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miR-19b is elevated in peripheral blood of schizophrenic patients and attenuates proliferation of hippocampal neural progenitor cells

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Abstract

MicroRNAs (miRNAs) have been investigated in neurodevelopmental and psychiatric disorders including schizophrenia (SZ). Previous studies showed miRNAs dysregulation in postmortem brain tissues and peripheral blood of SZ patients. These suggest that miRNAs may play a role in the pathophysiology of SZ and be a potential biomarker of SZ. Previous studies also showed that miRNAs regulated neurogenesis and that neurogenesis was involved in the pathophysiology of SZ. In addition, a recent study showed that miR-19a and 19b, enriched in neural progenitor cells (NPC) in adult hippocampus, were increased in human NPC derived from induced pluripotent stem cell derived from SZ patients. However, it remains unclear whether the levels of miR-19a and 19b are altered in peripheral blood of SZ patients and how miR-19a and 19b affects neurogenesis. To elucidate them, first we examined the levels of miR-19a and 19b in peripheral blood of SZ patients with quantitative RT-PCR and showed that the level of miR-19b, but not miR-19a, was significantly higher (miR-19a: p=0.5733, miR-19b: p=0.0038) in peripheral blood of SZ patients (N=22) than that of healthy controls (N=19). Next, we examined the involvement of miR-19b in proliferation and survival of mouse neonatal mice hippocampus-derived NPC with BrdU assay and TUNEL assay. The silencing of miR-19b significantly increased proliferation (N=5, p=0.0139), but not survival (N=5, p=0.9571), of neonatal mice hippocampus-derived NPC. These results suggest that the level of miR-19b in peripheral blood is a potential biomarker of schizophrenia and that the higher level of miR-19b may increase the vulnerability of SZ via attenuating proliferation of hippocampal NPC.

Keywords: schizophrenia, miRNA, miR-19b, neurogenesis, hippocampus

Introduction

Schizophrenia (SZ) is a debilitating disorder, which is characterized by positive (psychotic) symptoms and negative symptoms, as well as increasingly cognitive deficits (Lewis and Lieberman, 2000). The dysregulation of neurodevelopment is considered to play a role in the pathophysiology of SZ (Harrison, 1997). Recent studies have shown alterations in the expression of many genes in SZ patients (Gavin and Akbarian, 2012; Wu et al, 2012; Gardiner et al, 2013). These suggest that the dysregulation of gene expression at the transcriptional or post-transcriptional level may be involved in the pathophysiology of SZ.

MicroRNAs (miRNAs) are non-coding RNAs of an average length of 22 nucleotides that pairing to mRNAs of protein-coding genes by binding to their 3'UTR sequence to direct their posttranscriptional repression (Bartel, 2009). miRNAs regulate multiple biological pathways and are involved in neurogenesis, neuronal maturation and brain development (Kapsimali et al, 2007). The dysfunction of miRNA signaling contributes to neurodevelopment disorders such as fragile X and Rett syndromes, as well as complex behavioral disorders including SZ, drug addiction, and depression (Im and Kenny, 2012; Beveridge and Cairns, 2012). In addition, postmortem brain studies reported that altered miRNA biogenesis and expression in dorsolateral prefrontal cortex, hippocampus, and superior temporal gyrus is associated with SZ (Beveridge et al, 2010; Santarelli et al, 2011, Beveridge and Cairns, 2012). These suggest that miRNAs may be involved in the pathophysiology of SZ and that SZ-associated miRNAs are potential biomarkers for SZ (Schwarz and Bahn, 2008). Because brain tissue is not easily accessible for human examination, peripheral blood is usually used to search potential miRNA biomarkers for SZ (Sullivan et al, 2006). As miRNA molecules are highly stable (Mraz, 2009) and the levels of several miRNAs between peripheral blood and brain tissue are parallelly changed (Sullivan et al, 2006), it is expected that some abnormality of miRNA expressions in brain tissue may be detectable in peripheral blood (Gladkevich et al, 2004; Marques-Deak et al, 2005). In fact, SZ-associated alternations of miRNA expressions have been shown in human peripheral blood (He et al, 2018). However, it remains unclear whether such miRNAs functionally work in the pathophysiology of SZ.

