Human iPSC-Derived Neural Stem Cells with ALDH5A1 Mutation as a Model of Succinic Semialdehyde Dehydrogenase Deficiency

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Abstract

Background: Succinic semialdehyde dehydrogenase deficiency (SSADH-D) is an autosomal recessive gamma-aminobutyric acid (GABA) metabolism disorder that can arise due to *ALDH5A1* mutations, resulting in severe, progressive, untreatable neurodegeneration. The SSADH-D is primarily studied using simplified model symptoms such as HEK293 cells overexpressing genes of interest, but such overexpression can result in protein aggregation or pathway saturation that may not be representative of actual underlying disease phenotypes.

Methods: We utilized a CRISPR/Cas9 approach to generate human iPSC cell lines bearing *ALDH5A1* mutations. Through screening, two different mutant cell lines, namely NM_001080.3: c.727_735del (p.L243_S245del) and NM_001080.3: c.730_738del (p.A244_Q246del) were obtained. Then, we induced iPSCs to neural stem cells and analyzed the characteristics of *ALDH5A1* mutations in stem cells.

Results: The hiPSC and NSC cell lines presented typical stem cell-like morphology. We found exciting changes in *ALDH5A1* expression and GABA accumulation by comparing different cell lines. In addition, we found that the mutant cell lines had a splicing variant by analyzing the cDNA between the wild-type and the mutant cell lines.

Conclusions: iPSC represents a promising in vitro model of SSADH-D that can be used to study early central nervous system developmental alterations and pathogenic mechanisms.

Introduction

ALDH5A1 encodes a protein responsible for a step in the breakdown of gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter present in the neural cells. *ALDH5A1* mutations lead to a rare inborn error in the metabolism of GABA. Consequently, physiologic fluids from patients accumulate GHB, and succinic semialdehyde dehydrogenase deficiency (SSADH-D) occurs. Patients with SSADH-D present with late-infantile to the early-childhood onset of slowly progressive neurodegeneration or static encephalopathy [1, 2]. While many researchers have sought to understand this condition, knowledge about many aspects of the pathophysiology remains insufficient due to the difficulty of obtaining a nervous system model, which the disease mainly damages. [3]. The SSADH-D is primarily studied using simplified model symptoms such as HEK293 cells overexpressing genes of interest, but such overexpression can result in protein aggregation or pathway saturation that may not represent actual underlying disease phenotypes [3]. A short life span and low expression levels of SSADH further limit the application of dermal fibroblasts in a patient.

CRISPR/Cas9 approaches have recently been employed to edit mammalian cell genomes [4, 5], enabling researchers to precisely target genomic regions of interest with guide RNAs (gRNAs) that are recognized by the Cas9 nuclease, resulting in double-stranded break (DSB) generation in the genome, followed by subsequent non-homologous end-joining repair [6]. Such CRISPR/Cas9 approaches can also be leveraged to achieve homologous recombination (HR) and associated genomic editing by introducing DNA

templates and homologous flanking sequences into cells. Overall, CRISPR/Cas9 approaches are cost-effective and highly efficient, making them ideal for cellular manipulation in a research context. Therefore, editing human-induced pluripotent stem cells (hiPSCs) via CRISPR/Cas9 approach and then differentiating them into specific cell types of interest can be used to model various diseases, including SSADH-D.

NM_001080.3: c.727_735del, a mutation reported earlier in our department [7], was targeted in *ALDH5A1* in a healthy hiPSC line to generate mutated hiPSC lines by using a CRISPR/Cas9-mediated editing system. A single guide RNAs (sgRNA) targeting *ALDH5A1* NM_001080.3: c.727_735del and the ribonucleoprotein Cas9 were used to edit the gene via HR. The sgRNA was engineered with an endogenous fluorescent reporter-enhanced green fluorescent protein (sgRNA-eGFP). The edited knock-in cell lines showed a functioning fluorescent reporter system for successful cell transfection identifiable via flow cytometry. Through screening, two different mutant cell lines, namely NM_001080.3: c.727_735del (p.L243_S245del) and NM_001080.3: c.730_738del (p.A244_Q246del) were obtained. These two cell lines and the control iPSCs were further differentiated into neural stem cells (NSCs). *ALDH5A1*-mutated cell lines were then used with the wild-type control line for disease modelling. Our results indicated that the *ALDH5A1* expression significantly increased when iPSCs were to NSCs. The absolute amount of GABA in NSCs was less than that in iPSCs. The accumulation rate of GABA compared with the wild-type in the mutant NSCs was higher than that in the mutant iPSCs. Interestingly, the two cell lines with only one amino acid mutation difference exhibited various characteristic changes when iPSCs were induced to NSCs. This finding suggested that the construction of disease models with iPSC would be beneficial to studies on disease mechanisms because iPSC could be induced in different cell lines with a characteristic expression of disease-related genes.

