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Differential gene expression profiles in neurons generated from lymphoblastoid B-cell line-derived iPS cells from monozygotic twin cases with treatment-resistant schizophrenia and discordant responses to clozapine

Takanobu Nakazawa [a,b,c,1], Masataka Kikuchi [d,1], Mitsuru Ishikawa [e,1], Hidenaga Yamamori [f], Kazuki Nagayasu [b,c], Takuya Matsumoto [e,g], Michiko Fujimoto [f], Yuka Yasuda [f,h], Mikiya Fujiwara [b], Shota Okada [b], Kensuke Matsumura [b], Atsushi Kasai [b], Atsuko Hayata-Takano [b], Norihito Shintani [b], Shusuke Numata [i], Kazuhiro Takuma [a,j], Wado Akamatsu [e,k], Hideyuki Okano [e], Akihiro Nakaya [d], Hitoshi Hashimoto [b,c,j,l], Ryota Hashimoto [f,j,*]

[a] Department of Pharmacology, Graduate School of Dentistry, Osaka University, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] iPS Cell-Based Research Project on Brain Neuropharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[d] Department of Genome Informatics, Graduate School of Medicine, Osaka University, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan
[e] Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[f] Department of Psychiatry, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan
[g] Institute for Innovation, Ajinomoto Co., Inc., 1-1 Suzukicho, Kawasaki-ku, Kawasaki, Kanagawa 210-8681, Japan
[h] Oncology Center, Osaka University Hospital, 2-15, Yamadaoka, Suita, Osaka 565-0871, Japan
[i] Department of Psychiatry, Course of Integrated Brain Sciences, School of Medicine, University of Tokushima, 2-50-1 Kuramotocho, Tokushima, Tokushima 770-8503, Japan
[j] Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, D3, 2-2, Yamadaoka, Suita, Osaka, 565-0871, Japan
[k] Center for Genomic and Regenerative Medicine, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[l] Division of Bioscience, Institute for Datability Science, Osaka University, 1-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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ABSTRACT

Schizophrenia is a chronic psychiatric disorder with complex genetic and environmental origins. While many antipsychotics have been demonstrated as effective in the treatment of schizophrenia, a substantial number of schizophrenia patients are partially or fully unresponsive to the treatment. Clozapine is the most effective antipsychotic drug for treatment-resistant schizophrenia; however, clozapine has rare but serious side-effects. Furthermore, there is inter-individual variability in the drug response to clozapine treatment. Therefore, the identification of the molecular mechanisms underlying the action of clozapine and drug response predictors is imperative. In the present study, we focused on a pair of monozygotic twin cases with treatment-resistant schizophrenia, in which one twin responded well to clozapine treatment and the other twin did not. Using induced pluripotent stem (iPS) cell-based technology, we generated neurons from iPS cells derived from these patients and subsequently performed RNA-sequencing to compare the transcriptome profiles of the mock or clozapine-treated neurons. Although, these iPS cells similarly differentiated into neurons, several genes encoding homophilic cell adhesion molecules, such as protocadherin genes, showed differential expression patterns between these two patients. These results, which contribute to the current understanding of the molecular mechanisms of clozapine action, establish a new strategy for the use of monozygotic twin studies in schizophrenia research.

Abbreviations: CLZ, clozapine; iPS, induced pluripotent stem; FDR, false discovery rate; DAVID, Database for Annotation, Visualization and Integrated Discovery; GO, gene ontology.

* Corresponding author at: Molecular Research Center for Children's Mental Development, United Graduate School of Child Development, Osaka University, D3, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail address: hashimor@psy.med.osaka-u.ac.jp (R. Hashimoto).

[1] These authors contributed equally to this work.

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1. Introduction

Schizophrenia is a severe neuropsychiatric disease with an approximate worldwide prevalence of 1% (Gaebel and Zielasek, 2015; Millan et al., 2016; Wang et al., 2015). Common symptoms include delusions, hallucinations, impaired cognitive function, emotional blunting and incoherent behavior. Although many typical and atypical antipsychotic drugs have been developed and demonstrated to be effective in the treatment of schizophrenia, 20–30% of patients remain partially or fully unresponsive to two or more adequate trials with antipsychotic drugs and are therefore classified as treatment resistant (Kane et al., 1988; Meltzer, 1997, 2013). Patients with treatment-resistant schizophrenia suffer disability and have a poor quality of life. Despite the social and economic burden of treatment resistance, the molecular pathophysiology of treatment-resistant schizophrenia remains poorly understood.

