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ORIGINAL INVESTIGATION

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Impact of clozapine on the expression of miR-675-3p in plasma exosomes derived from patients with schizophrenia

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ABSTRACT

Objectives: Recently, the expression changes of microRNAs (miRNAs) in the serum exosomes (EXO) of schizophrenia (SCZ) have been reported. The aim of this study was to investigate the global expression changes of miRNA derived from the plasma EXO of patients with treatment-resistant schizophrenia (TRS) and the effects of clozapine on miRNA expression.

Methods: Global miRNA expression changes in plasma EXO between TRS and controls were studied using microarray analysis. Then, miRNA expressions among TRS, non-TRS, and controls were confirmed with quantitative qPCR experiments. We also studied changes in EXO miRNA expression with in-vitro SH-SY5Y cells.

Results: A microarray for miRNA expression analysis (nine controls vs. nine patients with TRS) revealed 13 up- and 18 downregulated miRNAs that were relevant to neuronal and brain development based on gene ontology analysis. Of those, upregulated miR-675-3p expression was successfully validated in the same cohort by qPCR experiments. Conversely, miR-675-3p expression levels were significantly decreased in the non-TRS cohort (50 controls vs. 50 patients without TRS without clozapine treatment).

Conclusions: We identified global miRNA changes in plasma EXO derived from patients with SCZ that were relevant to neuronal functions, among which, hsa-miR-675-3p expression was upregulated by clozapine treatment.

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Schizophrenia; treatment-resistant schizophrenia; exosome; MiRNA; clozapine

Introduction

Schizophrenia (SCZ) is a common mental illness known to impair social function that has an estimated prevalence of approximately 0.5%–1.0% (Saha et al. 2005; Owen et al. 2016). Genetic and environmental backgrounds are known to contribute to the onset of SCZ (Sullivan et al. 2003; Hannon et al. 2016). Although several hypotheses, including dopamine and glutamate hypotheses, have been explored (Seeman and Lee 1975; Javitt and Zukin 1991), the critical aetiology and pathogenesis of SCZ remain unclear. Approximately 30% of patients with SCZ have treatment-resistant schizophrenia (TRS), which is defined as having resistance to at least two conventional antipsychotics (Meltzer 1997; Howes et al. 2017). Clozapine, a multi-acting receptor-targeted antipsychotic, is considered the most effective agent for TRS (Numata et al. 2018); however, its unresponsiveness rate among patients with TRS has been reported to be as high as 40% (Siskind et al. 2017).

As an epigenetic modifier and part of the genetic background, microRNA (miRNA), a small single-stranded RNA (~23 bases), plays a key role in the pathogenesis of SCZ (Bartel 2009; Yoshino and Dwivedi 2020). Epigenetically, miRNAs regulate gene expression by binding to complementary sites to target messenger RNA (mRNA) mostly present in the 3′UTR (Bartel 2004, 2009). Recently, several reports have suggested that miRNA expression in the blood may be a diagnostic and/or treatment biomarker in psychiatric disorders (Gibbons et al. 2020; Khavari and Cairns 2020; Yoshino and Dwivedi 2020). Exosomes (EXO) are defined as particles with a diameter of about 40–100 nm that are released from cells and contain proteins, DNA, RNA, and miRNA (Simpson et al. 2012;

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Zhang et al. 2015). Tissue cell-cell communication is one of the reported functions of EXO (Valadi et al. 2007; Tominaga et al. 2015; Amoah et al. 2020), which can also cross the blood-brain barrier (Andjus et al. 2020). Based on these reports, we assumed that altered miRNA expression levels in plasma EXO might reflect the brain pathology of SCZ. Although EXO miRNA in serum has been shown to be a useful diagnostic biomarker in first-episode and drug-free patients with SCZ compared with controls (Du et al. 2019), few studies have examined the relationship between EXO and SCZ.

Given this background, the present study aimed to clarify the expression changes of miRNA in plasma EXO of patients with TRS and investigated whether these changes reflect the relationship between aberrant miRNAs and neuronal functions. To achieve this, we investigated the following: 1) the global miRNA expression changes in plasma EXO using microarray analysis in nine patients with TRS compared with nine controls, 2) the type of biological processes to which predicted target genes from up- and downregulated miRNAs belong based on gene ontology (GO) analysis, 3) the expression profiles of selected miRNAs in patients with and without TRS, and 4) the direct effect of clozapine on miRNA expression using an in vitro system.

Methods

Patients with SCZ and controls

According to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, we enrolled patients who had been diagnosed by at least two expert psychiatrists. Patients with TRS were defined as follows: those who did not respond to more than two sufficient doses of antipsychotics (more than 600 mg/day of chlorpromazine equivalent), including at least one atypical antipsychotic for more than 4 weeks. All patients with TRS were treated with clozapine during this study. On the other hand, patients with SCZ that had been diagnosed with non-treatment-resistant schizophrenia and treated with antipsychotic agents other than clozapine were defined as the non-TRS group. The demographic data and clinical parameters of the TRS and non-TRS groups are presented in Tables 1a and 1b, respectively. SCZ symptoms were evaluated using the Positive and Negative Syndrome Scale (PANSS) (Kay et al. 1987). The Drug-Induced Extrapyramidal Symptoms Scale (DIEPSS) was also used to evaluate antipsychotic-induced extrapyramidal symptoms (Peljto et al. 2017). We recruited control subjects who were free from psychiatric symptoms and had no history of mental illness or related medication. All participants were of Japanese origin and genetically unrelated. This study was approved by the institutional ethics committees of Ehime University Hospital (31-K8), and all participants provided written informed consent before the study began.

Table 1a. Demographic data of the microarray study.