Results

Generation of knock-in hiPSC lines with CRISPR/Cas9 technology

A CRISPR/Cas9 was developed to engineer one hiPSC line at the *ALDH5A1* locus. First, two different sgRNAs were designed in silico using the online CRISPR Design Tool (http://crispr.mit.edu/) [8]. The efficiency of each candidate was tested *in vitro* using hiPSC, evaluating the transfection efficiency with eGFP fluorescence and estimating the editing efficiency via sequencing to single out two sgRNAs. The Cas9 plasmid contained the following sequence: a nonviral vector of gRNA for mammalian cells and an eGFP-coding gene with an EF1A core promoter sequence flanked by T2A sites. The sgRNA plasmid contained the following sequence: a nonviral vector of gRNA for mammalian cells, the eGFP coding gene with U6 core promoter sequence flanked by P2A sites. hiPSCs were transfected with the sgRNA plasmid and an expression vector carrying Cas9 using Lipofectamine stem transfection reagent (Invitrogen, CAT# STEM00001). The successfully transfected cells were screened with flow cytometry (BD FACS Aria SORP, USA). The results revealed that secondary sorting could improve the acquisition of positive-edited cells.

Single hiPSC clones were obtained with a cloning ring. The single clones were selected, transferred to a Matrigel-coated 24-well plate, and amplified to obtain the DNA for sequencing and find homozygous mutated hiPSC clones. The clones were further characterized, and whole-genome sequencing was performed to confirm whether no undesired editing was present in the newly established cell lines.

Karyotype analysis before and after genome editing

The cell line before and after editing presented a normal karyotype (Fig. 1) and was free of mycoplasma. The mutated hiPSC was also confirmed to have a normal karyotype.

Whole-genome sequencing results

Genome sequencing revealed no other significant deletion changes in the mutant cell lines. The target fragment of *the ALDH5A1* gene was edited correctly.

Immunofluorescence of iPSCs

The cell line presented typical stem cell-like morphology. Immunocytochemistry assays showed the expression of the pluripotency markers, such as OCT4, SSEA-4, Tral-60, and NANOG (Table 1) in our hiPSC lines and our transfected hiPSC lines. Sox1 and Nestin (Table 1) were expressed in our NSCs, which contained the wild type and the two mutant cell lines (Fig. 2).

Table 1
Reagents and primers details

Antibodies used for immunocytochemistry Antibody		Dilution	Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:200	CST Cat.75463
	Rabbit anti-NANOG	1:200	CST Cat.4903T
	Mouse anti-TRA1-60	1:500	Abcam Cat.ab/6288
	Mouse anti-SSEA4	1:500	CST Cat.MC813
NSC differentiation Markers	Mouse anti-Nestin	1:200	ABcam.Cat.ab18102
	Rabbit anti-sox1	1:200	CST Cat.4194s
Secondary antibodies	Mouse anti-IgG	1:1000	CST Cat.4409
	Goat-anti-Rabbit	1:500	Servicebio. Cat.GB25303
Primers	**Target**	**Forward/Reverse primer (5'-3')**	
Targeted mutation	ALDH5A1-Exon5	ACGTGACTTTAGCACTAATAAGA	AGAGCTTTTAACACTCTGCTGGA
RT-qPCR Primers	ALDH5A1-RNA-1	GGCACCAGTTATCAAGTTCG	ACTCCACCAAAAGGGCACTC
	ALDH5A1-RNA-2	AGTCATCACCCCGTGGAATTT	GAGAAGGGCGTGTCTTCGG
	ALDH5A1-RNA-3	AGGGGAGGCAATTTGTACTGA	GTGGTGCAACAGGATCTTTCC

mRNA and protein expression levels in iPSC and NSC lines

p.A244_ Q246del may be more unstable than p.L243_ S245del because quantitative PCRs performed on the total RNA extracted from iPSC and NSC lines (containing wild type, p.L243_S245del, and p.A244_Q246del cell lines) showed a significant difference between the wild type and p.A244_ Q246del RNAs (Fig. 2B), the same result also appears in western blot analyses of iPSC cell lines (Fig. 2D).