Clozapine is the only effective drug for treatment-resistant schizophrenia (Hill and Freudenreich, 2013; Meltzer, 2013; Raja and Raja, 2014). At six months, the response rate to clozapine in treatment-resistant schizophrenia patients is 60–70%. Although the efficacy of clozapine for treatment-resistant schizophrenia is remarkable, clozapine has life-threatening side effects, such as agranulocytosis, which requires medical monitoring. These factors limit the use of this compound in clinical practice. To maximize the efficacy and minimize the side effects of clozapine treatment, biologically validated predictors of the clozapine treatment response should be identified. However, considering that inter-individual variability in the drug efficacy of clozapine treatment exists and that the molecular mechanisms of clozapine action remain largely unclear, the current molecular knowledge of drug response predictors of clozapine is extremely limited (Gressier et al., 2016; Kohlrausch, 2013; Muller et al., 2013; Sriretnakumar et al., 2015).

Considering the very limited accessibility to live neurons from patients with schizophrenia, the molecular defects in neurons leading to the initiation and progression of this disease remain obscure. Accordingly, schizophrenia is poorly understood at the molecular and cellular levels. Modeling schizophrenia using induced pluripotent stem (iPS) cells offers an emerging opportunity to examine the mechanisms underlying complex disease pathogenesis (Falk et al., 2016; Imaizumi and Okano, 2014; Okano and Yamanaka, 2014; Wright et al., 2014). Particularly, iPS cell technologies can produce live human neurons with the genetic backgrounds that lead to schizophrenia (Bundo et al., 2014; Hashimoto-Torii et al., 2014; Maekawa et al., 2015; Robicsek et al., 2013; Topol et al., 2016; Wen et al., 2014; Yoon et al., 2014). Considering the high heritability of schizophrenia, schizophrenia patient-derived live neurons with the same genetic information as the patient can be ideal experimental materials for studies of the molecular pathophysiology of schizophrenia.

Monozygotic twins have been useful resources for studying the genetic and environmental factors of schizophrenia (Boomsma et al., 2002; Kato et al., 2005). Molecular biological methods using blood samples and brain imaging methods have been conventionally used in discordant monozygotic twins to investigate the potential mechanisms of schizophrenia. Currently, to the best of our knowledge, there are no reports on the use of iPS cell technology in monozygotic twin studies of schizophrenia. In the present study, to elucidate the molecular mechanism of inter-individual variability in the clozapine response and clozapine action, we focused on monozygotic twin cases with treatment-resistant schizophrenia who were discordant for clozapine treatment, i.e., one twin responded well to clozapine treatment and the other twin did not. We established iPS cells from immortalized B cells of each patient and neurons were differentiated from these iPS cells. We subsequently performed RNA sequencing to compare the transcriptome profiles of the mock or clozapine-treated neurons as well as lymphoblastoid B-cell lines from these patients and observed that several genes, including cell adhesion molecules, showed differential expression patterns between these two patients.

2. Materials and methods

Following a description of the study, written informed consent was obtained from each subject. This study was conducted in accordance with the World Medical Association's Declaration of Helsinki and approved through the Research Ethics Committee of Osaka University. All recombinant DNA experiments were reviewed and approved by the Gene Modification Experiments Safety Committee of Osaka University.

2.1. Subjects

The monozygotic twin patients with treatment-resistant schizophrenia were recruited at Osaka University Hospital. Each subject was diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on a structured clinical interview. Treatment-resistant schizophrenia was defined according to the following criteria mentioned in the clozapine drug information in Japan: 1) No or little response to treatment from at least two adequately dosed antipsychotic trials for at least 4 weeks (including at least 1 second-generation antipsychotic, >600 mg/day of chlorpromazine equivalent) and Global Assessment of Functioning (GAF) scores never higher than 41; or 2) intolerance to at least two second-generation antipsychotics due to extrapyramidal symptoms. The subjects included in the present study met the criterion of little response. Symptoms of schizophrenia were assessed using the Positive and Negative Syndrome Scale (PANSS). Written informed consent was obtained from subjects after the procedures were fully explained.

2.1.1. Clozapine-responder (CLZ-responder)

The patient was a 59-year-old Japanese female diagnosed with treatment-resistant schizophrenia whose symptoms were improved after clozapine treatment. The PANSS and GAF scores prior to clozapine treatment were PANSS positive, 19; negative, 25; general, 44; and GAF, 23. After clozapine treatment, the PANSS and GAF scores were PANSS positive, 14; negative, 19; general, 40; and GAF, 33. Prior to clozapine treatment, the patient could not leave her house and could not perform housework due to delusions. However, after clozapine treatment, the patient could leave the house and perform housework, reflecting the improvement of positive and negative symptoms.

2.1.2. Clozapine-non-responder (CLZ-non-responder)

The patient was a 59-year-old Japanese female diagnosed with treatment-resistant schizophrenia whose symptoms were not improved after clozapine treatment. The PANSS and GAF scores prior to clozapine treatment were PANSS positive, 29; negative, 20; general, 48; and GAF, 23. After clozapine treatment, the PANSS and GAF scores were PANSS positive, 26; negative, 23; general, 51; and GAF, 21. The patient was constantly affected by delusions concerning God before and after clozapine treatment.