	Ct subjects ($n = 9$)	TRS patients ($n = 9$)	p value
Sex (male:female)	4:5	4:5	1
Age (years)	41.9 ± 9.4	41.9 ± 9.4	1
Characteristics			
Age at onset ($n = 9$)		19.4 ± 3.4	
Duration ($n = 9$)		22.4 ± 8.5	
CLO dose ($n = 9$)		363.9 ± 158.7	
PANSS ($n = 9$)			
Total		60.8 ± 19.9	
Positive		12.7 ± 4.0	
Negative		17.6 ± 8.8	
General		30.6 ± 10.1	
DIEPSS ($n = 9$)		4.7 ± 5.1	

Values denote mean ± standard deviation.
Abbreviations: CLO, clozapine; Ct, control; DIEPSS, Drug Induced Extra-Pyramidal Symptoms Scale; Duration, duration of illness; PANSS, Positive and Negative Syndrome Scale; TRS, treatment-resistant schizophrenia.

Collection of blood samples and isolation of EXO RNA

Peripheral blood was collected into potassium EDTA tubes and then centrifuged at about 700 × g (2,000 × rpm) for 10 min to separate the plasma fraction. The plasma samples were stored at −80 °C until further analysis. EXO was isolated by ultracentrifugation with 1.2 mL of plasma using an ultracentrifuge (Optima TLX; Beckman Coulter Inc., Brea, CA, USA) according to previous reports (Garcia-Contreras et al. 2017; Sanada et al. 2020). Briefly, the plasma samples were subjected to 10,000 × g (10,200 × rpm) centrifugation at 4 °C for 30 min. The supernatants were then collected into ultracentrifuge tubes (Beckman Coulter Polycarbonate Thickwall Tube; Beckman Coulter Inc.) and subjected to 102,716 × g (50,000 × rpm) ultracentrifugation at 4 °C for 90 min in the ultracentrifuge with a TLA3 rotor (Optima TLX; Beckman Coulter Inc.). The precipitate was resuspended in phosphate-buffered saline (PBS), filtered through a 0.22-μm filter (Merck-Millipore, Burlington, MA, USA), and centrifuged at 102,716 × g (50,000 × rpm) at 4 °C for 70 min. EXO was then resuspended in 200 μL of PBS. The particle size of isolated EXO ($n = 3$ from controls) was validated using a nanoparticle-tracking analyser (Nanosight NS 300; Malvern Panalytical, Malvern, UK). Total RNA containing EXO miRNA was extracted using

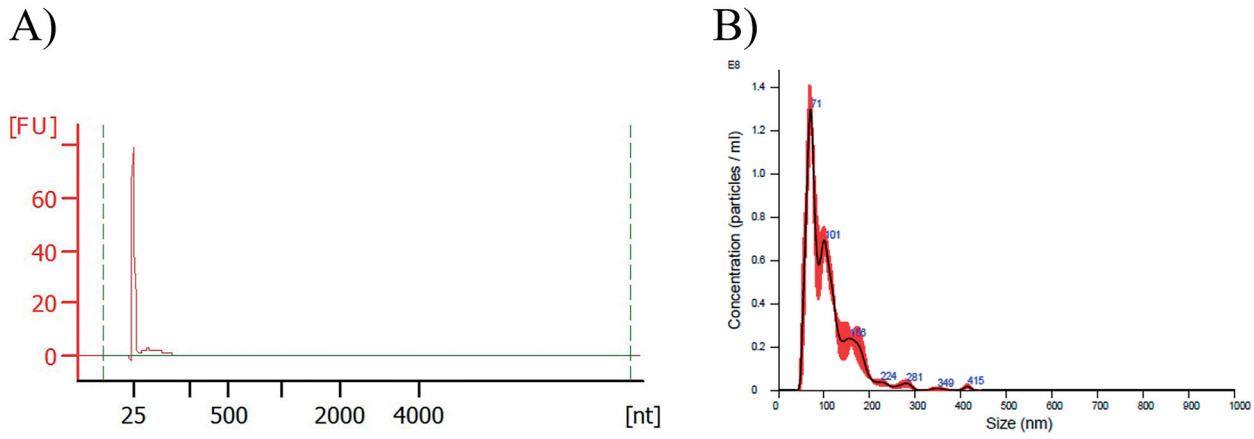

Figure 1. Characteristics of the extracted total RNA and exosomes. (A) Extracted total RNA, including small RNAs, was checked by a bioanalyzer. (B) The particle size distribution in the isolated exosomes ($n = 3$ from controls) was validated using a nanoparticle-tracking analyser. FU: fluorescence units; nt: nucleotides.

Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

MiRNA microarray analysis

Extracted total RNA was checked using a bioanalyzer (Agilent 2100; Agilent Technologies, Palo Alto, CA, USA) and labelled using a miRNA labelling kit (3 D-Gene; Toray Industries, Kamakura, Japan), as shown in Figure 1A. The total RNA content from these samples' isolated extracellular vesicles was analysed using a bioanalyzer (Agilent Technologies). Bioanalyzer was used with the RNA 6000 Pico Kit (5067-1513, Agilent Technologies). Labelled RNAs were hybridised onto 3 D-Gene Human miRNA Oligo chips (Toray Industries). The annotation and oligonucleotide sequences of the probes were confirmed to the miRBase Release 21 database (http://microrna.sanger.ac.uk/sequences/). After stringent washing, fluorescent signals were scanned using a 3 D-Gene scanner and analysed using 3 D-Gene extraction software (Toray Industries). The raw data for each spot were normalised by substitution, with the mean intensity and 95% confidence intervals of the background signal calculated based on the signal intensity of all blank spots. The measurements of spots with signal intensities greater than two standard deviations (SD) of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots throughout the microarray experiments. The normalised data were globally normalised per array so that the median of the signal intensity was adjusted to 25. MiRNAs including a global normalisation value of 0 in either the control or TRS group were excluded.