However, there was no significant difference in gene and protein expression of *ALDH5A1* between p.L243_ S245del and wild type. Interestingly, the expression of *ALDH5A1* in NSCs with the same mutation type was significantly higher than that in iPSCs because of the tissue-specific expression (Fig. 2C).

GABA accumulation in iPSCs and NSCs

The intracellular GABA accumulation was compared via HPLC (Fig. 2F and 2G), and the results showed that the GABA storage of the two mutant cell lines was higher than that of the wild type in iPSCs and NSCs. The two mutants in iPSCs were 50% higher than the wild type, whereas those in NSCs were threefold higher than the wild type. This finding explained why the *ALDH5A1* expression increased at multiple ratios after iPSCs were induced to NSCs.

mRNA sequence analysis after genome editing in iPSC

The first round of amplification was whole segment amplification, using F1 and R1 primers for amplification, and the fragment size was 1637 bp. F2-1sequencing of the A244_Q246del sample was successful, and F2-2 sequencing failed. The F2-1 sequencing result is the range of F2-2 sequencing. There may be splicing variants between the sequences between F2-1 and F2-2. Finally, after segmented amplification, the mutant of A244_Q246del has a large fragment splicing variant, and the mutant of L243_S245del only has 12 base splicing variants (Fig. 3).

Discussion

The biochemical hallmark of SSADH-D is an increase in the concentration of GABA and GHB in body fluids, such as blood, urine, and cerebrospinal fluid. These two substances mainly affect the nervous system. Thus, SSADH-D is often characterized by nervous system lesions [9, 10]. The SSADH-D is primarily studied using simplified model symptoms such as HEK293 cells overexpressing genes of interest, but such overexpression can result in protein aggregation or pathway saturation that may not represent actual underlying disease phenotypes[3]. A short life span and low expression levels of SSADH further limit the application of dermal fibroblasts in a patient. Thus, more *in vitro* approaches with cellular models closer to humans should be developed. For instance, iPSC models of various diseases have emerged [6, 11–14]. In the present study, a successful strategy was proposed to generate two SSADH-D iPSC lines via CRISPR/Cas9 genome engineering combined with flow cytometry and a clonal loop. The advantage of this model was that iPSCs could be differentiated into all cell types of the body, especially those representing the target tissue cells, such as NSCs. Through the induction of iPSCs, the NSCs of the wild type and mutants showed the characteristic changes in SSADH-D. Notably, comparing the expression of *ALDH5A1* and the amount of GABA between iPSCs and NSCs is attractive. (Fig. 2F and 2G) When iPSCs were induced into NSCs, the *ALDH5A1* expression increased in the wild type and mutant types, but the degree of increase differed, and the increase in mutant cell lines was more apparent (Fig. 2C). The GABA accumulation multiple of the mutants of the wild type NSC is more serious than that of the mutant than that of the wild-type in iPSC lines (Fig. 2F and 2G). However, in comparison with NSCs, iPSC likely tolerated higher GABA concentrations. This observation emphasizes that more SSADH was

needed to help metabolize GABA in NSCs. GABA accumulation could not be reduced even when the gene expression increased because of *ALDH5A1* mutation.

Furthermore, the generation of L243_S245del and A244_Q246del allowed us to conclude about the *in vitro* function of the splicing region between exons 4 and 5 in *ALDH5A1*. In Fig. 2B, the expression of the A244_Q246del mutant was only 30%-40% of that of the wild-type before and after iPSCs induced to NSCs. However, the decrease in the *ALDH5A1* expression in the L243_S245del mutant was not distinguishing (about 75%) in iPSC lines compared with that in the wild-type. Even after induction, the *ALDH5A1* expression of the L243_S245del mutant was higher than that of the wild-type (Fig. 2C). These results indicated that only the A244_Q246del mutation affected the gene expression, although only one amino acid difference existed between these two mutations in our study. This hypothesis was tested by sequencing the cDNA of the two different mutants in the target gene. As expected, the A244_Q246del mutant (exon 5) severely affected the RNA splicing, resulting in 545bp deletion encompassing exons 4–7 of *ALDH5A1* (Fig. 3B). Another mutation, i.e., L243_S245del, only affected the deletion of 12 bases (CTTGCAAGCCAG) in exon 5 (Fig. 3B). Thus, this observation was the fundamental reason for the different changes in the *ALDH5A1* expression before and after iPSC induction between the two mutant cell lines. Our literature search revealed that Akaboshi et al. [15] concluded that the mutations in a short stretch between aa 223 and 268 (encoded by exons 4 and 5) are not entirely random, and a vital region of the gene may be present. This finding was helpful for further studying the mechanism of the vital region of the *ALDH5A1* gene. Therefore, the construction of an iPSC disease model could be used as a good disease model for research on SSADH-D mechanisms.