2.2. Generation of iPS cells

The generation of iPS cells from lymphoblastoid B-cell lines was performed as previously described (Fujimori et al., 2016). The isolation of lymphocytes from patient blood samples and the immortalization of these cells using Epstein-Barr virus were performed by Special Reference Laboratories, Inc. (Tokyo, Japan) (Yamamori et al., 2011). Immortalized lymphoblastoid B-cell lines obtained from the schizophrenia monozygotic twin were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 20% fetal bovine serum. The immortalized lymphoblastoid B-cell lines (2×10^6 cells) were electroporated with 0.63 μg of pCE-hOCT3/4, 0.63 μg of pCE-hSK, 0.63 μg of pCE-hUL, 0.63 μg of pCE-mp53DD and 0.50 μg of pCXB-EBNA1 (Addgene, MA, USA) using the Nucleofector 2b Device (Lonza,

Basel, Switzerland) with the Amaxa Human T-cell Nucleofector Kit (Lonza) (Program, U-008). After electroporation, the immortalized lymphoblastoid B-cell lines were grown in RPMI medium supplemented with 10% fetal bovine serum for 1 week. Subsequently, the electroporated cells were seeded onto mitomycin C-treated mouse SNL feeder cells and maintained for 20–30 days in standard human ES cell medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 20% KnockOut serum replacement (Thermo Fisher Scientific, MA, USA), 1% of Non-essential Amino Acid Solution (NEAA) (Sigma-Aldrich, MO, USA), 2 mM of L-glutamine (Thermo Fisher Scientific), 0.1 mM of 2-mercaptoethanol (Wako Chemicals, Tokyo, Japan) and 4 ng/ml of bFGF (Peprotech, NJ, USA). Subsequently, the colonies similar to human ES cells were clonally isolated, morphologically selected, subjected to a PCR-based analysis of episomal vector loss and evaluated for the expression of pluripotent markers using immunohistochemistry (Oct-4A, Sox2, TRA-1-60 and TRA-1-81). Antibodies against Oct-4A, Sox2, TRA-1-60 and TRA-1-81 were purchased from Cell Signaling (MA, USA).

2.3. In vitro differentiation of iPS cells into excitatory neurons

For in vitro differentiation, iPS cells were transferred under feeder-free conditions onto Matrigel (Corning, NY, USA)-coated dishes and cultured in the chemically defined Essential 8 medium (Thermo Fisher Scientific) according to the manufacturer's instructions. The in vitro differentiation of iPS cells into neurons through Neurogenin2 (Ngn2) overexpression was performed according to Zhang et al. (Zhang et al., 2013), with minor modifications. On day 0, iPS cells were infected with recombinant lentiviruses expressing the reverse tetracycline transactivator (#20342 (Addgene)) and Neurogenin2-T2A-Puro under the TetO enhancer upstream of the minimal CMV promoter (#20321 and #34999 (Addgene); QM200PA-1 (System Biosciences, CA, USA)) in Essential 8 Medium containing 10 μM of Y-27632 (Wako Chemicals). On day 1, the culture medium was replaced with DMEM/F12/N2 containing 1% NEAA (Thermo Fisher Scientific), 10 μM of Y-27632, 10 ng/ml of human BDNF (R&D Systems, MN, USA), 10 ng/ml of human NT3 (R&D Systems), 200 ng/ml of mouse laminin (Thermo Fisher Scientific) and 2 μg/ml of human fibronectin (Thermo Fisher Scientific). Doxycycline (Clontech, CA, USA) (2 μg/ml) was added on day 1 to induce TetO gene expression. On day 2, the culture medium was replaced with the DMEM/F12/N2 medium containing 1 μg/ml of puromycin (Wako Chemicals). On day 3, the culture medium was replaced with Neurobasal medium (Thermo Fisher Scientific) supplemented with B-27 (Thermo Fisher Scientific), 1% Glutamax (Thermo Fisher Scientific), 10 μM of Y-27632, 10 ng/ml of human BDNF, 10 ng/ml of human NT3, 200 ng/ml of mouse laminin, 2 μg/ml of human fibronectin and 2 μg/ml of doxycycline. After day 3, 50% of the medium was exchanged every 2 days. On day 8, 10 μM of Ara-C (Sigma-Aldrich) and 1 μM of clozapine (Sigma-Aldrich) (or mock) were added to the medium. On day 15, Ngn2-induced neurons were stained with a neuron marker, class III β-tubulin (Tuj1, Biolegend, CA, USA) or were harvested to isolate total RNA for RNA sequencing. Neuronal nuclei were stained with Hoechst 33258 (Calbiochem, CA, USA).

2.4. Immunocytochemistry

Immunocytochemistry of iPS cells and differentiated neurons was performed as previously described (Nakazawa et al., 2016).

