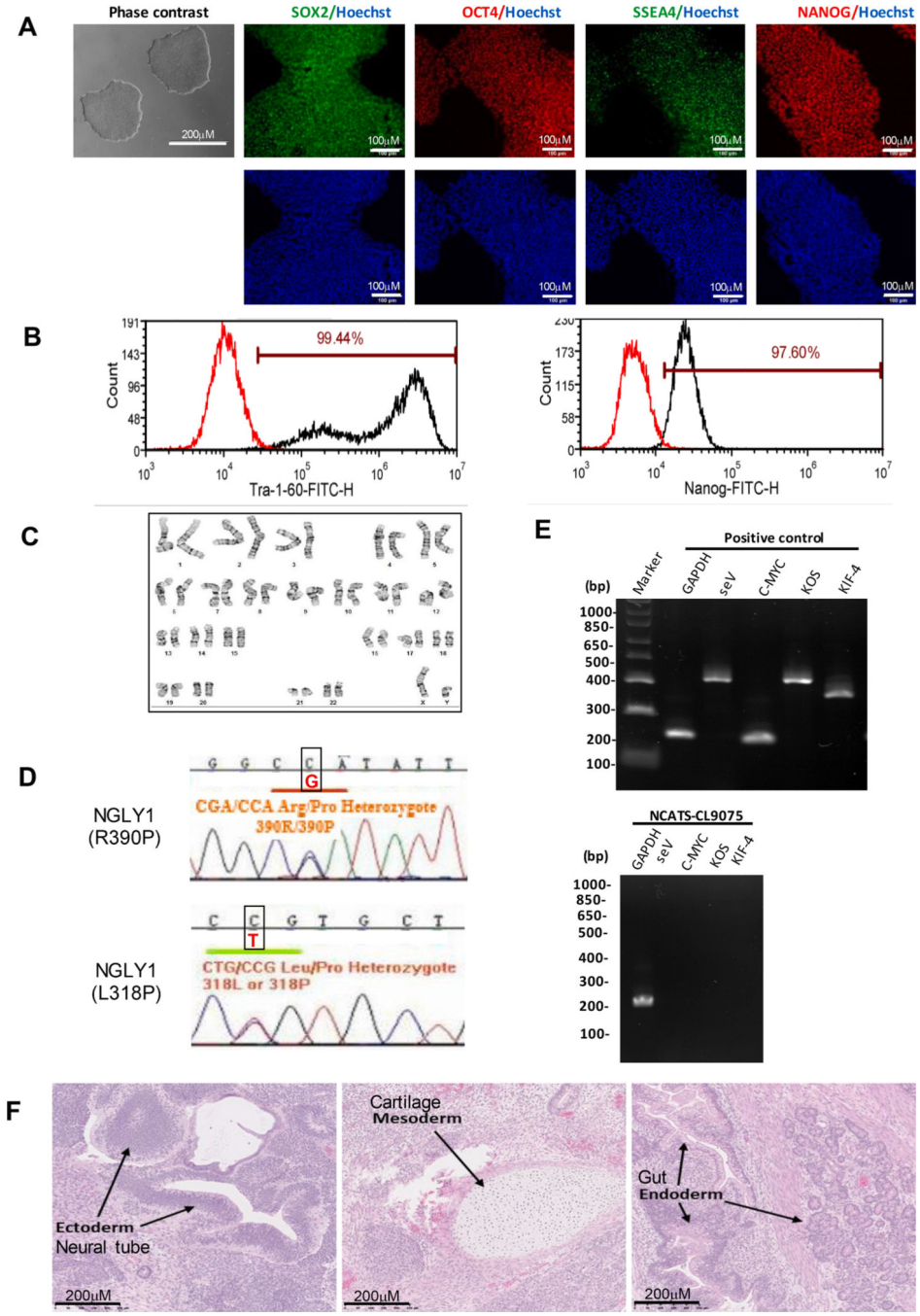

Fig. 1.

Characterization of NCATS-CL9075 iPSC line. A) Left: Phase contrast imaging of NCATS-CL9075 colonies on Matrigel coated plates at passage 12. Right: Immunofluorescent montage of iPSCs marker expression: SOX2, OCT4, TRA-1–60, NANOG, and SSEA4 with Hoechst 33,342 labelled nucleus (in blue). B) Flow cytometry analysis of pluripotency protein markers: TRA-1–60 and NANOG respectively. C) G-banding analysis showed a normal karyotype (46, XY). D) Detection of heterozygous gene mutations p.L318P p.R390P in the *NGLY1* gene. E) RT-PCR verification of SeV and factors clearance from

reprogrammed cells using sendai virus vector transduced fibroblasts as a positive control. F) Histopathological analysis of teratoma displaying normal ectodermal, mesodermal and endodermal differentiation.