A correct diagnosis is the first step in treating rare diseases, and it is the basis of disease mechanism research. For rare diseases, the availability of validated samples from patients with a specific disease is usually low, limiting the possibilities of using these samples. iPSC disease models can be differentiated into various target cells because of the pluripotency of iPSCs. In our study, the iPSC disease model could be differentiated into NSCs. This model could be utilized to detect changes in the *ALDH5A1* expression and the GABA accumulation in the two cell lines, which should be a good cell model for drug screening because the therapy-induced reduction of GABA in the periphery may be a vital issue for the development of future therapies for SSADH-D [16].

In conclusion, the CRISPR-based genome editing of iPSCs shows potential for future studies on the pathogenicity of diseases. Our research concluded that the iPSCs could be helpful for SSADH-D disease modelling.

Materials And Methods

Cell lines

Genetic engineering was carried out on one hiPSC line (DYR0100, procured from Hunan Fenghui Biotechnology Company) derived from a human prepuce cell of a healthy boy donor (ATCC-ACS-

1011TM).

Gene edit

sgRNA design and testing

sgRNAs were designed in silico via the CRISPR Design Tool (http://crispr.mit.edu/). All sgRNAs were designed to target the NM_001080.3: c.727_735del region of *ALDH5A1*. Two sgRNAs with a high fraction of efficacy were selected for the test. Two oligonucleotides for sgRNAs were cloned into two VB UltraStable vectors, respectively.

Three hundred thousand hiPSC cells were plated in a 6-well culture plate for transfection. On the following day, the cells were transfected with 2 µg sgRNA1 and Cas9-carrying plasmid DNA Lipofectamine™ Stem transfection reagent (ThermoFisher, Cat# STEM00003). They were kept in the same media for 24 h. Then, the successfully transfected cells were screened through flow cytometry (BD FACS Aria Sorp, USA). sgRNA1 was more efficient than sgRNA2, and they had editorial effects. The sgRNA1 sequence was: 5'-TATAGCTTGCAAGCCAGGCT-3', and the protospacer adjacent motif (PAM) sequence was GGG. The sgRNA2 sequence was: 5'-TTATAGCTTGCAAGCCAGGC-3', and the PAM sequence was TGG.

ssDNA for homologous directed repair (HDR)

Single-stranded donor oligonucleotides were synthesized at Vector Builder company. The concentration was 10 µM, and their sequence was:
TTTTTTTTTTTTTTCAGTTTGGTAAATTTTGGCAAGTTTGCTTTTCTCTTTATAGCAGGCTGG
GATTCCTTCAGGTGTATACAATGTTTTCCCTGTTCTCGAAAGAATGCCAAGGA. At the time of transfection, 2µl was added to each well of the 6-well plate.

Cell culture

hiPSCs culture and transfection

All hiPSCs were cultured in a PGM1 medium (Cellapy, CAT# CA3001500) with 0.5% Plasmocin prophylactic on matrix-coated (Corning Matrigel hESC-Qualified) plates and maintained at 37°C in humidified air with 5% CO_2. Cells were passaged with Cellapy Cell Dissociation Reagent (Stem cell, CAT# 07174) every 3–4 days and plated at a density of 2×10^4 cells/cm^2 and a split ratio of around 1:6. During cell generation, 10 µM Y-27632 (STEMCELL Technologies, CAT# 72302) was needed to add to a PGM1 complete medium.

Cells were transfected with a complex formed with the Cas9 plasmid and two sgRNA plasmids using Lipofectamine stem transfection reagent (Invitrogen, CAT #STEM00001).