2.5. RNA sequence

Total RNA from lymphoblastoid B-cell lines (P0052 and P0070) and iPS cell-derived differentiated neurons (P0052-1B, -2B, -4B, P0070-2B, -6B and -9B) was isolated using the PureLink RNA Micro Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Equal amounts of total RNAs from each patient were combined (for iPS cell-derived differentiated neurons) and sequenced using the Illumina HiSeq2000 system (BGI, Beijing, China). The generated fastq files were subjected to quality control. Subsequently, the reads were aligned to the human reference genome hg19 and the reference genes using BWA and Bowtie software (BGI, Beijing, China), respectively. The aligned reads were processed using RSEM (Li and Dewey, 2011). The gene expression levels were measured based on Fragments Per Kilobase of exon per Million mapped (FPKM). We compared the gene expression levels between the clozapine- and mock-treated neurons and lymphoblastoid B-cell lines of each patient to identify clozapine responsive genes. We also compared the gene expression levels of the mock-treated neurons and lymphoblastoid B-cell lines to identify differentially expressed genes between each patient in the basal state. We performed differential gene expression analysis based on a Poisson distribution to identify differentially expressed genes (BGI, Beijing, China). The false discovery rate (FDR) was used to correct for multiple testing. The significance level was set at FDR < 0.05. The *Z*-score was calculated as $(x_{i,j} - \mu_i) / \sigma_i$, where $x_{i,j}$ is the expression level of gene i in sample j, and μ_i and σ_i are the average expression level and standard deviation, respectively, of gene i across samples. We calculated Fisher's exact p values for the identification of genes showing differential expression patterns in the same direction between the iPS-derived neurons and lymphoblastoid B-cell lines.

2.6. Gene ontology and pathway analysis

We used the Database for Annotation, Visualization and Integrated Discovery (DAVID) to perform Gene Ontology (GO)-based functional classification of differentially expressed genes. Fisher's exact p values were calculated to search for overlapped GO terms. The calculated p values were adjusted using FDR to correct for multiple testing. The significance level was set at FDR < 0.05.

3. Results

3.1. Generation of iPS cells from a pair of monozygotic twins with treatment-resistant schizophrenia

We used a non-integrating approach with episomal vectors expressing OCT3/4, SOX2, KLF4, L-MYC, LIN28, dominant negative TP53 and EBNA1 to establish iPS cell lines using the immortalized B cells obtained from monozygotic twin patients with treatment-resistant schizophrenia and discordant clozapine responses, i.e., one twin responded well to clozapine treatment (patient number, P0070, hereafter clozapine (CLZ)-responder) and the other twin did not (patient number, P0052, hereafter CLZ-non-responder) (see Materials and Methods for patient information). Each iPS cell line was clonally isolated, morphologically selected, subjected to PCR-based analysis of episomal vector loss and evaluated for the expression of pluripotent markers (Oct-4A, Sox2, TRA-1-60 and TRA-1-81) (Fig. 1). Based on these criteria, we selected three clones from the CLZ-non-responder (P0052-1B, -2B and -4B) and three clones from CLZ-responder (P0070-2B, -6B and -9B) for further analysis.

3.2. Clozapine responsive genes in iPS cell-derived differentiated neurons

Using the forced expression of the neuronal transcription factor Ngn2 (Zhang et al., 2013), we generated differentiated neurons from each iPS cell clone obtained from the CLZ-non-responder and CLZ-responder (Fig. 1, *right*). To identify differentially expressed genes in response to clozapine treatment in each patient, we conducted RNA-seq and compared the global gene expression data obtained for the differentiated neurons treated with mock or 1 μM of clozapine for 1 week. For the RNA-seq experiments, total RNA (P0052-1B, -2B, -4B, P0070-2B, -6B and -9B) was isolated from each clone and equal amounts of total RNAs from each patient were combined and sequenced using the