A lot of miRNAs are coded in polycistronic miRNA clusters on genomic DNA. Multiple miRNAs, belonging to a particular polycistronic miRNA cluster, were transcribed as a single RNA from genomic DNA (Concepcion et al, 2012). In such polycistronic miRNA clusters, miR17-92 cluster is well characterized. Its partial deletion results in Feingold syndrome-2, which causes mild intellectual disability and psychiatric symptoms (Ganjavi et al, 2014) and its partial duplication results in autism spectrum disorder (Hemmat et al, 2014). In addition, miR17-92 cluster mediates the proliferation and survival of neural progenitor cells (Liu et al, 2013). These suggest that miR17-92 cluster may be associated with psychiatric disorders. In miR17-92 cluster-included miRNAs, Han et al (2016) showed that miR-19a and miR-19b, which are enriched in neural progenitor cells (NPC) in adult hippocampus, are increased in human NPC derived from induced pluripotent stem cell (iPSC) derived from SZ patients than the healthy control. In addition, past

studies showed the possibility that hippocampal neurogenesis is involved in the pathophysiology of SZ (Kusumi et al, 2015). These suggest that miR-19a and miR-19b may functionally work in the pathophysiology of SZ. However, it remains unclear whether the expressions of miR-19a and miR-19b are altered in peripheral blood of SZ patients. Although Han et al showed that miR-19a and miR-19b may be involved in neural differentiation and migration of NPC. it remains unclear whether miR-19a and miR-19b are involved in NPC proliferation and survival.

Here, we examined the levels of miR-19a and miR-19b in peripheral blood of SZ patients and showed that the level of miR-19b, but not miR-19a, was higher in peripheral blood of SZ patients than the healthy controls. Moreover, we examined the involvement of miR-19b in NPC proliferation and survival with the culture system of neonatal mice hippocampus-derived NPC.

Materials and Methods

Ethical statements

This study was conducted in accordance with the latest version of the declaration of Helsinki. The clinical part of this study was conducted with the approval of the ethical committee for genetic studies of Kobe University Graduate School of Medicine. The informed consent of all participants was obtained after the details of the procedures of the clinical part of this study had been fully explained. The basic part of this study was performed in strict accordance with the guidelines for animal experiments of Kobe University Graduate School of Medicine. The protocols of this study were approved by the animal care and use committees of Kobe University Graduate School of Medicine. All efforts were done to minimize suffering of mice.

Subjects

All subjects were Japanese bloodline and recruited in the suburbs of Kobe city, Japan. All of SZ subjects were assessed with the DSM-5 criteria for SZ by two psychiatrists. The healthy volunteers collaborated as the control subjects. The presence or absence of psychiatric disorders in all of the control subjects was assessed with an unstructured interview performed by a psychiatrist. All of the control subjects have no present, past or family (first-degree relatives) histories of psychiatric disorders, neuroleptic medication and substance abuse outside of nicotine. The subjects of this study consisted of 22 SZ patients and 19 controls. The antipsychotic dose was estimated at blood examination and converted to chlorpromazine equivalents. The status of SZ was estimated with global assessment of functioning (GAF) and brief psychiatric rating scale (BPRS) at blood examination. The demographic and clinical characteristics are shown in Table 1.

RNA extraction and quantitative RT-PCR

Whole-blood samples derived from the subjects were stocked in PAXgene RNA tubes (BD Biosciences, Franklin Lakes, NJ, USA). Total RNA was isolated from the stocked whole-blood samples in PAXgene RNA tubes with miRCURY RNA Isolation Kit – Biofluid (Qiagen, Hilden, Germany). Single-strand cDNA was synthesized from the isolated total RNA with miRCURY LNA Universal RT microRNA PCR Universal cDNA Synthesis Kit ***II*** (Qiagen). The PCR reaction was

performed with triplicate using miRCURY LNA Universal RT microRNA PCR ExiLENT SYBR Green Master Mix (Qiagen) in ABI 7500 Real Time PCR system (Applied Biosystems) as follows; 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 15 sec and 60 ℃ for 60 sec. miRCURY LNA UniRT PCR primer mix has-miR-19a and 19b (Qiagen) were used as the primers for miR-19a and miR-19b. miRCURY LNA UniRT primer mix has-miR-93 (Qiagen) was used as an endogenous control. The data were analyzed using the 7500 software v2.3 (Applied Biosystems).

Culture of neonatal mice hippocampus-derived NPC

The culture of NPC was performed as our past study (Boku et al, 2018). In Brief, the hippocampus was isolated from neonatal C57BL/6J mice. The isolated hippocampus was dissociated by pipetting. The dissociated cells were suspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12; Thermo Fishers, Waltham, MA, USA) supplemented with Penicillin/Streptomycin (PS, Thermo Fishers), B27 (Thermo Fishers), N2 (Thermo Fishers) and EGF (R&D systems, Minneapolis, MN, USA). The suspended cells were cultured in a 10% CO_2 incubator at 37℃. Every 5-7 days, the well-grown neurospheres were dissociated by pippeting and suspended in DMEM/F12 supplemented with PS, B27, N2 and EGF.