The detailed steps were as follows:

Steps	Tube	Component	Each well of a 6-well plate
1	1	Opti-MEM™ I Medium	100 µl
		Lipofectamine™ Stem Reagent	4 µl
2	2	Opti-MEM™ I Medium	100 µl
		DNA	2 µg*
3	Add diluted DNA to diluted Lipofectamine Stem Reagent.		
4	Incubate for 10 min at room temperature.		
5	Add DNA–lipid complex to cells (200 µl/per well).		
6	Incubate and monitor the transfected stem cells at 37°C for 2 days.		

*Equimolar amounts of Cas9 plasmid DNA and gRNA plasmid DNA were added.

Screening of positive clones with targeted homozygosity

After 48 h of transfection, the cells were harvested with StemPro Accutase (Stem cell, CAT. #At-104) and sorted on a BD FACS Aria SORP (BD Biosciences). eGFP-positive cells were collected, re-sorted to remove false-positive cells, and cultured in 12-wells plates. For HDR screening, one week after cultivation, a portion of cells of each colony was extracted from hiPSCs with a DNeasy Blood & Tissue Kit (Qiagen) for PCR amplification and sequencing. Sequencing results showed that cells with editing were successful and were selected in a 10-cm dish by clone ring (Sigma, Aldrich, CAT# C7983-50EA).

Chromosome analysis and whole-genome sequencing for iPSC

Cultured cells were incubated in 50ng/ml Colcemide (Gibco, CAT# 15210-040) solution for 1 h, subjected to hypotonic treatment in 0.075M KCl for 20min at 37°C, fixed with Carnoy solution (3:1 v/v methanol/acetic acid) twice for 20min at each time and spread on a wet cooling microscopic slide with a plastic transfer pipette to obtain chromosome preparations for karyotype analysis. After the specimen was air-dried, the slide was incubated on a heating plate at 82°C for 2.5 hours. Then, changes in karyotype were observed under a phase-contrast microscope after 10 min of Giemsa staining.

The two successfully edited homozygous cell lines and the unedited cell lines were sent to BGI for genome-wide sequencing to confirm whether no undesired editing was present in the newly established cell lines.

Immunofluorescence staining

The cells were fixed in 4% PFA for 20 min and washed three times with phosphate-buffered saline (PBS). Then, the cells were permeabilized with 1 ml of 0.5% Triton-X 100 for 20min and blocked with 5% BSA for 1 h at room temperature. Primary antibodies (OCT4, Tral-60, SSEA, and NANOG) were incubated at 4°C for

overnight and were washed three times with PBS. Then, secondary antibodies were incubated in the dark at room temperature for 2 h. Nuclei were stained with 0.5 μg/ml DAPI, and iPSC images were acquired with a ZOE fluorescence cell imager (Bio-Rad).

Induction and identification of neural stem cells

iPSCs were induced to NSCs to produce various neural cells from iPSCs. A serum-free neural induction medium (gibco Neurobasal® Medium, life technologies) was used to differentiate human iPSCs into NSCs following the manufacturer's instructions. The staining was completed to identify NSCs as in Section 2.7. Primary antibodies included Sox1 and Nestin.

Real-time PCR analysis

Total mRNA was extracted after the treatment for the indicated time. First-strand cDNA synthesis was generated from 1μg of the total RNA. Target and two references (*GAPDH/β-ACTIN*) genes were quantified in triplicate on ChamQ™ Universal SYBRqPCR Master Mix (version7.1, Vazyme). Three pairs of primers were designed to detect *ALDH5A1* expression. The primers used in each reaction were listed as follows: *ALDH5A1*-1, forward 5' -GGC ACC AGT TAT CAA GTT CG-3'and reverse 5' -ACT CCA CCA AAA GGG CAC TC-3'; *ALDH5A1*-2, forward 5' -AGT CAT CAC CCC GTG GAA TTT-3' and reverse 5'-GAG AAG GGC GTG TCT TCG G-3'; *ALDH5A1*-3, forward 5'-AGG GGA GGC AAT TTG TAC TGA-3' and reverse 5'-GTG GTG CAA CAG GAT CTT TCC-3'; and *GAPDH*, forward 5'-GCACCGTCA AGG CTG AGA AC-3' and reverse 5'-TGG TGA AGA CGC CAGTGG A-3'. The target gene expression levels were calculated using the comparative threshold cycle (Ct) method. The ΔCt values were determined with the following formula: ΔCt = Ct (gene of interest) − Ct(GAPDH), and the $2^{-\Delta\Delta Ct}$ was calculated with the following formula: ΔΔCt = ΔCt (control group) −ΔCt (experimental group) to determine the relative expression.