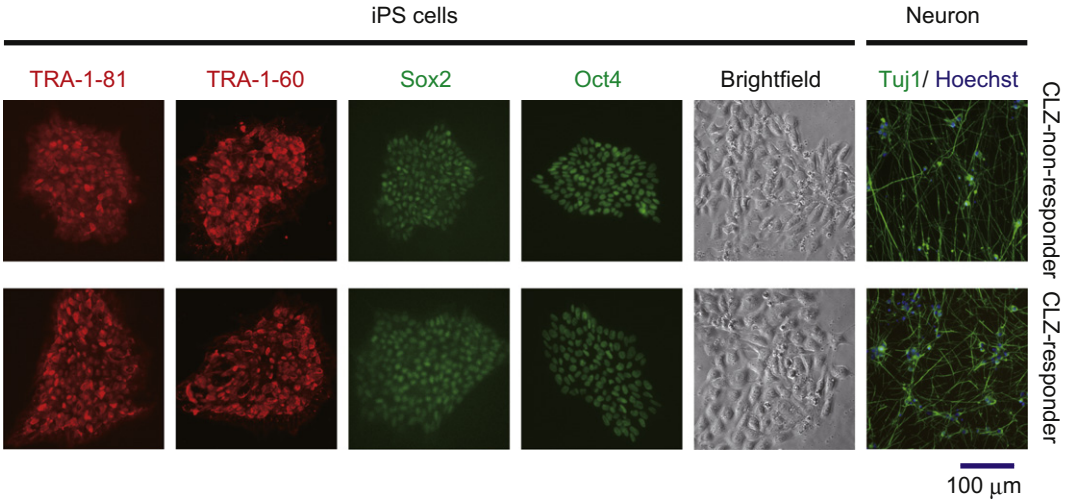

Fig. 1. Representative images of iPS cells and Ngn2-induced neurons from the CLZ-non-responder and CLZ-responder. iPS cells were stained for pluripotency markers (Oct4, Sox2, TRA-1-60 and TRA-1-81). Ngn2-induced neurons were stained with a neuron marker, Tuj1 (green). Neuronal nuclei were stained with Hoechst 33258 (blue). Scale bar, 100 μm.

Illumina HiSeq2000 system. In response to clozapine treatment, a total of 22 and 46 genes were upregulated and downregulated, respectively, in the mixed sample from the differentiated neurons from the CLZ-responder (|FC| > 1, FDR < 0.05) (Table 1 and Supplementary Table 1). Likewise, a total of 28 and 24 genes were upregulated and downregulated, respectively, in the mixed sample from the differentiated neurons from the CLZ-non-responder (|FC| > 1, FDR < 0.05) (Table 1 and Supplementary Table 2). Among the 68 genes that were clozapine-responsive in the CLZ-responder, 20 and 44 genes were uniquely upregulated and downregulated, respectively, in the CLZ-responder but not in the CLZ-non-responder (Table 1 and Supplementary Table 3). A Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of the 64 genes that were uniquely upregulated or downregulated in the CLZ-responder showed that these genes were modestly enriched in the GO annotations for cell adhesion/biological adhesion (*TNR*, *PCDHA13*, *PCDHGA4*, *PCDHGC5*, *GP1BB* and *ITGA9*) and homophilic cell adhesion (*PCDHA13*, *PCDHGA4* and *PCDHGC5*) (Table 2); however, the enrichment of these GO annotations was not statistically significant (FDR > 0.05). We next conducted RNA-seq and compared the global gene expression data obtained for the patients' lymphoblastoid B-cell lines treated with mock or 1 μM of clozapine for 1 week. In response to clozapine treatment, a total of 22 and 38 genes were upregulated and downregulated, respectively, in the sample from the lymphoblastoid B-cell lines from the CLZ-responder (|FC| > 1, FDR < 0.05) (data not shown). Likewise, a total of 38 and 61 genes were upregulated and downregulated, respectively, in the sample from the lymphoblastoid B-cell lines from the CLZ-non-responder (|FC| > 1, FDR < 0.05) (data not shown). We then focused uniquely upregulated or downregulated genes in the iPS cell-derived neurons from CLZ-responder that were modestly enriched in the GO annotations for cell adhesion/biological adhesion and homophilic cell adhesion (cell adhesion/biological adhesion, *TNR*, *PCDHA13*, *PCDHGA4*, *PCDHGC5*, *GP1BB* and *ITGA9*; homophilic cell adhesion, *PCDHA13*, *PCDHGA4* and *PCDHGC5*) (|FC| > 1, FDR < 0.05) (Table 2). We found that none of these genes showed a differential expression pattern in the same direction upon clozapine treatment between the iPS cell-derived neurons and lymphoblastoid B-cell lines from the CLZ-responder (Supplementary Table 4), suggesting that their lymphoblastoid B-cell lineage may not contribute to the differential gene expression patterns in the iPS cell-derived neurons between the patients (FDR > 0.05).

3.3. Gene expression analysis in iPS cell-derived differentiated neurons in the basal state

Monozygotic twins are derived from the same single cell and therefore share their genetic variations (Boomsma et al., 2002). As expected, exome sequencing did not reveal differences in the DNA sequence between the twins (data not shown). Nevertheless, monozygotic twins generally show discordant global gene expression profiles, potentially leading to phenotypic discordance (Boomsma et al., 2002; Kato et al., 2005). Therefore, we compared the global gene expression data obtained for the mock-treated differentiated neurons. We observed that the expression levels of a total of 167 genes were higher in the CLZ-responder compared with those in the CLZ-non-responder (|FC| > 1, FDR < 0.05) (Supplementary Table 5). Similarly, the expression levels of a total of 95 genes were lower in the CLZ-responder compared with those in the CLZ-non-responder (|FC| > 1, FDR < 0.05) (Supplementary Table 6). DAVID analysis of the 262 genes revealed an enriched functional group in homophilic cell adhesion (FDR = 4.76×10^{-4}) (higher, *PCDHGB3*, *CDH8*, *PCDHB15*, *PCDHGB1*, *PCDHGA11*, *PCDHGA6* and *DSC3*; lower, *PCDHGC3*, *PCDHA7*, *PCDHGA7* and *PCDHA13*) (Table 3 and Fig. 2). We also observed that these genes were modestly enriched in the GO annotations for cell adhesion/biological adhesion (*PCDHA7*, *PCDHGA11*, *CNTNAP5*, *TNC*, *PCDHB15*, *PCDHGA7*, *PCDHGA6*, *FER*, *PCDHGC3*, *PCDHGB3*, *CDH8*, *ITGA9*, *PCDHGB1*, *GP1BB*, *TNR*, *DSC3*, *RELN*,

Table 1
The number of clozapine-responsive genes in the CLZ-non-responder and CLZ-responder.

