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COLLECTION OF PLURIPOTENT STEM CELL LINES

Passport of the pluripotent stem cell line

Generation of an Induced Pluripotent Stem Cell Line TNRMCi001-A by Reprogramming Fibroblasts from a Homozygous F508del Cystic Fibrosis Patient

D. I. Zhigalina[a, *], T. N. Kireeva[a], T. V. Nikitina[a], O. N. Odinokova[a], N. A. Kolesnikov[a], A. A. Malakhova[b, c], R. R. Savchenko[a], I. Zh. Zhalsanova[a], N. R. Valiahmetov[a], A. E. Postrigan[a], S. L. Vovk[a], N. B. Torkhova[a], A. A. Frolova[a, d], V. A. Stepanov[a], and N. A. Skryabin[a]

[a] *Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, 635050 Russia*

[b] *Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, 630090 Russia*

[c] *Meshalkin Medical Research Center, Ministry of Health of the Russian Federation, Novosibirsk, 630055 Russia*

[d] *National Research Tomsk State University, Tomsk, 634050 Russia*

**e-mail: darya.zhigalina@medgenetics.ru*

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Abstract—Cystic fibrosis (CF) is a hereditary disease that leads to impaired functioning of chloride channels in cells, and, as a result, to a decrease in the viscoelastic properties of the secretion of all exocrine glands. Cystic fibrosis is the result of mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes the CFTR protein. In this study, induced pluripotent stem cells (iPSCs) were obtained from the skin fibroblasts of a patient with a homozygous mutation F508del *CFTR* (NM_000492.3(CFTR):c.1521_1523del). This deletion is the most common for cystic fibrosis. The resulting iPSC line had a normal karyotype, retained the original genotype, and also demonstrated the presence of pluripotency markers (OCT4, SOX2, NANOG, SSEA4, TRA-1-60) and the ability to differentiate into derivatives of three germ layers.

Keywords: cystic fibrosis, induced pluripotent stem cells, reprogramming, fibroblasts

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INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (Rafeeq and Murad, 2017). The *CFTR* gene encodes a transmembrane protein located on the surface of the majority of epithelial cells; this protein functions as a chloride channel necessary for the transport of chloride and other ions through cell membranes. The disease is characterized by abnormal mobility of fluid and electrolytes in the epithelium of many tissues (Riordan et al., 1989). The first manifestations of disease are expressed in damage to the respiratory tract and then spread to other organs. In the present study, the skin fibroblasts of a patient with a homozygous mutation F508del *CFTR* NM_000492.3(CFTR):c.1521_1523del (p.Phe508del) were reprogrammed. This deletion of three base pairs is the most common mutation for CF (Stanke et al., 2014). The line of induced pluripotent stem cells (iPSCs) TNRMCi001-A with homozygous deletion F508del *CFTR* can be used in studies on the pathological effects of this mutation to find optimal drug therapy.

MATERIALS AND METHODS

Obtaining iPSC Cells and Culturing Conditions

Skin fibroblasts were obtained from a 17-year-old man with CF. Fibroblasts were cultured in a medium with the following composition: HiGlutaXL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HiMediaLabs) with 10% FBS (fetal bovine serum HyClone), 1× L-glutamine (Gibco), 1× MEM NEAA (Gibco), 1× MEM Vitamins Solution (100×) (Gibco), and 1× penicillin-streptomycin (Gibco). The cells were cultured at 37°C, 5% CO_2. We reprogrammed 5×10^5 fibroblasts using a set of episomal vectors encoding the reprogramming factors OCT4, KLF4, L-MYC, SOX2, LIN28, and mp53DD (Addgene ID #41855-56, #41813-14) as well as encoding the *GFP* and *EBNA-1* genes (Addgene ID #41857-58). Electroporation was performed using the NEON

transfection system in accordance with the manufacturer's protocol (Thermo Fisher Scientific). Primary iPSC colonies were collected manually and plated into six-well plates coated with 0.1% gelatin and containing a feeder layer of mouse embryonic fibroblasts (MEF) mitotically inactivated by treatment with mitomycin. The culture medium for iPSC included HiGlutaXL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HiMediaLabs) with 15% KnockOut Serum Replacement (knockout serum substitute, Gibco), 1× MEM NEAA solution (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 1× penicillin-streptomycin (Gibco), 1× GlutaMAX (Gibco), and 10 ng/mL of basic fibroblast growth factor (bFGF) (SCI-STORE). The cells were cultured at 37°C in 5% CO_2. iPSCs were replated every 4–5 days in a 1 : 10 ratio using TripLE (Life Technologies). A ROCK inhibitor (ROCK inhibitor Y-27632 dihydrochloride, Tocris Bioscience) was used for cell passaging and freezing.