Transfection of LNA inhibitor for miR-19b, Proliferation assay and Survival Assay

miRCURY LNA miRNA inhibitor for miR-19b (Qiagen) was used for silencing of miR-19b. 2×10^5 cells/well were seeded into six-well plates in DMEM/F12 supplemented with PS, B27, N2 and EGF. After overnight incubation, miRCURY LNA miRNA inhibitor for miR-19b was transfected using Lipofectamine RNAi MAX (Thermo Fisher). miRCURY LNA miRNA inhibitor control (Qiagen) was used as a transfection control. miRCURY LNA inhibitor Negative Control A (Qiagen) was used as a negative control.

In the case of proliferation assay, 1 mM bromodeoxyuridine (BrdU, BD Biosciences) was administrated to cells after 24 hours of transfection. After 30 minutes of BrdU administration, BrdU staining was performed with BrdU Flow Kit (BD Biosciences) as described in the manufacturer's manual. Fluorescent signals were detected with Accuri C6 Plus flowcytometer (BD Biosciences). Data were analyzed with the software attached to Accuri C6 Plus flowcytometer.

In the case of survival assay, apoptosis was induced with 1 μM staurosporine (Sigma, St. Louis, MO, USA) after 24 hours of transfection. After 24 hours of staurosporine administration, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with Apo-Direct Kit (BD Biosciences) as described in the manufacturer's manual. Fluorescent signals were detected with Accuri C6 Plus flowcytometer (BD Biosciences). Data were analyzed with the software attached to Accuri C6 Plus flowcytometer.

Statistical analysis

χ^2-test and Students' t-test for the difference between two groups were performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Multiple linear regression analyses for the relationship of the level of miR-19b (a response variable) and explanatory variables, such as sex, age, onset age, phenotype, antipsychotic dose and BPRS score (total, positive symptoms, and

negative symptoms) were performed with JMP 14 (SAS Institute, Cary, NC, USA). In multiple linear regression analysis, dummy variables were used (sex, male=1 and female=2; phenotype, control=1 and SZ=2). Receiver operating characteristics (ROC) curve analysis was also performed with JMP 14. Significance was defined as $p<0.05$. The data are expressed as the means ± SEMs.

Results

The levels of miR-19a and miR-19b in peripheral blood

The levels of miR-19a and miR-19b in in peripheral blood were examined with quantitative RT-PCR and compared between 22 SZ patients (10 males and 12 females) and 19 control subjects (9 males and 10 females). There was no significant difference in the level of miR-19a between the control group and SZ group (Control: 1.000 ± 0.1053, SZ: 0.9402 ± 0.03670, p=0.5733, Figure 1A). On the other hand, the level of miR-19b was significantly higher in SZ group than the control group (Control: 1.000 ± 0.06859, SZ: 1.315 ± 0.07430, p=0.0038, Figure 1A). In addition, multiple linear regression analysis was performed to examine the relationship between the level of miR-19b (a response variable) and explanatory variables such as sex, age and phenotype in participants (Table 2). In addition, another multiple linear regression analysis was performed to examine the relationship between the level of miR-19b (a response variable) and explanatory variables such as sex, age, onset age, antipsychotic dose and BPRS score (total, positive symptoms and negative symptoms) in SZ patients (Table 3). These multiple linear regression analysis showed that only phenotype was significantly correlated with the level of miR-19b in participants (Table 2), and that antipsychotic dose and BPRS score were not significantly correlated with the level of miR-19b in SZ patients (Table 3).

Next, ROC curve analysis was performed to estimate the diagnostic power of miR-19b for SZ. The area under the curve (AUC) was 0.7751 (95% confidence interval: 0.6259-0.9244, $p < 0.01$) with sensitivity of 81.8% and specificity of 73.7% (Figure 1B). Therefore, the level of miR-19b in peripheral blood is considered to have the moderate diagnostic power for SZ.

The effects of silencing of miR-19b on NPC proliferation and survival

Silencing of miR-19b was performed with miRCURY LNA miRNA inhibitor for miR-19b, which almost completely silenced the expression of miR-19b (Supplementary Figure 1). Subsequently, BrdU assay and TUNEL assay were performed to investigate the roles of miR-19b on NPC proliferation and survival, respectively. Silencing of miR-19b significantly increased the ratio of S-phase cells in BrdU assay (N=5, Control: 6.380 ± 1.048, miR-19b SI: 10.46 ± 0.7698, p=0.0139, Figure 2), but not the ratio of TUNEL-positive cells in TUNEL assay (N=5, Control: 30.04 ± 2.560, miR-19b SI: 29.82 ± 3.021, p=0.9571, Figure 3). These results suggest that miR-19b may negatively affect NPC proliferation and not be involved in NPC survival.