	Upregulated	Downregulated
CLZ-responder	22	46
CLZ-non-responder	28	24
CLZ-responder-specific	20	44

Table 2
Top GO annotations for specifically upregulated or downregulated genes in the clozapine-treated CLZ-responder.

Annotations	No. of genes	*p* value
GO:0007155 cell adhesion	6	5.65E-03
GO:0022610 biological adhesion	6	5.68E-03
GO:00007156 homophilic cell adhesion	3	2.06E-02

Table 3
Differentially expressed genes annotated as being involved in homophilic cell adhesion (GO:0007156) between the CLZ-non-responder and CLZ-responder in the basal state.

Symbol	P0070 (FPKM)	P0052 (FPKM)	Fold changes
PCDHGB3	0.35	0.05	7.00
CDH8	4.48	0.96	4.66
PCDHB15	24.7	7.81	3.16
PCDHGB1	0.97	0.44	2.20
PCDHGA11	2.55	1.18	2.16
PCDHGA6	4.17	1.94	2.15
DSC3	0.76	0.38	2.00
PCDHGC3	36.6	75.5	0.48
PCDHA7	0.73	1.53	0.47
PCDHGA7	0.30	1.97	0.15
PCDHA13	0.11	0.82	0.13

|FC| > 1, FDR < 0.05.

Table 4
Top GO annotations for differentially expressed genes between the CLZ-responder and CLZ-non-responder in the basal state.

Annotations	Gene number	*p* value	FDR q value
GO:0007156 homophilic cell adhesion	11	3.05E-07	4.76E-04
GO:0007155 cell adhesion	19	6.02E-05	0.093
GO:0022610 biological adhesion	19	6.13E-05	0.095
GO:0045449 regulation of transcription	43	6.32E-05	0.098

PCDHA13 and *ENG*) and regulation of transcription (*ONECUT1*, *TBX20*, *SOX5*, *SOX7*, *WTIP*, *ZBTB37*, *GTF2H2C*, *LHX4*, *ZSCAN23*, *DMRTC1/1B*, *PER3*, *SIM2*, *HSFX1/2*, *GMEB1*, *OTX2*, *IGF2*, *ZFP3*, *NPAS4*, *SIGIRR*, *MAFIP*, *IGSF1*, *VEGFA*, *ZFPM2*, *NEUROD4*, *ENG*, *TMEM189-UBE2V1*, *INS-IGF2* and 17 *ZNF* genes); however, the enrichment of these GO annotations was not statistically significant (cell adhesion, FDR = 0.094; biological adhesion, FDR = 0.096; regulation of transcription, FDR = 0.099) (Table 4). We next conducted RNA-seq and compared the global gene expression data obtained for the mock-treated patients' lymphoblastoid B-cell lines. We observed that the expression levels of a total of 1170 genes were higher in the CLZ-responder compared with those in the CLZ-non-responder (|FC| > 1, FDR < 0.05) (data not shown). Similarly, the expression levels of a total of 754 genes were lower in the CLZ-responder compared with those in the CLZ-non-responder (|FC| > 1, FDR < 0.05) (data not shown). We then focused upregulated or downregulated genes in the iPS cell-derived neurons from the CLZ-responder that were enriched in the GO annotation for homophilic cell adhesion (|FC| > 1, FDR < 0.05) (Table 3). We found that *DSC3*, but not other genes, showed a differential expression pattern in the same direction between the iPS cell-derived neurons and lymphoblastoid B-cell lines from the CLZ-responder, which is not statistically significant (FDR > 0.05) (Table 5 and Supplementary Table 7). We also found that several differentially expressed genes enriched in the GO annotation for cell adhesion/biological adhesion and regulation of transcription showed differential expression patterns in the same direction between the iPS cell-derived neurons and lymphoblastoid B-cell lines from the CLZ-responder, which is not statistically significant (FDR > 0.05) (Supplementary Table 7). These results suggest that their lymphoblastoid B-cell lineage may generally not contribute to the differential gene expression patterns in the iPS cell-derived neurons between the patients. The information of differentially expressed genes in the lymphoblastoid B-cell lines may be useful for eliminating noise from the gene expression patterns in the iPS cell-derived neurons.

