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Screening of schizophrenia associated miRNAs and the regulation of miR-320a-3p on integrin β1

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Abstract

Schizophrenia is one of the most severe chronic psychiatric disorders, which lacks of objective and effective diagnosis and observation indicators.

In this work, the serum miRNA profiles of schizophrenic patients were analyzed. Targets of abnormal miRNAs, and their regulatory mechanisms were studied. A miRNA array was used to analyze the serum from 3 schizophrenic patients without treatment, 3 clinically cured patients and 3 healthy controls. The findings from the array were confirmed by real-time PCR in a larger cohort, including 59 patients and 60 healthy controls. The candidate miRNAs were analyzed using bioinformatics tools. Their potential targets were studied through *in vitro* cellular experiments.

MiR-320a-3p and miR-320b were found to be down-regulated in patients compared with cured patients and controls in the miRNA array, which was also confirmed by real-time PCR in the larger cohort. Integrin β1 (ITG β1) was found to be one of the targets of miR-320a-3p. An enzyme-linked immune sorbent assay demonstrated that the ITG β1 concentration increased significantly in the patients' serum, and the *in vitro* study confirmed that miR-320a-3p targeted the 3′ UTR of ITG β1 mRNA and reduced its expression.

Our results demonstrated that the regulatory effect of miR-320a-3p on its target ITG β1 might play an important role in schizophrenia pathogenesis, which could be a potential pathway for schizophrenia diagnosis and therapy.

Abbreviations: ECM = extracellular matrix, ITG β1 = integrin β1, miRNAs = MicroRNAs, PNN = perineuronal net.

Keywords: integrin beta1, MIRNA-320a-3p, schizophrenia

1. Introduction

Schizophrenia is a serious, sustained and usually chronic mental illness, which has a major impact on the patient's behavior and emotions. Its incidence is increasing year by year.[1] Schizophrenia is a multifactorial disease, which is composed of a group of clinical symptoms and syndromes. Although studies into all aspects of schizophrenia have been conducted, the etiology and pathogenesis of schizophrenia are not yet clear. The diagnosis of schizophrenia is currently dependent on symptom-based criteria and lacks objective indicators.[2] The current difficulties in diagnosing and monitoring schizophrenia are major factors contributing to the severity and clinical burden of the disease.[3]

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MicroRNAs (miRNAs) are endogenous ~22-nt RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression.[4] Mitchell et al[5] found that miRNAs were present in human plasma in a remarkably stable form that was protected from endogenous RNase activity. Gilad et al[6] confirmed that changes in blood miRNAs could reflect the pathological physiology of the human body. Chen et al[7] found that the abundance of specific miRNAs in the plasma or serum could serve as a biomarker for cancer or other diseases. MiRNAs in plasma are readily available to be measured. Kroh et al[8] reported a procedure for RT-qPCR analysis of circulating miRNAs as biomarkers and discussed the relevant issues of sample preparation, experimental design, and data analysis, which has provided methods for our study of circulating miRNAs. Previous studies have indicated the possibility of circulating miRNAs being considered as biomarkers for different pathological conditions[9–11] Compared to conventional biomarkers, such as proteins and mRNAs, miRNAs are less complex, more stable in bodily fluids[5] and easier to measure.[12]

In recent years, research on miRNAs and schizophrenia has been increasing. Emerging evidence suggests that miRNAs are closely associated with schizophrenia[13,14] and could be novel markers for schizophrenia diagnosis.[15] miRNAs regulate cell signaling pathways via epigenetic mechanisms, thus affecting the function of brain cells.[16] This research might have important clinical value for schizophrenia. Some recent studies found that there were a variety of altered miRNAs in the peripheral blood of patients with schizophrenia. Among them, miR-34a,[17] miR-130-b[2] and miR-193-a-3p[2] were up-regulated, and miR-107,[18] miR-98,[19] miR-183[19] and miR-30a-5p[20] were down-regulated. Several significant correlations between miRNAs and mRNAs linked to schizophrenia and brain function have suggested

potential miRNA-mRNA interactions that may be associated with disease pathophysiology.[21] A meta-analysis including 6 studies reported that the measurement of blood-derived miRNA levels might be a promising objective method for schizophrenia diagnosis.[22] miRNAs might be involved in the regulation of multiple cell signaling pathways and may affect cellular physiological functioning, and thus may be involved in the onset of schizophrenia.