Discussion

Based on a past study (Han, 2016), we hypothesized that miR-19a and/or miR-19b have

possibilities to be a biomarker of SZ. To test this hypothesis, we compared the levels of miR-19a and miR-19b in peripheral blood between SZ group and the control group, and showed that the level of miR-19b, but not miR-19a, was higher in SZ group than the control group. In addition, the level of miR-19b was related with phenotype, but not age, sex, antipsychotic dose and BPRS score. These results suggest that miR-19b has a possibility as a trait marker, rather than a state marker, of SZ. In addition, ROC curve analysis showed that miR-19b has the moderate diagnostic power for SZ. Therefore, miR-19b is expected as a potential biomarker for SZ.

Several past studies examined the association of the levels of miRNAs in peripheral blood with SZ and the diagnostic power of miRNAs for SZ (Lai et al, 2011; Fan et al, 2015; Sun et al, 2015; Yu et al, 2015; Lai et al, 2016; Wu et al, 2016; Liu et al, 2017a; Liu et al, 2017b; Ma et al, 2018). In many cases, single miRNA has insufficient diagnostic power of SZ. On the other hand, some past studies showed that the combinations of multiple miRNAs remarkably improved the diagnostic power of SZ（Fan et al, 2015; Liu et al, 2017a; Ma et al, 2018）. In addition, Liu et al (2017b) showed that the combination of single miRNA and mRNA of its target gene also improved the diagnostic power of SZ. Although our current results showed that miR-19b has moderate diagnostic power of SZ, it is not still enough in terms of usefulness. Therefore, to develop useful biomarkers for SZ, it is necessary to examine the combinations of multiple miRNAs and/or those of single RNA and its target genes.

We also examined the involvement of miR-19b in NPC proliferation and survival with neonatal mice hippocampus-derived NPC. Neurogenesis consists of four phenomena, such as proliferation, differentiation, survival and migration. In these phenomena, Han et al (2016) focused on only differentiation and migration. Therefore, we focused on proliferation and survival in this study, and showed that miR-19b may negatively affect NPC proliferation. This result suggests that the proliferation of NPC may be decreased in SZ patients compared with healthy controls. Actually, a human postmortem study showed that Ki-67, a proliferative marker of neurogenesis, was decreased in the hippocampus of SZ patients compared with that of healthy patients (Reif et al, 2006). Therefore, the increase of miR-19b in SZ patients may be involved in the decrease of the proliferation of NPC in the hippocampus of SZ patients, which suggests that oligonucleotide therapeutics to inhibit miRNA-19b are expected to lead to the development of new therapeutics for SZ. To test its possibility, further investigations with in vivo model and human samples are required.

Phosphatase and tensin homolog (PTEN), a tumor suppressor, is a well-known target of miR-19b, which inhibits PTEN expression (Jia et al, 2013; Tian et al, 2013). However, PTEN deletion enhances proliferation of hippocampal neural progenitor cells (Amiri et al, 2012). Therefore, miR-19b may not decrease NPC proliferation via inhibition of PTEN expression. On the other hand, miR-19b decreased cell proliferation via inhibition of fibroblast growth factor (FGF) receptor 2 (Yin et al, 2012). Vertebrates have 22 members of FGF family, which mediate cell proliferation (Ornitz and Itoh, 2001). In FGF family, FGF2 is well known to promote proliferation of neural progenitor cells (Parmer et al, 1995). We previously showed that FGF2 increased proliferation of neural

progenitor-like cells via activating GSK3β/β-catenin pathway (Boku et al, 2013), which is a main pathway to regulate proliferation of neural progenitor cells (Boku et al, 2009). These studies suggest that miR-19b might decrease NPC proliferation via inhibiting FGF2/GSK3β/β-catenin pathway. Elucidating how miR-19b decrease NPC proliferation is expected to lead to the further understanding of the pathophysiology of SZ and the development of new therapeutics for SZ.

There are several limitations in this study. First, the number of subjects in this study is limited and not enough to clarify whether miR-19b can be a biomarker of SZ as a practical manner. Therefore, as a next step, further large-scale study is necessary to clarify the possibility of miR-19b as a biomarker of SZ. Next, most of SZ subjects in this study were in chronic stage and continued medication for a long time. Therefore, it is difficult to exclude the effects of disease duration and antipsychotics on the level of miR-19b completely, despite of the results of multiple linear regression analysis (Table 3). Therefore, as a next step, it is necessary to examine the level of miR-19b in the drug-free SZ subjects in acute stage to exclude the effects of disease duration and antipsychotics on the level of miR-19b as far as possible.