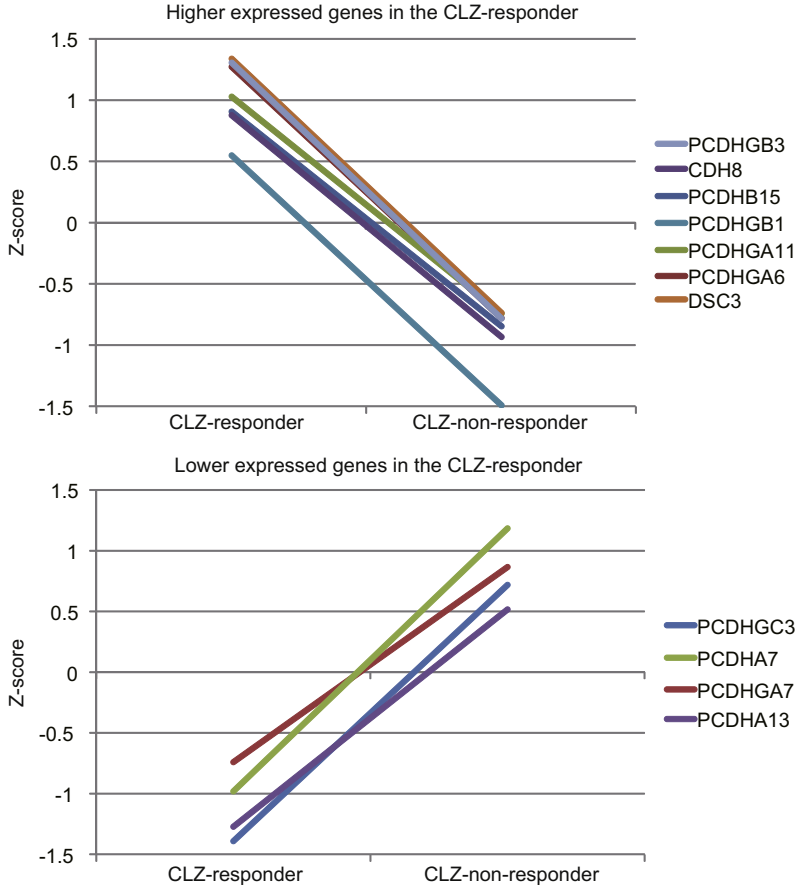

Fig. 2. Differentially expressed genes annotated as being involved in homophilic cell adhesion (GO:0007156) between the CLZ-non-responder and CLZ-responder in the basal state. (*upper*) Higher expressed genes in the CLZ-responder. (*lower*) Lower expressed genes in the CLZ-responder. The expression level of each gene is expressed as a Z-score.

Table 5
Comparison of differential expression of genes enriched in the GO annotation for homophilic cell adhesion between lymphoblastoid B-cell lines and iPS cell-derived differentiated neurons.

Symbol	P0070 (FPKM) (neuron)	P0052 (FPKM) (neuron)	Fold changes (neuron)	P0070 (FPKM) (B cell)	P0052 (FPKM) (B cell)	Fold changes (B cell)	FDR q value (B cell)
PCDHGB3	0.35	0.05	7.00	1.06	0.48	2.21	0.090
CDH8	4.48	0.96	4.66	ND	ND		
PCDHB15	24.7	7.81	3.16	0.12	0.001	120	0.27
PCDHGB1	0.97	0.44	2.20	0.1	0.2	0.5	0.61
PCDHGA11	2.55	1.18	2.16	0.41	0.96	0.43	0.023
PCDHGA6	4.17	1.94	2.15	0.89	0.45	1.98	0.24
DSC3	0.76	0.38	2.00	0.99	0.42	2.36	4.7E-04
PCDHGC3	36.6	75.5	0.48	6.04	3.99	1.51	3.7E-05
PCDHA7	0.73	1.53	0.47	0.11	0.18	0.61	0.76
PCDHGA7	0.30	1.97	0.15	0.49	0.09	5.44	0.074
PCDHA13	0.11	0.82	0.13	0.001	0.05	0.02	0.66

ND, not detected.

4. Discussion

The molecular identification of drug response predictors of clozapine is directly linked to the development of new, effective treatment strategies. However, clozapine has complex effects on various neurotransmitter receptors, such as dopamine, serotonin, adrenaline, acetylcholine and histamine receptors, preventing the understanding of the molecular mechanisms of clozapine action and the identification of drug response predictors. In the present study, we applied iPS-cell-based technology in a monozygotic twin study and observed that several proteins involved in homophilic cell adhesion are differentially expressed between the patients. To the best of our knowledge, there are no reports on the use of iPS cell technology in monozygotic twin studies of schizophrenia; however, the present study is necessarily preliminary due to the observational nature and its being conducted with a single pair of patients.