This study aimed to identify a potential serum miRNA signature for schizophrenia by comparing genome-wide miRNA expression profiles from treatment-naive patients with schizophrenia, clinically cured patients and healthy controls. Targets of abnormal miRNAs and their regulatory mechanisms were studied. The experiment demonstrated that miR-320a-3p and miRNA-320b in the peripheral blood of patients were down-regulated. We found that miR-320a-3p might negatively regulate the mRNA expression of integrin β1 (ITG β1) and thus affect its protein concentration. The signaling pathways regulated by miR-320a-3p may provide potential targets for the early diagnosis and treatment of schizophrenia.

2. Methods

2.1. Patients

In this study, we enrolled 59 patients (28 males, 31 females, mean age: 45.2 [range from 19 to 63] years) with schizophrenia who had not received treatment (Sz) between October 2013 and January 2015 at the Department of Psychiatry, The Affiliated Hospital of Southwest Medical University (Luzhou, China). All members of the Sz group met the inclusion criteria:

1. Conformity to the 10th edition of the International Classification and Diagnostic Criteria of Mental Disease (ICD-10);
2. schizophrenia at an early stage; and
3. never having received any antipsychotic drugs.

Participants with psychiatric disorders caused by any other organic cause or disease were excluded. Clinical treatment was assessed using the Brief Psychiatric Rating Scale (BPRS), with a total of 18 items. 3 cured patients (total score <35) were classified as the clinical treatment group (Sz-H). A total of 60 age- and gender-matched healthy volunteers (29 males, 31 females, mean age: 44.1 [range from 18 to 60] years) were taken as the control group (H), who were screened to confirm the absence of a current or previous psychiatric history, and had not taken any antipsychotic drugs in the previous 3 months.

The study was approved by the ethical committee of our hospital, which abides by the revised World Declaration of Helsinki on ethical principles for medical research involving human subjects. All participants or their guardians gave informed written consent after a complete description of the study.

2.2. RNA extraction

Blood samples were collected from all subjects at the start of the study. After blood coagulation, serum was prepared by centrifuging the samples at 1600 × g at 4°C for 15 minutes to remove particulate matter. The resulting supernatants were stored at -80°C in Low Binding Eppendorf tubes (Qiagen GmbH, Hilden, Germany) before the analysis.

The serum was dissolved at 37°C before RNA extraction. A MiRNeasy Serum/Plasma kit (Qiagen GmbH) and miRNeasy Serum/Plasma Spike-In Control (Qiagen GmbH) were used for miRNA extraction. High-quality miRNA was extracted, with an RNA integrity number (RIN) of 8 and an OD260/OD280 ratio of 1.9-2.0.

2.3. MiRNA array analyses

The GeneChip microRNA 2.0 chip (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA), which can completely detect all mature miRNAs from 131 species in Sanger miRBase version 15.0, was used to detect the expression profiles of the samples (4 schizophrenia patients without treatment, in which 1 patient's sample did not pass the quality control checks for microarray analyses, 3 cured patients and 3 healthy controls). Significance Analysis of Microarrays[23] (SAM) was used to analyze the different expression profiles of miRNAs among the 3 groups. SAM software was used to screen differential miRNA. Screening criteria include: $|\text{Score (d)}| \geq 1.5$; the reliability value (q-value) is controlled within 5%; the variation multiple is ≥1.5 or Fold Change is ≤0.667 and the sample size is ≥3.