Extraction of Genomic DNA and RNA

Genomic DNA from iPSCs and skin fibroblasts was isolated using the Quick-RNA Miniprep Kit (Zymo Research) according to the manufacturer's protocol. RNA was extracted using Trizol according to the manufacturer's protocol (Thermo Fisher Scientific).

Genetic Analysis

The mutation was confirmed by Sanger sequencing using the BigDye®Terminator v3.1 cyclic sequencing kit (Thermo Fisher Scientific) and the ABI Prism 3130XL genetic analyzer (Applied Biosystems). The *CFTR* gene locus containing the mutation was amplified using PCR primers presented in Table 1.

Staining with Alkaline Phosphatase

The activity of alkaline phosphatase was confirmed using a set of BCIP/NBT alkaline phosphatase substrates (Vector).

Detection of Episomes and Mycoplasma

Detection of episomal DNA and mycoplasma DNA in cells were performed using PCR (95°C for 5 min; 32 cycles: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 5 min) with HS-TaqPCR Color (2×) (Biolabmix) in the T100 thermal cycler (BioRad) as described earlier (Van Kuppeveld et al., 1992; Okita et al., 2011). Primers for the detection of mycoplasma DNA and episomal DNA are presented in Table 1.

Karyotype Analysis

The analysis of the iPSC karyotype was carried out on the 17th passage on the basis of the Genetics Clinic of the Institute of Medical Genetics of the Tomsk NRMC using Ikaros software (MetaSystems). The G-staining of slides was performed based on the protocol described by M. Seabright (Seabright, 1971). The interpretation of the results of the analysis of 40 metaphase plates was carried out in accordance with ISCN 2016.

Spontaneous Differentiation In Vitro

iPSC colonies were separated from the surface of the Petri dishes using 0.15% collagenase type IV (Thermo Fisher Scientific). Then they were transferred to plates with a diameter of 30 mm, coated with 1% agarose. iPSCs were cultivated in a nonadherent state for 20 days in iPSC medium without growth factor bFGF: HiGlutaXL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HiMediaLabs) with 15% KnockOut Serum Replacement (Gibco), 1× MEM NEAA solution (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 1× penicillin-streptomycin (Gibco), and 1× GlutaMAX (Gibco). The embryonic bodies were then transferred to six-well plates with 0.1% gelatin and a feeder layer of mitotically inactivated embryonic mouse fibroblasts for another 8 days.

Immunofluorescence Staining

iPSC colonies were fixed with 3% paraformaldehyde for 20 min at 4°C, permeabilized in 0.1% Triton X-100 (Sigma) solution for 5 min, and incubated in a blocking solution (5% FBS (HyClone) in Phosphate-buffered saline, PBS (Sigma)) for 30 min. Fixed cells were washed with PBS (Sigma) and incubated with primary antibodies overnight at 4°C (Table 1). After that, iPSC cells were washed with 0.2% Tween20/PBS (Sigma) solution for 5 min at 37°C and incubated with secondary antibodies conjugated with Alexa Fluor (Thermo Fisher) (Table 1) in a blocking solution for 1 h at room temperature. The nuclei were stained with an antifade (Vectashield antifade-Vector) with DAPI (Sigma) at room temperature. The fluorescent images were obtained using the Axio Imager Z2 (Zeiss) with ISIS (MetaSystems) software.

Flow Cytofluorometry

Flow cytofluorometry was performed after immunofluorescence staining of cells on a Cytoflex equipment (Becman Coulter) according to the manufacturer's protocol.