It is generally impossible to obtain live neurons from schizophrenia patients. Accordingly, imaging studies of patient brains, genetic studies of patient lymphocytes and postmortem studies of patient brains have primarily been used in schizophrenia research (Falk et al., 2016). While these studies significantly contribute to the current understanding of schizophrenia, the precise molecular pathogenesis of schizophrenia remains unclear. Because schizophrenia is a genetically complex disorder, it is important to use live patient-derived neurons with genetic backgrounds of the disorder to identify the neuronal defects associated with schizophrenia. The present and several previous studies have reported the establishment of disease-specific iPS cells from patients with schizophrenia and identified some of the pathogenic mechanisms of schizophrenia (Brennand et al., 2011; Bundo et al., 2014; Falk et al., 2016; Hook et al., 2014; Imaizumi and Okano, 2014; Okano and Yamanaka, 2014; Wen et al., 2014; Wright et al., 2014). Among these studies, the present study is unique because we used samples from monozygotic twins with treatment-resistant schizophrenia. Importantly, one twin responded well to clozapine treatment and the other twin did not. Because monozygotic twins share almost all of their genetic variants, the iPS cell-derived neurons established in the present study may be ideal experimental materials to dissect the molecular mechanisms underlying the inter-individual variability in the clozapine response.

Using iPS cell-derived neurons, we observed that the expression levels of several genes were different between the patients. Although the precise mechanisms behind these differences are currently unknown, epigenetic mechanisms, such as DNA methylation and chromatin remodeling, are likely some of the main mechanisms underlying the differential transcriptional events. Alternatively, although we did not identify genetic variations in exons using whole exome sequencing, somatic de novo mutations might occur in specific organs, such as the brain. Rare de novo CNVs should also be considered.

Previous genetic association studies have reported that variations in several genes, such as *DRD2*, *DRD3*, *HTR2A*, *HTR3A*, *NRXN1*, *KCNH2*, *GSK3* and *BDNF*, are candidate drug response predictors for schizophrenia (Apud et al., 2012; Gressier et al., 2016; Ikeda et al., 2008; Jenkins et al., 2014; Kohlrausch, 2013; Muller et al., 2013; Souza et al., 2010; Souza et al., 2008; Sriretnakumar et al., 2015; Zai et al., 2012; Zhang et al., 2010). These genes converge on the dopamine, serotonin pathways and the regulation of synaptic functions; however, the current molecular knowledge of drug response predictors for clozapine is limited. We observed that the expression levels of homophilic cell adhesion molecules, such as *CDH8*, *DSC3* and protocadherin genes, were different in the iPS cell-derived neurons derived from each patient (Table 3 and Fig. 2). The differential expression of homophilic cell adhesion molecules potentially results in alterations in the number and structure of synapses and dendrite morphology, leading to altered neural circuit formation in the brain. Importantly, taking advantage of recently developed iPS technology, we can investigate the functional significance of the differential expression of homophilic cell adhesion molecules in patient-derived live neurons. Additional studies using the monozygotic twins with schizophrenia are required for validating the present findings. However, it is generally very hard to find another monozygotic twin cases with treatment-resistant schizophrenia and discordant responses to clozapine, because the estimated incidence of a monozygotic twin of patients with treatment-resistant schizophrenia and discordant responses to clozapine among schizophrenia patients is only about 0.13 to 0.33 pairs per million patients with schizophrenia (Supplementary text). The estimation implies that the expected number of monozygotic twin pairs with treatment-resistant schizophrenia and discordant responses to clozapine is under about 0.013, even if we investigate schizophrenia patients in the large genetic study (36,989 patients, Ripke et al., 2014). To the best of our knowledge, our current study is the first report of an extremely rare case of monozygotic twin with treatment-resistant schizophrenia and discordant responses to clozapine. In combination with recently developed iPS cell technology, although there are many limitations in our current study, our study proposes a new approach for schizophrenia research.

Instead of monozygotic twin cases, a large number of responder and non-responder schizophrenia cases who are not monozygotic twin cases can be useful for validating the expression data; however, with current technologies, the generation of iPS cells from a large number of schizophrenia cases would require tremendous cost, time and manpower.

In the present study, we used iPS-cell-based technology to identify homophilic cell adhesion molecules as potential candidates for the molecular basis of clozapine response. These results not only contribute to the current understanding of the molecular mechanisms of clozapine action but also provide information for the development of new, effective treatment strategies.

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The funding sources had no involvement in study design, the collection and the interpretation of the data, and the preparation of the manuscript.

Author contributions

T.N., M.K., H.O., A.N., H.H. and R.H. designed the study and drafted the manuscript. T.N., H.Y., K.N., M. Fujimoto, Y.Y., M. Fujiwara, S.O., K.M., A.K., A. H-T., N.S., S.N., K.T., H.H. and R.H. designed and performed the biochemical analysis. M.K. and A.N. designed and performed the gene expression analysis. T.N., M.I., T.M., W.A. and H.O. generated the iPS cells from the patients. H.Y., M. Fujimoto, Y.Y. and R.H. recruited and characterized the schizophrenia patients. All authors interpreted the results, participated in the critical revision of the manuscript to assess the important intellectual content, and read and approved the manuscript.

Conflicts of interest

H.O. is a paid Scientific Advisory Board Member of SanBio Co., Ltd. The other authors declare no conflicts of interest.

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