Here we showed that the level of miR-19b in peripheral blood was elevated in SZ patients. The elevated level of miR-19b was related with phenotype, but not other factors including antipsychotic dose and clinical scores of SZ, and has the moderate diagnostic power of SZ. In addition, we also showed that miR-19b attenuates the proliferation of hippocampal NPC. Although a lot of miRNAs have been shown to be associated with SZ (He et al, 2018), it remains unclear whether such miRNAs functionally work in the pathophysiology of SZ. Therefore, miR-19b is one of the few miRNAs which are shown to have both the possibility of biomarkers for SZ and the potential functional role in the pathophysiology of SZ. Estimating the availability of SZ as a biomarker of SZ and elucidating the functional role of miR-19b in the pathophysiology of SZ may lead to the development of the translational research for SZ.

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Disclosure statement

All the authors declare that they have no conflict of interest.

Author contributions

Conceived and designed the experiments: SB, SO, AH. Performed the experiments: TH, SB, SO, NY, TH. Analyzed the data: TH, SB, SO. Contributed regents/materials/analysis tools: TH, SB, SO, IO, RW, HS. Wrote the paper: TH, SB, SO, AH.

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Figure legends

Figure 1. The levels of miR-19a and miR-19b in peripheral blood

(A) There was no significant difference in the serum level of miR-19a between the control group and SZ group. On the other hand, the serum level of miR-19b was significantly higher in SZ group than the control group. Values are shown as the ratio of miR-19a vs hsa-miR-93. Data are shown as the means ± SEMs. Statistical significance is shown at $^{\#\#}p < 0.01$.

(B) ROC curve analysis of the diagnostic value of the serum level of miR-19b. AUC: area under curve.

Figure 2. The effects of silencing of miR-19b on NPC proliferation

(A) The typical charts of FACS analysis of BrdU assay. R1 is the area corresponding to S-phase cells. (B) Silencing of miR-19b significantly increased the ratio of S-phase cells. Values are shown as the ratio of S-phase cells. Data are shown as the means ± SEMs of five dependent cultures. Statistical significance is shown at $^{\#}p < 0.05$.

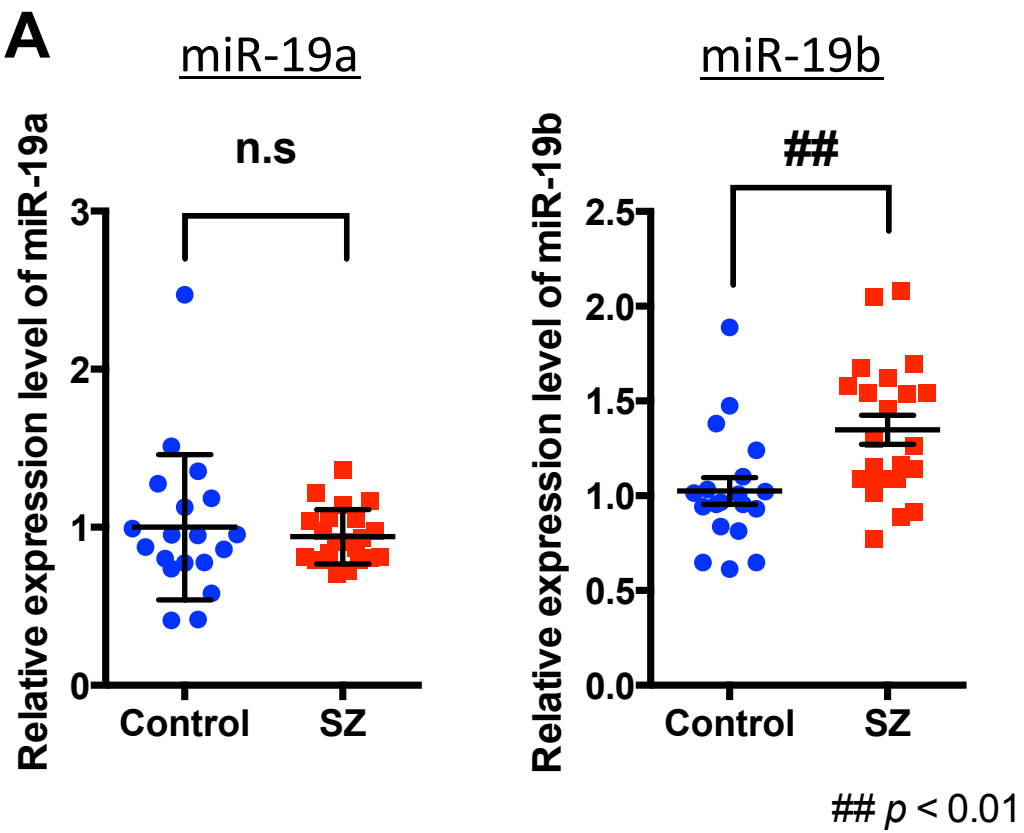
Figure 3. The effects of silencing of miR-19b on NPC proliferation