Western blot and biochemical diagnosis

The mutant cell lines and the wild type were lysed with RIPA lysis buffer (Beyotime, Beijing, China) containing 1% PMSF (Beyotime, Beijing, China). Protein (15 μg) was loaded on 10% SDS-PAGE gels for electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBS, containing 0.1% Tween 20 for 1.5 h at room temperature, and incubated with the respective primary antibody of SSADH (AffinitY, Cat# DF12820) and *β*-ACTIN (Multi Sciences, Hangzhou, China) at 4°C overnight. The membranes were incubated with the HRP secondary antibody (Multi Sciences, Hangzhou, China) for 2 h at room temperature. Signals were detected using a Western blot detection reagent (Pierce, Rockford, IL, USA).

GABA in iPSC and NSC cell lines were determined with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) [17].

cDNA sequencing

First, wild-type and mutant cell lines RNA was extracted and reverse transcribed to cDNA. Second, by designing primers, F1: TTCCTGTCGCCGTCGTTGC, R1TTTGTAAAAGCATTCGCCG, we amplified the

cDNA. Then, for segmented sequencing analysis, we use the primers as follows, F2-1：GGTCCTCAAGCAGCCCATA, F2-2：TTTGTAAAAGCATTCGCCG, R2：AAAATAACCTGCTAACCCAACATC;F3：GATGCCGTTTCTAAAGGTGC, R3：TTACAAGGACTGGATGAGTTCTG. (the location of the primers was shown in Fig. 3)

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were following the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/ Submission ID: SUB11566734)

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Li Liu contributed to the conception of the article. Xiaodan Chen contributed to the study's design and drafted and revised the manuscript. Yanna Cai and Chengcheng Zhou contributed to the acquisition of data. Minzhi Peng contributed to the analysis of data. All authors read and approved the final manuscript.

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Figures

Figure 1

Characterization of *ALDH5A1*-mutated iPSC lines (p.L243_S245del and p.A244_Q246del).

(A) Illustration showing CRISPR/Cas9 strategy for the ALDH5A1 gene, with the sgRNA targeting the two mutations marked by the red arrows in exon-5. **(B)** Images showing typical stem cell morphology and cell efficiency of transfection (×10 objective). **(C)** Both mutated cell lines' Sanger-sequencing and alignment show deletions (p.L243_S245del mutant on the upper left and p.A244_Q246del mutant on the upper right). **(D)** Representative images of the Immunofluorescence staining for pluripotency markers OCT4, Tra1-60, SSEA-4 and NANOG, and DAPI for nuclei. Scale bar = 100 μm. **(E)** Results of the karyotype analysis for three cell lines.

Figure 2

Characterization of *ALDH5A1*-mutated NSC lines (p.L243_S245del and p.A244_Q246del).

(A) In vitro differentiation assay to the three neural stem cell lines (NSCs): Sox1 (scale bar = 50 μm), Nestin (scale bar = 50 μm). **(B)** Real-time quantitative PCR analysis showed expression of *ALDH5A1* genes normalized to GAPDH and *β*-actin expression in iPSC lines and induced neural stem cells. The p.A244_Q246del mutant represented significantly reduced gene expression of *ALDH5A1* compared to the wild-type. However, the gene expression of *ALDH5A1* of p.L243_S245del mutant represented a slight

decrease, and it increased significantly and was higher than that of wild-type when induced to NSCs. (Each experiment was repeated three times) **(C)** The expression of *the ALDH5A1* gene was increased significantly when human pluripotent stem cells were induced into neural stem cells. **(D/E)** Western blots analyses revealed that two mutants of iPSC cell lines caused significant differences in the SSADH protein level concerning the wild type. The iPSC mutants, but not NSC mutants, result appeared to be less abundant. **(F)** Analysis of GABA accumulation in iPSC cell lines by denaturing high-performance liquid chromatography (D-HPLC). **(G)** Analysis of GABA accumulation in NSC cell lines by denaturing high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).

Figure 3

The cDNA sequencing of *ALDH5A1*-mutated iPSC lines (p.L243_S245del and p.A244_Q246del) and the wild-type

(A) The letters F1, R1, f2-1, f2-2, R2, F3, and R3 indicate the location of each primer for cDNA sequencing. (B) Comparing cDNA sequencing results among two mutant cell lines and the wild type.

Supplementary Files

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