Bioinformatics analysis

In silico analysis was carried out to confirm the target gene prediction of miRNA using the comprehensive miRNA database miRWalk (http://mirwalk.umm.uni-heidelberg.de/) (Dweep et al. 2011). The predicted target genes of each miRNA were defined as genes included in all seven selected databases (miRwalk, miRDB, MicroT4, RNA22, miRanda, Targetscan, and Pictar2). GO analyses regarding the terms Biological Process (BP), Molecular Function, and Cellular Component (CC) were conducted using the ClueGO plugin (Bindea et al. 2009) in the Cytoscape program (ver. 3.8.0). Statistical significance was set at $p < 0.05$ using Bonferroni correction. Graphical networks of significant GO terms were created by using GO term fusion based on the following criteria: visual style = Groups, and GO Term/Pathway Network Connectivity = Medium (kappa score = 0.40).

Synthesis of cDNA and qPCR analysis

We performed the reverse transcription and real-time qPCR to examine miRNA expression by using the Mir-X miRNA First-Strand Synthesis Kit (Takara Bio Inc., Tokyo, Japan) and StepOne software (Applied Biosystems, Foster City, CA, USA). MiRNA expression levels were examined in duplicate because the standard deviation of Ct values in the same sample was small enough. Based on the miRNA microarray data, we confirmed the suitable internal standard for the EXO qPCR experiment by using Normfinder (https://moma.dk/normfinder-software). Briefly, less than 10% of the coefficient of variation was listed and subjected to Normfinder. U6 was used as the internal standard (the cDNA from SH-SY5Y RNA). The qPCR forward primer sequences are shown in Supplementary Table 1.

The mRQ 3′ Primer supplied with the kit (Takara Bio Inc., Tokyo, Japan) was used as reverse primer. The U6 primers were also supplied with this kit (primer sequences were not provided). All qPCR experiments were performed with 1.0 μL miRNA-specific cDNA in a reaction mixture containing TB Green Advantage qPCR Premix (Takara Bio Inc.). Each thermal cycling condition was performed according to the manufacturer's protocol, and the primer's specificity was judged by dissociation curve analysis. Relative miRNA levels were analysed using Livak's ΔΔCt method (Livak and Schmittgen 2001).

In vitro cell line-based study

SH-SY5Y neuroblastoma cells were purchased from ECACC (Salisbury, UK) and cultured in DMEM/F12 containing 10% foetal bovine serum with penicillin and streptomycin (10,000 U/mL). The cells were incubated at 37 °C in a 5% CO_2 atmosphere. Next, 10 μM and 50 μM of clozapine, which was purchased from the Tokyo Chemical Industry (Tokyo, Japan), were dissolved in 0.65% DMSO. Cells were harvested 48 h after exposure to clozapine, vehicle (0.65% DMSO only), or sham (normal saline) to investigate the effect of clozapine on miR-675-3p. EXO of SH-SY5Y cells were collocated using ExoQuick-TC (System Biosciences, Palo Alto, CA, USA) according to the manufacturer's protocol. EXO and SH-SY cell RNAs were isolated using Trizol Reagent (Invitrogen).

Statistical analysis

The mean miRNA expression levels between the control and TRS groups were compared with unpaired two-tailed t-tests using GeneSpring GX software (Tomy Digital Biology, Tokyo, Japan) in the microarray study. Benjamini–Hochberg multiple tests corrected *P*-values. Other statistical analyses were conducted using SPSS 27 software (IBM Japan, Tokyo, Japan). The Shapiro–Wilk test of normality was used to assess whether the data were distributed normally. The miRNA expression levels of plasma samples in the replication set did not follow the normal distribution by using the Shapiro–Wilk test. TRS patients and cell line samples could not assess whether the data were distributed normally due to the small sample size. For that reason, the miRNA expression levels of two groups in the qPCR analysis were compared using the Mann–Whitney *U* test. Other averages of the two groups were compared using the Mann–Whitney *U* test or Student's *t*-test. The averages of more than three groups were compared by the Kruskal–Wallis test with Bonferroni corrections. Sex differences were compared using Fisher's exact test. Correlations between each clinical parameter and miRNA expression levels were assessed by Spearman's rank correlation coefficients. Statistical significance was defined at the 95% level ($p = 0.05$).

Table 1b. Demographic data of the replication set.

	Ct subjects ($n = 50$)	Non-TRS patients ($n = 50$)	p value
Sex (male:female)	24:26	24:26	1
Age (years)	55.1 ± 16.4	55.1 ± 15.7	1
Characteristics			
Age of onset ($n = 48$)		25.8 ± 10.1	
Duration ($n = 48$)		30.3 ± 14.9	
CP equation ($n = 50$)		819.4 ± 715.2	
PANSS ($n = 50$)			
Total		77.2 ± 18.0	
Positive		18.0 ± 5.7	
Negative		20.1 ± 5.2	
General		39.1 ± 9.6	
DIEPSS ($n = 49$)		4.4 ± 3.6	

Values denote mean ± standard deviation.
Due to lack of the clinical data, we could not analyse some of non-TRS patients.
Ct: control; CP equation: chlorpromazine equation; DIEPSS: Drug Induced Extra-Pyramidal Symptoms Scale; Duration: duration of illness; PANSS: Positive and Negative Syndrome Scale; SCZ: schizophrenia; TRS: treatment-resistant schizophrenia.