(A) The typical charts of FACS analysis of TUNEL assay. R1 is the area corresponding to TUNEL-positive cells. (B) Silencing of miR-19b had no significant effect on the ratio of TUNEL-positive cells. Values are shown as the ratio of TUNEL-positive cells. Data are shown as the means ± SEMs of five dependent cultures.

Supplementary Figure 1. The silencing effect of LNA inhibitor for miR-19b on the expression of miR-19b

miRCURY LNA inhibitor for miR-19b remarkably silenced the expression of miR-19b. Values are shown as the ratio of miR-19b vs miRCURY LNA inhibitor Negative Control A. Data are shown as the means ± SEMs of three dependent culture. Statistical significance is shown at $^{\#\#\#\#}p < 0.0001$.

Figure 1


A
miR-19a
n.s
Relative expression level of miR-19a
3
2
1
0
Control
SZ
miR-19b
##
Relative expression level of miR-19b
2.5
2.0
1.5
1.0
0.5
0.0
Control
SZ
p < 0.01

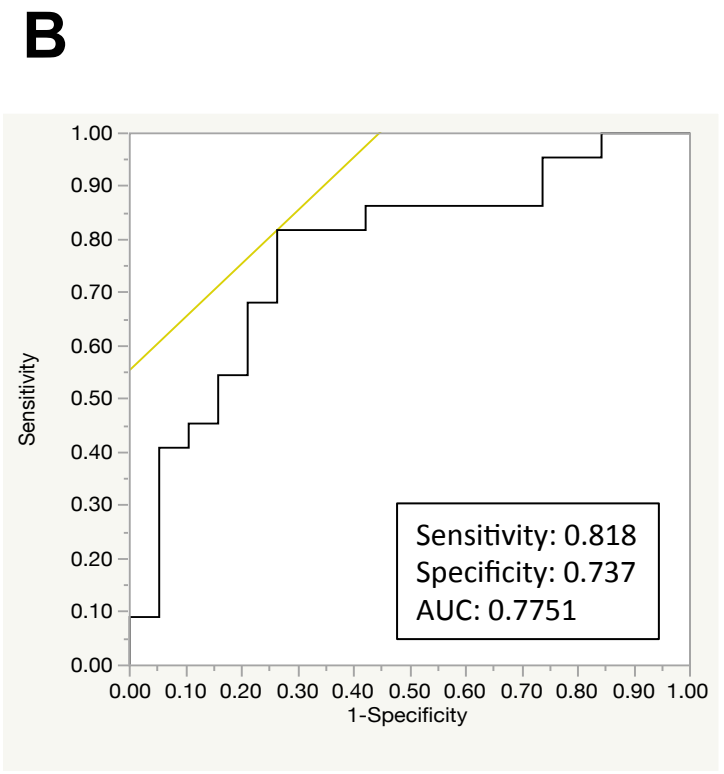
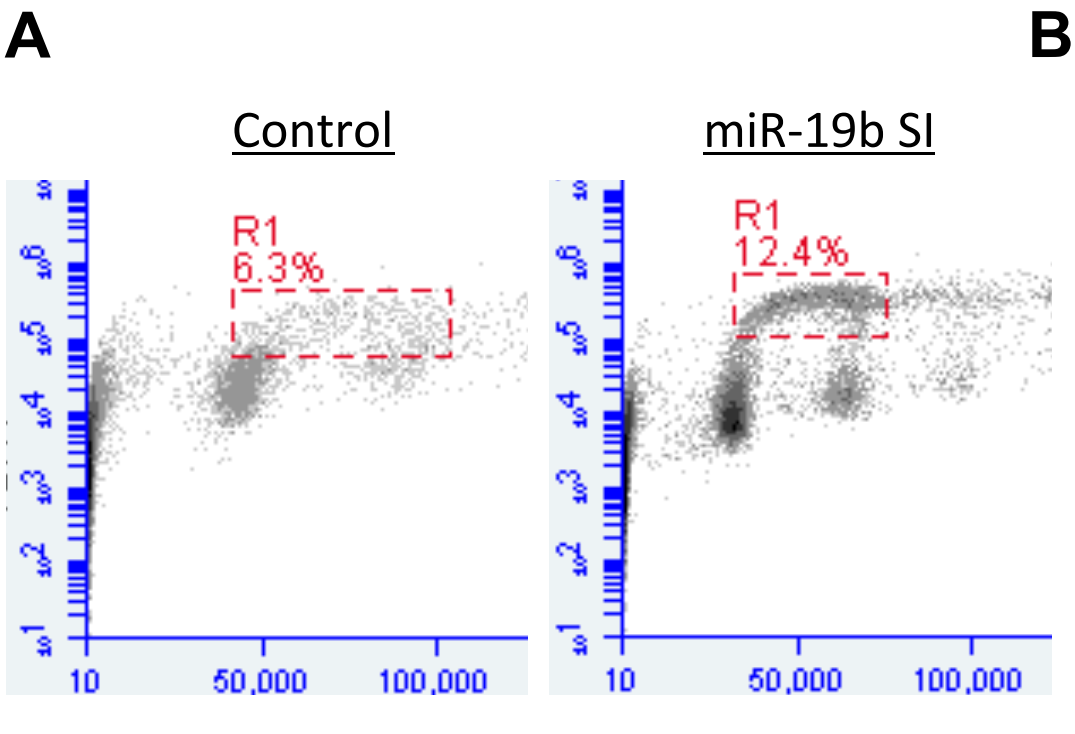