Quantitative RT-PCR

Reverse transcription was performed using the M-MuLV-RH First Strand cDNA synthesis kit (Biolabmix). Quantitative PCR was performed using LightCycler 480 Real-Time PCR System (Roche) device with a set of BioMaster HS-qPCR SYBR Blue 2× (Biolabmix) using the following program: 95°C for 5 min; 40 cycles: 95°C for 10 s, 62°C for 1 min. CT val-

Table 1. Antibodies and oligonucleotides used in the study

	Antibodies			
	antibody	dilution	manufacturer, cat. no.	RRID
Markers of pluripotency	Rabbit IgG anti-NANOG	1 : 200	Abcam Cat# ab62734	AB_956161
	Mouse IgG3 anti-SSEA4	1 : 200	Abcam Cat# ab16287	AB_778073
	Mouse IgM anti-TRA-1-60	1 : 200	Abcam Cat# ab16288	AB_778563
	Rabbit IgG anti-SOX2	1 : 150	Novus Cat# NB110-37235	AB_792070
	Rabbit IgG anti-OCT4	1 : 200	Abcam Cat# ab18976	RRID:AB_444714
Markers of differentiated derivatives	Rabbit IgG anti-NF200	1 : 1000	Sigma-Aldrich Cat# N4142	AB_477272
	Rabbit IgG anti-collagen I	1 : 200	Abcam Cat# ab34710	AB_731684
	Mouse IgG1 anti-AFP-01	1 : 200	Abcam Cat# ab3980	AB_304203
Secondary antibodies	Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	1 : 400	Thermo Fisher Scientific Cat# A-11035	AB_2534093
	Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1 : 400	Thermo Fisher Scientific Cat# A-11001	AB_2534069
	Oligonucleotides			
	gene/locus	size, bp	direct/reverse primer (5'–3')	
Detection of episomal vectors	*EBNA-1*	61	ATCAGGGCCAAGACATAGAGATG/ GCCAATGCAACTTGGACGTT	
Reference genes (PCR, qRT-PCR)	*FBXO15*	100	GCCAGGAGGTCTTCGCTGTA/ AATGCACGGCTAGGGTCAAA	
	Beta-2-Microglobulin	280	TAGCTGTGCTCGCGCTACT/ TCTCTGCTGGATGACGTGAG	
Markers of pluripotency (qRT-PCR)	*NANOG*	115	TTTGTGGGCCTGAAGAAAACT/ AGGGCTGTCCTGAATAAGCAG	
	OCT4	79	CTTCTGCTTCAGGAGCTTGG/ GAAGGAGAAGCTGGAGCAAA	
	SOX2	100	GCTTAGCCTCGTCGATGAAC/ AACCCCAAGATGCACAACTC	
Detection of mycoplasma	Ribosomal 16S RNA gene	270–276	GGGAGCAAACAGGATTAGATACCCT/ TGCACCATCTGTCACTCTGTTAACCTC	
Confirmation of the genetic variant	Mutation NM_000492.3(CFTR): c.1521_1523del (p.Phe508del) / Chr7: 117559591–117559593 (assembling GRCh38)	341	GAGGCAAGTGAATCCTGAGC/ ACCGATTGAATATGGAGCCAA	

ues were normalized to beta-2-microglobulin using the ΔΔCT method.

STR-Analysis

STR markers were analyzed using the Promega PowerPlex Fusion System (Promega Corp.) at 24 loci: D3S1358, TH01, D12S391, D5S818, TPOX, Yindel, D2S441, D7S820, D13S317, FGA, D22S1045, D18S51, D16S539, D8S1179, CSF1POD, D6S1043, vWA, D21S11, SE33, D10S1248, D1S1656, D19S433, D2S1338, DYS391. The PCR products were analyzed using the ABI Prism 3730 genetic analyzer (Applied Biosystems).