Results

Characteristics of the patients with SCZ and controls

The patients with TRS and controls did not differ in terms of sex distribution ($p = 1.0$) or age ($p = 1.0$), as shown in Table 1a. In addition, no significant differences were seen in sex ($p = 1.0$) or age ($p = 1.0$) between non-TRS and control subjects (Table 1b). Controls of each cohort represented an independent population.

miRNA microarray analysis

The highest particle size concentration was 40–100 nm, which is defined as the particle size of EXO (Figure 1B). We confirmed the EXO isolated from the control plasma sample with Western blots (Supplementary Data). Among the 2,632 measured miRNAs in the microarray data, 1,230 met the criteria described earlier. Of those miRNAs, 13 significantly up- and 18 significantly downregulated miRNAs were found in the TRS compared with the control group. However, by Benjamini–Hochberg multiple tests corrected *P*-values, and all miRNAs did not meet *q* value <0.05 (Table 2). Volcano plots of EXO miRNA expression levels are shown in Figure 2A. A heat map of the 13 significantly up- and 18 significantly downregulated

Table 2. Significantly changed miRNAs in the microarray data.

microRNA	Chromosome location	Accession (miRBase)	Fold change	*P*-value	Benjamini-Hockberg test (*q* value)
Upregulated miRNAs					
hsa-miR-5193	chr3: 49806137-49806245 [–]	MIMAT0021124	1.391549	0.005092	0.98948216
hsa-miR-2113	chr6: 98024531-98024621 [+]	MIMAT0009206	1.159817	0.006895	0.98948216
hsa-miR-675-3p	chr11: 1996759-1996831 [–]	MIMAT0006790	1.240657	0.014218	0.98948216
hsa-miR-6131	chr5: 10478037-10478145 [+]	MIMAT0024615	1.876869	0.023263	0.98948216
hsa-miR-4646-3p	chr6: 31701029-31701091 [–]	MIMAT0019708	1.156625	0.025119	0.98948216
hsa-miR-1229-3p	chr5: 179798278-179798346 [–]	MIMAT0005584	1.167771	0.027231	0.98948216
hsa-miR-99b-5p	chr19: 51692612-51692681 [+]	MIMAT0000689	1.204721	0.03022	0.98948216
hsa-miR-6852-5p	chr9: 35710676-35710741 [–]	MIMAT0027604	1.272459	0.033121	0.98948216
hsa-miR-6815-3p	chr21: 45478266-45478326 [+]	MIMAT0027531	1.237723	0.036356	0.98948216
hsa-miR-8064	chr3: 52846463-52846552 [–]	MIMAT0030991	1.094344	0.034757	0.98948216
hsa-miR-579-5p	chr5: 32394378-32394475 [–]	MIMAT0026616	1.247525	0.042641	0.98948216
hsa-miR-1468-5p	chrX: 63786002-63786087 [–]	MIMAT0006789	1.28007	0.047496	0.98948216
hsa-miR-6727-3p	chr1: 1312502-1312566 [–]	MIMAT0027356	1.142142	0.046402	0.98948216
Downregulated miRNAs					
hsa-miR-3675-5p	chr1: 16858949-16859021 [–]	MIMAT0018098	0.630808	4.41E-05	0.054266077
hsa-miR-939-3p	chr8: 144394149-144394230 [–]	MIMAT0022939	0.901634	0.001702	0.98948216
hsa-miR-4730	chr17: 80419418-80419493 [+]	MIMAT0019852	0.81215	0.008662	0.98948216
hsa-miR-4665-5p	chr9: 6007826-6007904 [+]	MIMAT0019739	0.644194	0.016485	0.98948216
hsa-miR-125b-2-3p	chr21: 16590237-16590325 [+]	MIMAT0004603	0.734137	0.017565	0.98948216
hsa-miR-6820-3p	chr22: 37967563-37967624 [+]	MIMAT0027541	0.883044	0.020113	0.98948216
hsa-miR-3120-3p	chr1: 172138808-172138888 [+]	MIMAT0014982	0.809726	0.025275	0.98948216
hsa-miR-6776-5p	chr17: 2692861-2692919 [–]	MIMAT0027452	0.863856	0.025675	0.98948216
hsa-miR-302c-5p	chr4: 112648363-112648430 [–]	MIMAT0000716	0.819129	0.033592	0.98948216
hsa-miR-150-3p	chr19: 49500785-49500868 [–]	MIMAT0004610	0.855819	0.038345	0.98948216
hsa-miR-711	chr3: 48578902-48578977 [–]	MIMAT0012734	0.788717	0.043983	0.98948216
hsa-miR-199b-5p	chr9: 128244721-128244830 [–]	MIMAT0000263	0.88312	0.045458	0.98948216
hsa-miR-4283	chr7: 56955785-56955864 [–]	MIMAT0016914	0.830486	0.045841	0.98948216
hsa-miR-500a-3p	chrX: 50008431-50008514 [+]	MIMAT0002871	0.837063	0.044591	0.98948216
hsa-miR-1267	chr13: 107531171-107531248 [–]	MIMAT0005921	0.889389	0.048024	0.98948216
hsa-miR-4251	chr1: 3127975-3128035 [+]	MIMAT0016883	0.804741	0.048668	0.98948216
hsa-miR-1292-3p	chr20: 2652777-2652842 [+]	MIMAT0022948	0.911227	0.0487	0.98948216
hsa-miR-5581-3p	chr1: 37500935-37500994 [–]	MIMAT0022276	0.729484	0.049218	0.98948216

Bold indicates the miRNAs used in the qPCR experiments. hsa, *Homo sapiens*; miR, microRNA

miRNAs between the TRS and control groups were created using the differential hierarchical clustering distribution method (Figure 2B).