B
Sensitivity
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00
1-Specificity
Sensitivity: 0.818
Specificity: 0.737
AUC: 0.7751

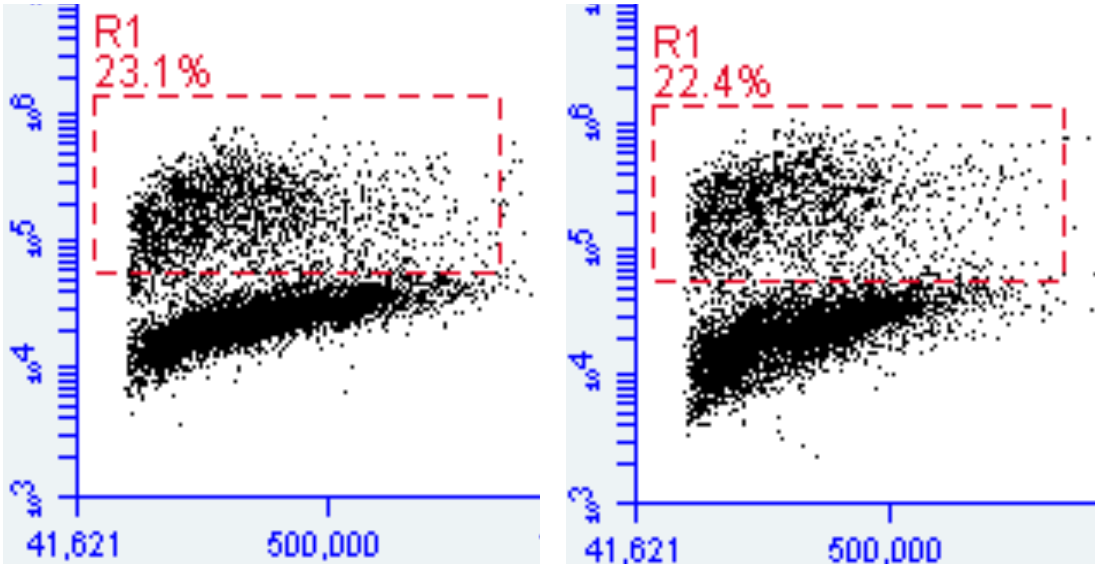
Figure 2



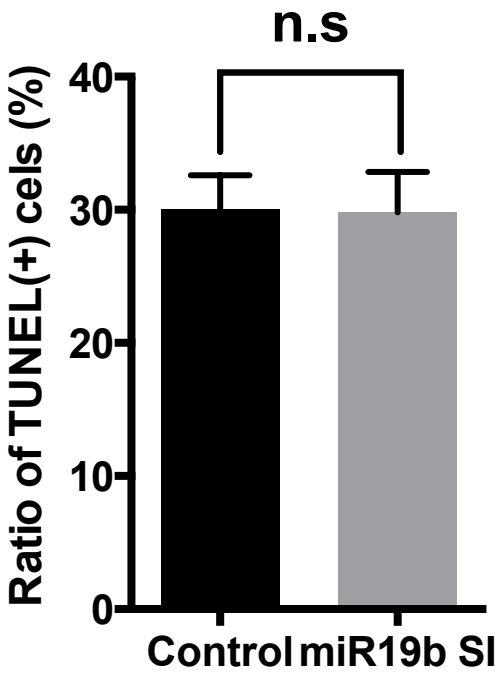
# $p < 0.05$

Figure 3

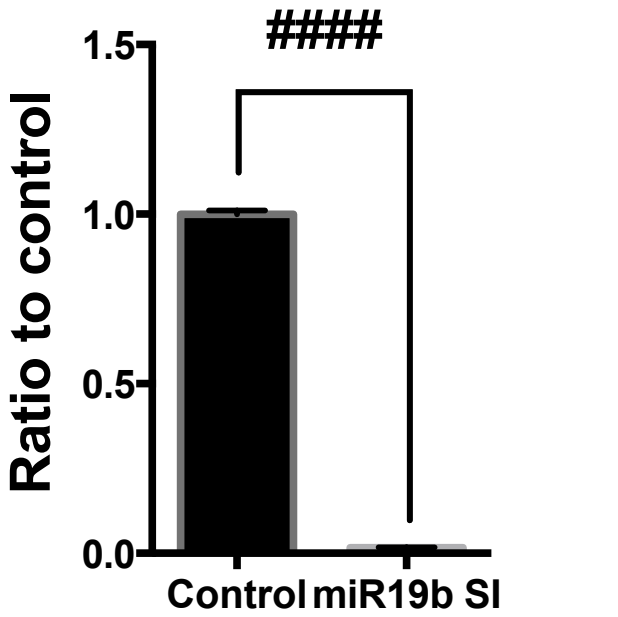
A


Control
miR-19b SI
R1
23.1%
R1
22.4%
41,621
500,000
41,621
500,000

B


n.s
Ratio of TUNEL(+) cels (%)
40
30
20
10
0
Control
miR19b SI

Supplementary Figure 1



$p < 0.0001$

Table 1

Demographic and clinical characteristics of participants

	Control (N = 19)	Schizophrenia (N = 22)	P-value
Sex (Male/female)	9/10	10/12	0.90[a]
Age (years)	38.0 ± 7.5	40.9 ± 13.3	0.49[b]
Onset Age	-	22.5 ± 8.32	-
Antipsychotic dose[c] (mg/day)	-	896.7 ± 642.4	-
GAF[d] score	-	39.5 ± 11.3	-
BPRS[e] total score	-	46.7 ± 12.8	-
BPRS[e] positive symptoms	-	18.7 ± 6.94	-
BPRS[e] negative symptoms	-	19.9 ± 5.29	-

[a] P-value was calculated using χ^2-test between the schizophrenia and control groups.

[b] P-value was calculated using Student's t-test between the schizophrenia and control groups.

[c] Antipsychotic doses were calculated with chlorpromazine equivalents.

[d] GAF, Global Assessment of Functioning

[e] BPRS, Brief Psychiatric Rating Scale