RESULTS

The passport of the iPSC cell line TNRMCi001-A is presented in Table 2, the full characteristics of this line are shown in Fig. 1 and Table 3. A skin biopsy,

Table 2. Passport of the human iPSC cell line TNRMCi001-A

Parameters	Description
Unique identifier	TNRMCi001-A
Alternative name of the line	iTAF32
Institution	Tomsk National Research Medical Center of the Russian Academy of Sciences, Tomsk, Russia
Approval by the Ethics Committee	The study was approved by the Committee on Biomedical Ethics of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center of the Russian Academy of Sciences, Russia, protocol no. 10 (February 15, 2021)
Cell type	iPSC
Species of organism	*Homo sapience*
Additional information about the origin of the cell line	Age: 17 Sex: male Ethnicity: Caucasian race
Original cell type	Fibroblasts
Date of collection of biomaterial	2022
Reprogramming method	Nonintegrating episomal plasmid vectors
Reprogramming factors	OCT4, KLF4, L-MYC, SOX2, LIN28, p53 shRNA, GFP, and EBNA-1
Clonality	Clonal
Genetic modification	No
Type of genetic modification	No
Confirmation of elimination/silencing of reprogramming transgenes	PCR, not detected
Disease	Cystic fibrosis
Gene/locus	The gene of cystic fibrosis transmembrane conductance regulator (*CFTR*); 11p13; NM_000492.3(CFTR):c.1521_1523del (p.Phe508del)
Morphology	Monolayer colonies similar to human pluripotent stem cells
Pluripotency	Confirmed in tests for the formation of embryoid bodies
Karyotype	46,XY
Checking for contamination	Bacteria, fungi, and mycoplasma were not detected
Area of application	In vitro model of cystic fibrosis
Method of cultivation	Monolayer on gelatin-coated plastic on a feeder layer (MEF cells)
Cultivation medium	HiGlutaXL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (HiMediaLabs), KnockOut Serum Replacement, MEM NEAA (Gibco) solution, 2-mercaptoethanol (Gibco), 1× penicillin-streptomycin (Gibco), 1× GlutaMAX (Gibco), basic fibroblast growth factor (bFGF) (SCI-STORE)
Temperature, °C	37
Concentration CO_2, %	5
Concentration O_2, %	20
Method of passaging	TrypLE (Life Technologies)
Multiplicity of passaging	1 : 10 using ROCK inhibitor Y-27632 dihydrochloride (Tocris Bioscience)
Cryopreservation	90% KnockOut Serum Replacement, 10% DMSO
Storage conditions	Liquid nitrogen
Account in the registry	https://hpscreg.eu/cell-line/TNRMCi001-A
Date of certification/deposit	August 23, 2023