Validation of real-time qPCR assays

We selected hsa-miR-4507 as a suitable internal standard for the EXO qPCR experiment because the coefficient of variation was low in both groups (control vs. TRS group = 6.95 vs. 8.48, respectively), and the expression was abundant (stability value = 0.014, Supplementary Table 2). Based on the microarray data, we selected two up- and three downregulated miRNAs, which were relatively abundantly expressed and measured accurately according to the dissociation curve analysis. First, we compared these miRNA expression levels between the nine control subjects and nine patients with TRS. The results revealed that hsa-miR-675-3p expression levels were significantly higher in the TRS compared with the control group (1.84 ± 1.03 vs. 1.00 ± 0.59, respectively, $p = 0.040$, Figure 3A). This result was consistent with that of the miRNA microarray analysis. However, no significant differences in hsa-miR-4665-5p ($p = 0.387$), hsa-miR-939-3p ($p = 0.190$), hsa-miR-4730 ($p = 0.489$), or hsa-miR-6131 ($p = 0.077$) expression levels were observed. Next, we compared miRNA expression levels between 50 non-TRS and 50 control subjects. The results revealed that hsa-miR-675-3p expression levels were significantly lower in the non-TRS than in the control group (0.79 ± 0.46 vs. 1.00 ± 0.33, respectively, $p < 0.001$, Figure 3B). In addition, hsa-miR-4665-5p expression levels were significantly higher in the non-TRS than in the control group (1.29 ± 1.52 vs. 1.00 ± 0.72, respectively, $p = 0.039$). No significant differences in hsa-miR-939-3p ($p = 0.469$), hsa-miR-4730 ($p = 0.989$), or hsa-miR-6131 ($p = 0.457$) expression levels were observed. In terms of the correlations between miRNA expression levels and clinical parameters in the non-TRS group, no changes were associated with covariates except for age ($r = -0.301$, $p = 0.033$) and PANSS negative score ($r = 0.302$, $p = 0.033$) in hsa-miR-6131, as shown in Supplementary Table 3.

Target gene prediction of miRNA and GO analysis

Based on the 18 downregulated miRNAs, 251 genes were listed as predicted target genes under the stringent criteria mentioned earlier (Supplementary Table 4).