Table 2

Multiple linear regression analysis in the serum level of miR-19b in participants

Explanatory variable	Response variable (the serum level of miR-19b)			
	B[a]	SE[b]	t	P
Sex (Male:1, Female:2)	-0.016881	0.053545	-0.32	0.7543
Age	0.0025961	0.004968	0.52	0.6044
Phenotype (Control:1, Schizophrenia:2)	-0.156574	0.054159	-2.89	**0.0064**[c]

[a] B, the unstandardized partial regression coefficient.

[b] SE, standard error

[c] Boldface type indicates significance.

Table 3

Multiple linear regression analysis in the serum level of miR-19b in SZ patients

Explanatory variable	Response variable (the serum level of miR-19b)			
	B[a]	SE[b]	t	P
Sex (Male:1, Female:2)	0.019231	0.182809	0.11	0.9177
Age	0.001097	0.007768	0.14	0.8897
Onset age	0.003177	0.011096	0.29	0.7788
Antipsychotic dose[c] (mg/ml)	0.000136	0.000153	0.89	0.3898
BPRS[d] total score	-0.0075652	0.056160	-1.35	0.1994
BPRS[d] positive symptoms	0.059046	0.062976	0.94	0.3643
BPRS[d] negative symptoms	0.102129	0.071039	1.44	0.1725

[a] B, the unstandardized partial regression coefficient.

[b] SE, standard error

[c] Antipsychotic doses were calculated with chlorpromazine equivalents.

[d] BPRS, Brief Psychiatric Rating Scale