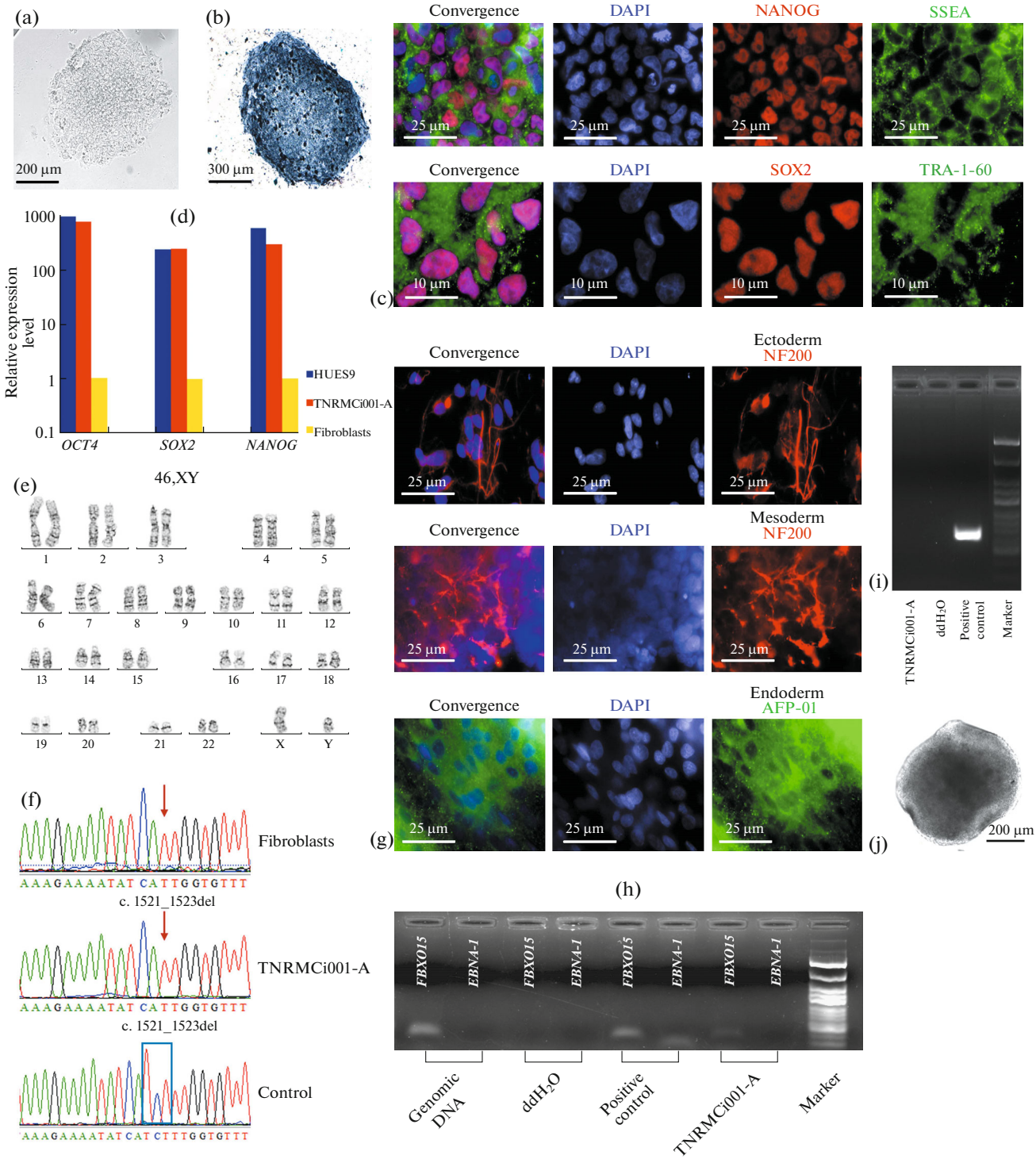

Fig. 1. Characteristics of the human iPSC line TNRMCi001-A. (a) Morphology of colonies; (b) histochemical detection of alkaline phosphatase; (c) immunofluorescence staining for pluripotency markers: NANOG, SOX2 (red), SSEA-4, and TRA-1-60 (green); (d) relative expression of pluripotency markers *OCT4*, *SOX2*, and *NANOG* for iPSC line TNRMCi001-A, control cell line HUES9 (HVRDe009-A) and fibroblasts of the patient; (e) G-staining of iPSC chromosomes on the 17th passage (46,XY); (f) chromatograms of the NM_000492.3 (CFTR):c.1521_1523 del (p.Phe508del) gene region in iPSCs and in the fibroblasts of the skin of a patient with CF and control DNA from an individual without CF; (g) immunofluorescence staining for markers of three germ layers: endoderm (alpha-1 fetoprotein, AFP-01, green), ectoderm (neurofilament 200, NF200, red), and mesoderm (Collagen I, red); (h) DNA of episomes was not detected by PCR in the iPSC line on the 14th passage; (i) on the 14th passage, mycoplasma contamination was not detected by PCR results; (j) embryoid body. Designations: HUES9 is a line of human embryonic stem cells that served as a positive control of the expression of pluripotency markers.

Table 3. Characteristics of the human iPSC line TNRMCi001-A

Parameter	Method	Result	Data
Morphology	Phase contrast microphotography	Characteristic of human pluripotent cells	Fig. 1a
Phenotype	Qualitative analysis *Staining for alkaline phosphatase*	Positive	Fig. 1b
	Qualitative analysis *Immunofluorescence staining*	Positive staining for pluripotency markers: NANOG, SOX2, SSEA4, TRA-1-60	Fig 1c
	Quantitative analysis *Flow cytofluorometry*	OCT4 (95.7%)	–
	Quantitative analysis *Real-time PCR*	Increased expression of pluripotency markers: NANOG, OCT4, SOX2	Fig. 1d
Genotype	Karyotyping	46,XY, Resolution 450–500 bands	Fig. 1e
Identification	STR analysis	24 of 24 polymorphic loci coincide with the loci of patient's fibroblasts	Data available upon request from the authors
Genotyping	Sanger sequencing	Presence of a homozygous mutation NM_000492.3(CFTR):c.1521_1523del (p.Phe508del) has been confirmed	Fig. 1f
Contamination	Mycoplasma	Absent	Fig. 1i
Differentiation potential	Formation of embryoid bodies, immunofluorescence staining	Positive staining on markers of three germ layers: Collagen I (mesoderm); NF200 (ectoderm); AFP-01 (endoderm)	Fig. 1g, Fig. 1j
Donor's infections	HIV, hepatitis B, hepatitis C	No data	No data
Additional information about the genotype	Blood type	No data	No data
	HLA-typing	No data	No data