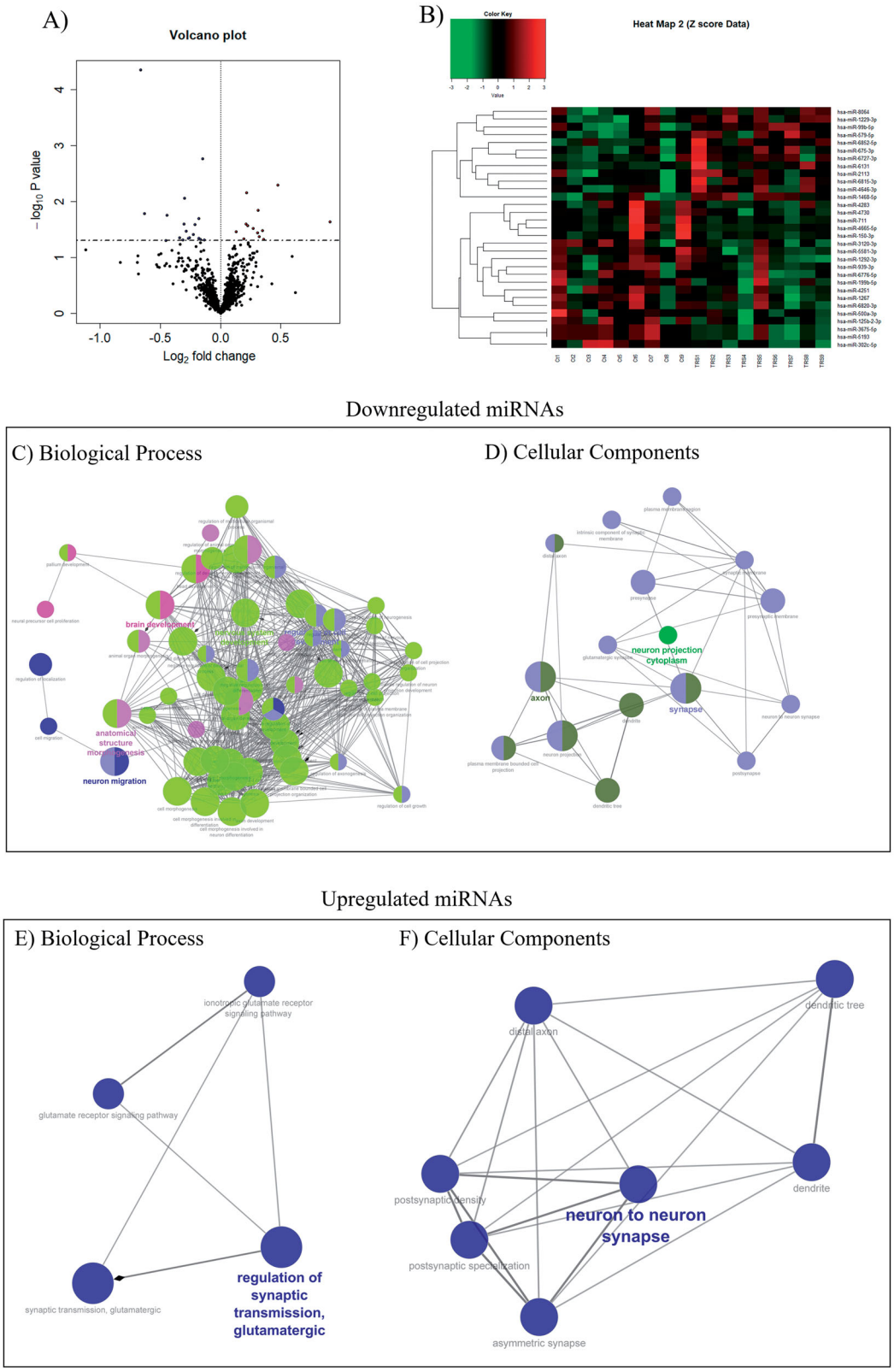

Figure 2. Volcano plot and heatmap from microarray data, and the results of gene ontology analysis from miRNAs with significant changes. (A) A total of 1,230 miRNAs meeting the criteria in the microarray data were plotted in a volcano plot. The y-axis corresponds to the significance level represented with the $\log_{10}$P value, and the x-axis displays the $\log_2$ (FC) value. The red and blue dots represent the significantly up- and downregulated miRNAs in the patients with TRS, respectively. (B) High-expression miRNAs are shown in red on the map; low-expression genes are shown in green. The 13 up- and 18 downregulated miRNAs were used to create the heatmap. The network was constructed by neuronal function related to the BP (C) and CC (D) terms based on the predicted target genes from the 13 upregulated miRNAs and the BP (E) and CC (F) terms based on the predicted target genes from the 18 downregulated miRNAs by using ClueGo in the Cytoscape program. hsa: *Homo sapiens*; miRNA: microRNA; TRS: treatment-resistant schizophrenia; Ct: control subjects.

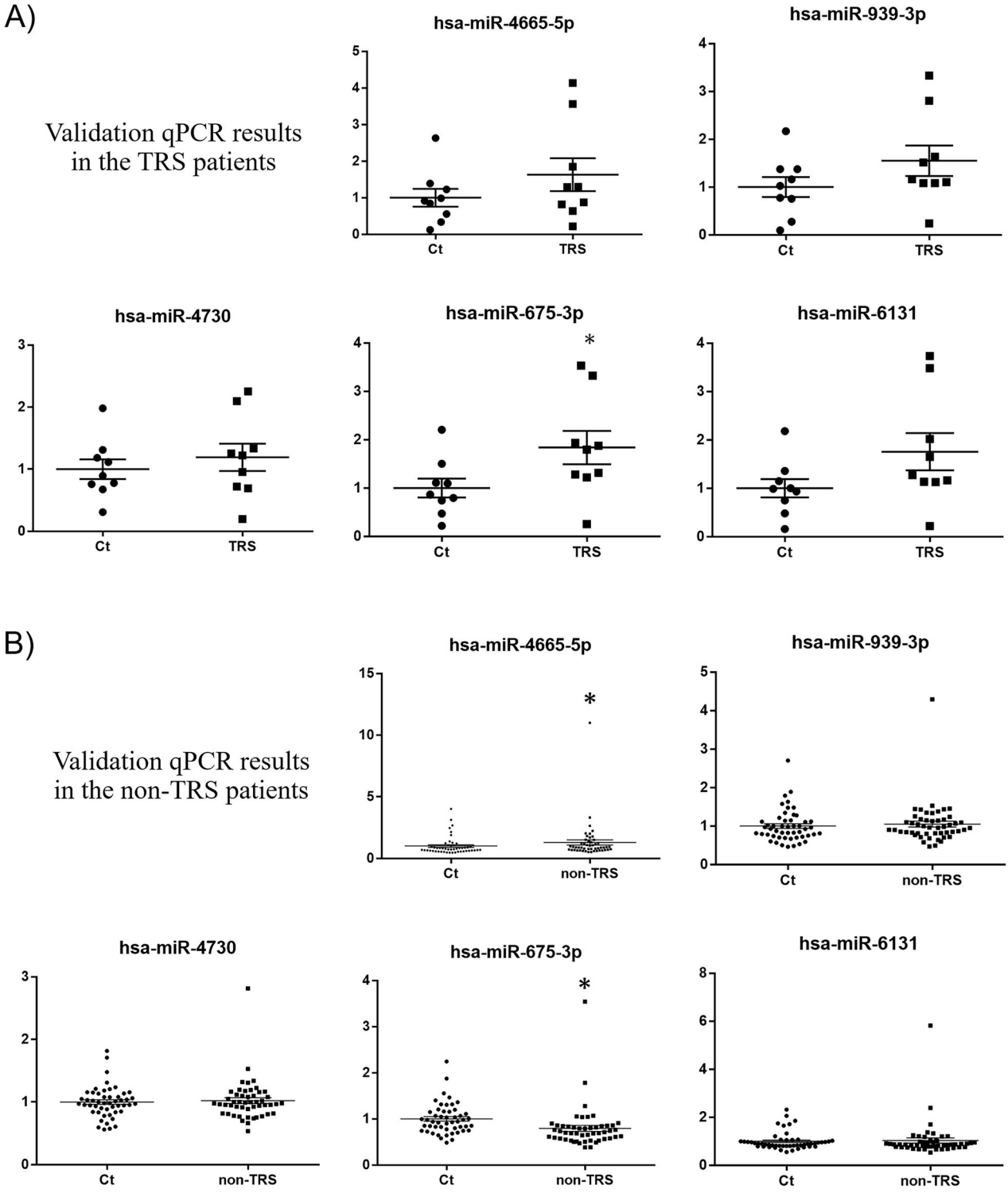

Figure 3. Validation qPCR results in the TRS and non-TRS groups. The y-axis represents the ratio of the relative expression value compared with control subjects in regard to the five miRNAs selected from the microarray data. qPCR was performed for (A) TRS ($n = 9$, each) and (B) non-TRS ($n = 50$, each). We used hsa-miR-4507 as the internal standard and statistical significance was defined at $p = 0.05$ (Mann-Whitney *U* test). hsa: *Homo sapiens*; miRNA, microRNA; TRS: treatment-resistant schizophrenia; Ct: control subjects. $^{*}p < 0.05$.