Table 1

Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 Panel A
Phenotype	Immunocytochemistry	SOX2, OCT4, NANOG, SSEA-4, TRA-1-60	Fig. 1 Panel A
	Flow cytometry	TRA-1-60 (99.44%); NANOG (97.60%)	Fig. 1 Panel B
Genotype	Karyotype (G-banding) and resolution	46XY Resolution: 450-550	Fig. 1 Panel C
Identity	Microsatellite PCR (mPCR)	Not performed	N/A
	STR analysis	18 Loci tested, all sites matched	Supplemental Fig. S1
Mutation analysis (IF APPLICABLE)	Sequencing	Heterozygous mutations LEU318PRO (L318P), ARG390PRO (R390P)	Fig. 1 Panel D
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence. Negative result	Supplemental Fig. S1
Differentiation potential	Teratoma formation	Three germ layer formation: ectoderm (neural tube), mesoderm (cartilage) and endoderm (gut)	Fig. 1 Panel F
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2

Reagents details.

Antibodies for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Primary Marker	Mouse anti-SOX2	1:50	R & D systems, Cat# MAB2018, RRID: AB_358009
Pluripotency Primary Marker	Rabbit anti-NANOG	1:400	Cell signaling, Cat# 4903, RRID: AB_10559205
Pluripotency Primary Marker	Rabbit anti-OCT4	1:400	Thermo Fisher, Cat# A13998, RRID: AB_2534182
Pluripotency Primary Marker	Mouse anti-SSEA4	1:1000	Cell signaling, Cat# 4755, RRID: AB_1264259
Secondary Antibodies	Donkey anti-Mouse IgG (Alexa Fluor 488)	1:400	Thermo Fischer, Cat# A21202, RRID: AB_141607
Secondary Antibodies	Donkey anti-Rabbit IgG (Alexa Fluor 594)	1:400	Thermo Fischer, Cat# A21207, RRID: AB_141637
Flow Cytometry Antibodies	Anti-Tra-1-60-DyLight 488	1:50	Thermo Fischer, Cat# MA1-023-D488X, RRID: AB_2536700
Flow Cytometry Antibodies	Anti-Nanog-Alexa Fluor 488	1:50	Millipore, Cat# FCABS352A4, RRID: AB_10807973
Flow Cytometry Antibodies	Mouse-IgM-DyLight 488	1:50	Thermo Fischer, Cat# MA1-194-D488, RRID: AB_2536969
Flow Cytometry Antibodies	Rabbit IgG-Alexa Fluor 488	1:50	Cell Signaling, Cat# 4340S, RRID: AB_10694568
Primers			
	Target	**Forward/Reverse primer (5′–3′)**	
SeV specific primers (RT-PCR)	SeV /181 bp	F: GGATCACTAGGTGATATCGAGC R: ACCAGACAAGAGTTTAAGAGATATGTATC	
SeV specific primers (RT-PCR)	KOS/528 bp	F: ATGCACCGCTACGACGTGAGCGC R: ACCTTGACAATCCTGATGTGG	
SeV specific primers (RT-PCR)	Klf4/410 bp	F: TTCCTGCATGCCAGAGGAGCCC R: AATGTATCGAAGGTGCTCAA	
SeV specific primers (RT-PCR)	C-Myc/523 bp	F: TAACTGACTAGCAGGCTTGTCG R: TCCACATACAGTCCTGGATGATGATG	
House-Keeping gene (RT-PCR)	GAPDH/197 bp	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	
Targeted mutation analysis (PCR)	NGLY1^{L318P} /467 bp	F: GCACCTGTAGTCACAGATACTCTGGAGG R: GGTCAGACTGACAAGGCCAAAAAGTAAC	
Targeted mutation analysis (PCR)	NGLY1^{R390P}/571 bp	F: TATAGTCCCAGCTACTCAGGAGGCTG R: CTTTGAAATGAGACAGTTTAATCCAAAATAACTC	

Resource table

Unique stem cell line identifier	NCATS-CL39075
Alternative name(s) of stem cell line	HT594C
Institution	National Institutes of Health National Center for Advancing Translational Sciences Bethesda, Maryland, USA
Contact information of distributor	Dr. Wei Zheng Wei.Zheng@nih.gov
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: 2-year-old Sex: Male Ethnicity: Caucasian
Cell Source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	Integration-free Sendai viral vectors
Genetic Modification	No
Type of Modification	Hereditary
Associated disease	NGLY1 Deficiency
Gene/Locus	NGLY1 gene, located at 3p24.2, mutations L318P and R390P
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	4/20/2020
Cell line repository/bank	N/A
Ethical approval	NIGMS Informed Consent Form was obtained from patient at time of sample submission. Confidentiality Certificate: CC-GM-15-004