from which fibroblasts were obtained, was taken from a 17-year-old man with CF. Skin fibroblasts were reprogrammed into a pluripotent state using transfection with episomes encoding pluripotency factors OCT4, SOX2, KLF4, L-MYC, and LIN28; the episomes also contained the short-hairpin RNA shRNA p53 to reduce cell death during reprogramming (Okita et al., 2013); the *EBNA-1* gene to increase reprogramming efficiency; and the *GFP* gene to visually assess the effectiveness of electroporation. iPSC colonies of TNRMCi001-A line grew on gelatin-coated plastic on the feeder layer of MEF cells; they had a typical iPSC-like morphology (Fig. 1a) and were positive for alkaline phosphatase activity (Fig. 1b). The pluripotency of iPSCs was confirmed by immunocytochemical staining for transcription factors NANOG and SOX2 and surface markers SSEA-4 and TRA-1-60 (Fig. 1c). Quantitative RT-PCR showed high levels of *OCT4*, *SOX2*, and *NANOG* expression in the cell line TNRMCi001-A, as well as in the HUES9 (HVRDe009-A) human embryonic stem cell line (Cowan et al., 2004), compared with the expression of these genes in the patient's skin fibroblasts (Fig. 1d). iPSCs had a normal karyotype: 46,XY (Fig. 1e). The presence of NM_000492.3(CFTR):c.1521_1523del (p.Phe508del) mutation associated with the disease and most common in many human populations in this iPSC line was confirmed by Sanger sequencing (Fig. 1f). The differentiation potential of the obtained iPSC cells was evaluated by analyzing the formation of embryoid bodies in vitro (Fig. 1j). According to the results of immunofluorescence staining of embryoid bodies, endoderm cells (alpha-1 fetoprotein, AFP-01), ectoderm cells (neurofilament 200, NF200) and mesoderm cells (collagen I, ColI) were identified (Fig. 1g). The iPSC line TNRMCi001-A was not contaminated with mycoplasma (Fig. 1i). Elimination of the vectors of episomal reprogramming was confirmed

by PCR (Fig. 1h). The analysis of short tandem repeats (STR) in iPSC cells demonstrated an identical DNA profile by 24 polymorphic STR loci with primary fibroblasts (data available from the authors). The full description is presented in Table 3.

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AUTHOR CONTRIBUTION

D.I. Zhigalina, T.N. Kireeva, and T.V. Nikitina performed the tissue culture and molecular genetic part of the work, namely, generation of iPSC cells from the patient's skin fibroblasts as well as part of their detailed characterization. O.N. Odinokova and N.R. Valiahmetov performed sequencing and discovered and confirmed the mutation. N.A. Kolesnikov conducted STR analysis. R.R. Savchenko, I.Zh. Zhalsanova, and A.E. Postrigan isolated genomic DNA and RNA. A.A. Malakhova performed PCR analysis. A.A. Frolova performed flow cytofluorometry. Medical support of the patient, skin biopsy, and isolation of primary fibroblast culture were performed by T.V. Nikitina and N.A. Skryabin. Karyotyping of iPSCs was performed by S.L. Vovk and N.B. Torkhova. D.I. Zhigalina and N.A. Skryabin worked on the design of the study, analysis of the results, and writing of the article. V.A. Stepanov carried out the general management of the work.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Research Ethics Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center (Tomsk, Russia), protocol no. 10 (February 15, 2021). The patient was provided with all information about the study, and an informed consent was signed with his own hand.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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