After subjecting all 251 genes to GO analysis, 146 BP terms reached a significant level (Supplementary Table 5). Of these BP terms, neuron- and brain development-related terms were abundant and had functionally interacted with each other (Figure 2C). In addition, the results of the CC terms indicated that neuron- and

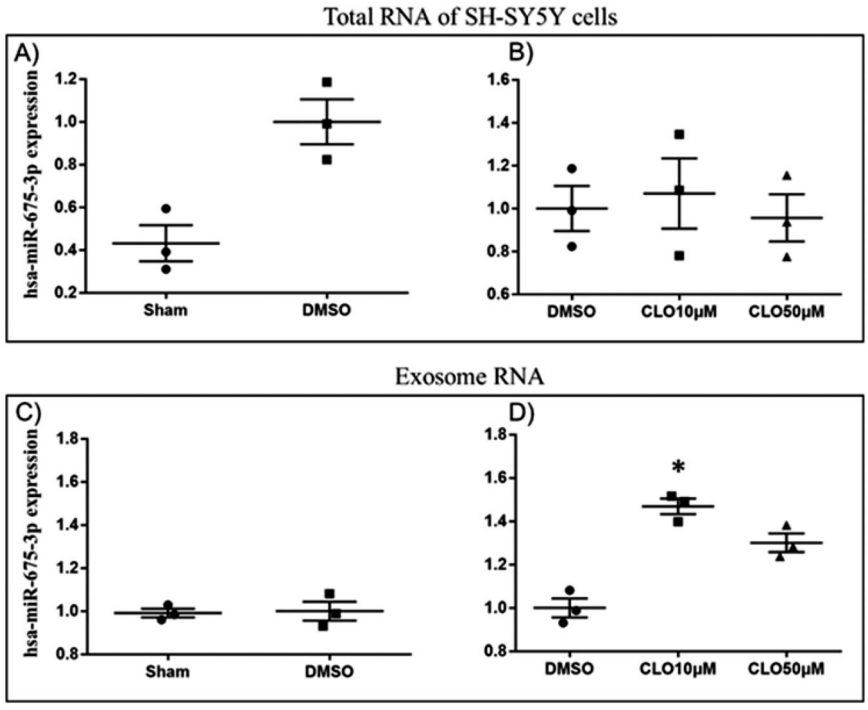

Figure 4. Validation of clozapine treatment on miR-675-3p expression using an in vitro cell model. The y-axis represents the ratio of the relative expression value of hsa-miR-675-3p. For the total RNA of SH-SY5Y cells, relative expressions were measured by qPCR to investigate the effects of (A) DMSO and (B) clozapine treatment. For EXO RNA, relative expressions were measured by qPCR to investigate the effects of (A) DMSO and (B) clozapine treatment. 3 samples in each group were analysed. U6 was used as the internal standard (A and B). Hsa-miR-4507 was used as the internal standard (C and D). Statistical significance was each defined at $p = 0.05$ (Mann-Whitney *U* test or Kruskal-Wallis test with Bonferroni corrections). CLO: clozapine; hsa: Homo sapiens; miR: microRNA. $^{*}p < 0.05$: significant change compared with DMSO.

synapse-related CC terms had functionally interacted with each other (Figure 2D).

Regarding the 13 upregulated miRNAs, four genes (ATXN1, YTHDF2, SRSF2, and TME123) were predicted as target genes (from miR-675-3p only). To assess the function of the 13 upregulated miRNAs by GO analysis, we set the following target gene criterion: those included more than six databases within all seven selected databases. Next, 177 genes were predicted as target genes (Supplementary Table 6) and subjected to GO analysis. Interestingly, nine BP terms reached a significant level (Supplementary Table 7), four of which were associated with glutamate signalling (Figure 2E). When considering all 11 CC terms (Supplementary Table 7), neuron- and synapse-related CC terms had functionally interacted with each other (Figure 2F).

In vitro cell line-based study

In the analysis of SH-SY5Y cell lines, 3 samples in each group were analysed. In the analysis of SH-SY5Y RNA, hsa-miR-675-3p expression levels did not significantly change in the DMSO compared with the sham group ($p = 0.100$, Figure 4A). The averages among the DMSO, 10 μM, and 50 μM clozapine groups had not significantly changed ($p = 0.733$, Figure 4B). In terms of EXO miRNA expression, no significant change in miR-675-3p expression levels was found between the sham and DMSO groups ($p = 1.0$, Figure 4C). However, miR-675-3p expression levels were significantly changed after clozapine treatment ($p = 0.027$, comparison between groups; $p = 0.022$; DMSO vs. clozapine 10 μM, $p = 0.539$; DMSO vs. clozapine 50 μM), as shown in Figure 4D.

Discussion

This study investigated global miRNA expression levels in the plasma EXO of patients with TRS. Predicted target genes from 13 up- and 18 downregulated miRNAs were found to be relevant to neuronal and brain development. Among these, upregulated miR-675-3p expression was successfully validated in the same cohort (9 control subjects vs. 9 patients with TRS) by qPCR analysis. Conversely, miR-675-3p expression levels were significantly decreased in the non-TRS group (50 control vs. 50 non-TRS subjects). The results of an

in vitro study using SH-SY5Y cells revealed that clozapine treatment induced the upregulation of miR-675-3p in EXO.

Although neuron-derived EXO in plasma may be more suitable to assess the expressional changes of miRNAs related to CNS function because EXO is secreted from several types of cells (Saeedi et al. 2019), several studies have shown that miRNA expression in plasma EXO is changed in a number of neuropsychiatric diseases, including Alzheimer's disease, Parkinson's disease, and SCZ (Du et al. 2019; Xia et al. 2019; Nie et al. 2020). When considering that EXO can cross the blood-brain barrier (Andjus et al. 2020), the expressional change of miRNAs in plasma EXO may reflect the pathophysiology of neuropsychiatric diseases. Indeed, the in silico analysis performed in this study revealed that the 13 up- and 18 downregulated miRNAs were related to neuronal functions. Despite numerous reports showing expressional changes of miRNAs in the peripheral blood of patients with SCZ (Roy et al. 2020), only one investigated miRNA expression changes in blood EXO. Du et al.(Du et al. 2019) investigated global expression changes of miRNA contained in serum EXO in SCZ by miRNA sequencing and validated significantly changed miRNAs among 100 control subjects, 57 patients with first-episode SCZ (drug-free), and 43 medicated patients with SCZ in qPCR experiments. One of the conclusions of their study was that hsa-miR-206 expression was upregulated in first-episode SCZ (drug-free) and decreased in medicated SCZ. None of the significantly changed miRNAs overlapped between their study and the present study. The different types of samples (serum and plasma) and demographic backgrounds (e.g. antipsychotic type, race, duration of illness) may have contributed to these inconsistencies. In addition, these inconsistencies may be due to the different techniques of collecting EXO. Because of heterogeneity and low productivity of EXO, it is difficult to compare results with each other studies. Ultracentrifugation method is the most typical approach for EXO isolation and can be collected without containing a reagent in an EXO preparation kit.

The upregulation of hsa-miR-675-3p in the microarray data was successfully replicated in the same samples of patients with TRS by qPCR. Conversely, hsa-miR-675-3p expression was downregulated in the non-TRS group. First, we hypothesised that this opposite result might reflect the different pathophysiologies of TRS and non-TRS because patients with TRS generally show more severe symptoms (Howes et al. 2017), and miRNAs clusters contained in serum EXO have been reported to be correlated with SCZ symptoms (Du et al. 2019); however, hsa-miR-675-3p expression was not correlated with any symptoms in this study. Second, we hypothesised that hsa-miR-675-3p expression was affected by clozapine treatment, which is more effective for TRS compared with other atypical antipsychotics (Nucifora et al. 2017). Actually, in a cell line study, hsa-miR-675-3p upregulation was induced by clozapine treatment in EXO RNA, but not in the total RNA of SH-SY5Y. Amoah et al. (Amoah et al. 2020) reported that antipsychotics (haloperidol and olanzapine) changed the expression of miR-223 in neuronal and astrocytic EXO based on in vivo and in vitro studies. At the functional level of miR-675-3p, two previous studies in the field of cancer reported that miR-675-3p may regulate apoptosis through the regulation of immune-related genes (Li et al. 2020; Shen et al. 2020). Indeed, the predicted target genes of miR-675-3p (e.g. PTRO, SRSF2, CPEB4) have been shown to be relevant to apoptosis (Liang et al. 2017; Shu et al. 2017; Zhu et al. 2020). In addition, MEF2C was predicted as one of the target genes of miR-675-3p. MEF2C, one of the transcription factors in the MEF2 family, has been reported to be a risk gene for SCZ (Harrington et al. 2016), and functional deficits have been found to be associated with cognitive function through epigenetic modification (Mitchell et al. 2018). In addition, MEF2C is relevant to the inflammatory response (Deczkowska et al. 2017) and neuroinflammation (Harrington et al. 2020). When considering the mechanism underlying the effects of clozapine and adverse effects related to immune reactions (Roge et al. 2012), there may be no contradiction in terms of changing miR-675-3p expression levels by clozapine treatment. The H19/IGF2 imprinted domain on chromosome 11 contains miR-675 matured from the H19 transcript expressed from the maternal chromosome (Girardot et al. 2012). Because hsa-miR-675-3p is located in this imprinted region, this miRNA might be associated with parent of origin (Gardiner et al. 2012). However, as far as we know, precise abnormal imprinting mechanisms of this miRNA remain unknown.

This study had several limitations. First, the number of samples in the microarray study was small ($n = 9$ in each group). The inconsistent results between the microarray and validation studies may have been due to a type I error. However, the backgrounds of the two groups, including disease severity and medication history, were different. These inconsistent results also could be a problem due to the lack of the best suitable internal standard in the EXO study. However, the best internal standard in EXO remains unknown in

previous studies. This also could be a limitation in the qPCR analysis because there were no miRNAs in microarray analysis that were significantly changed after multiple comparisons. Further research is needed to reveal the relationship between EXO and SCZ. Second, we isolated plasma EXO derived from various cell types. Because the pathogenesis of SCZ is considered to originate from the central nervous system, neuron-derived EXO should be analysed in future studies. It should be noted that changes in miRNA expression in brain EXO have been already reported (Banigan et al. 2013; Amoah et al. 2020). The connectivity between brain and blood EXO should be explored in a future study by using neuron-derived EXO or brain and blood EXO together.

Conclusions

In conclusion, the results of this study revealed that global miRNA changes in plasma EXO in patients with TRS are relevant to their neuronal functions. Among these changes, hsa-miR-675-3p expression was regulated by clozapine treatment. These findings suggest a new approach to understanding the pathogenesis of SCZ and the effects of clozapine on miRNA expression using plasma EXO.

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Disclosure statement

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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