2.4. Real-time PCR analyses for miRNAs and targeted mRNAs

Quantitative PCR analysis of miRNA. A large cohort included 59 schizophrenia patients without treatment and 60 healthy controls were included in miR-320a and miR-320b quantitative PCR test. Reverse transcription was performed to obtain cDNA from the miRNAs using miScript II RT kit (Qiagen GmbH). The reaction mixture consisted of 4 μL 5 × miScript HiSpec buffer, 2 μL 10 × miScript Nucleics mix, 2 μL Enzyme mix, 10 μL miRNA, and water to 20 μL. It was performed at 37°C for 60 minutes followed by inactivation of the reverse transcriptase at 95°C for 5 minutes. Then, synthesized cDNA was amplified by PCR under the following condition: 95°C for 15 minutes; 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 35 seconds, using miScript SYBR Green PCR kit (Qiagen GmbH) and miScript Primer (Qiagen GmbH). SnRNA RNU6B (U6), a small nuclear RNA, was selected as an internal control for the quantification analyses. The reaction mixture consisted of 12.5 μL 2 × QuantiTect SYBR Green PCR mix, 2.5 μL 10 × miScript general primer, 2.5 μL 10 × miScript specific primers (miR-320a, miR-320b and U6), 2.5 μL cDNA (diluted 5-fold), and 5 μL water.

Quantitative PCR analysis of target mRNA. Total RNAs were extracted from the SK-N-SH cells (Suer Biological Technology, China) using TRIzol, as per the manufacturer's instructions. A UV spectrophotometer was used to detect RNA quality. Reverse transcription was performed using PrimeScript RT Reverse Transcription reagents (Takara Bio, Inc., Otsu, Japan). Reaction mixture consisted of 2 μL 5 x PrimeScript RT mix, 2 μL RNA (500 ng), and water to 10 μL. Reaction condition: 37°C for 15 minutes and 85°C for 5 seconds. PCR quantification was performed using SYBR Premix Ex Taq II (Takara Bio). The primers ITG β1-P1: 5′-ATG CCT ACT TCT GCA CGA TG-3′, ITG β1-P2: 5′-TGT AAA TGT CTG TGG CTC CC-3′, ITG β2-P1: 5′-CAT TGG CTT CGG GTC CTT CG-3′, ITG β2-P2: 5′-GC CGT CAT CAG TGG CAA ACA-3′, HPRT-P1: 5′-CTT TGC TGA CCT GCT GGA TTAC-3′, and HPRT-P2: 5′-ATC TTT GGA TTA TAC TGC CTG ACC-3′ were used for PCR. HPRT-P1 and HPRT-P2 were selected as internal controls for the quantification analyses. Reaction mixture consisted of 10 uL 2 x SYBR Premix Ex Taq II, 1 μL primer (10 μmol/L), 4 μL cDNA (diluted 5-fold), 5 μL deionized water. Reaction condition: 95°C for 5 minutes; and 35 cycles of 95°C for 25 seconds, 60°C for 30 seconds.

Real-time quantitative PCR was performed using standard protocols on an Applied Biosystems 7500 Sequence Detection System. All assays were performed in triplicate. The $2^{-\Delta\Delta CT}$ method was used as a relative quantification strategy for data analysis.

2.5. Bioinformatics analyses

Potential target genes associated with schizophrenia signaling pathways, receptors, and cytokines, which were regulated by the aberrantly expressed miRNAs, were filtrated for the following experiment using online bioinformatics tools[24] and PubMed.

2.6. ELISA detection of serum proteins

The ITG β1 serum concentration was detected by an ITG β1 ELISA (Catalog No. SEB042Hu; Cloud Clone Corp, Houston, TX), following the standardized operation process. All assays were performed in triplicate.

2.7. miRNA mimics and inhibitor transfection in cells

miRNA mimics and miRNA inhibitors for miR-320a-3p and miR-320b were purchased from Qiagen. SK-N-SH cells (Suer Biological Technology, China) were transfected with miRNA mimics or miRNA inhibitors of miR-320a-3p or miR-320b or controls, using HiPerFect Transfection Reagent (Qiagen GmbH), as follows:

1. SK-N-SH cells were inoculated in 12-well plates at a concentration of 3×10^4 cells/pore and cultured overnight;
2. Working solution of miRNA mimics and miRNA inhibitors were prepared following the instructions. Optimization experiments were performed using various miRNA mimic or inhibitor concentrations. In this study, 0.6 μL miRNA mimics or 6 μL miRNA inhibitors at a concentration of 100 nM were transfected into SK-N-SH cells; and
3. the whole RNAs and proteins were extracted, respectively, using TRIzol and RIPA Cell Lysis solution.

2.8. Luciferase reporter gene assays

Fragments of 3 recognition sites in the ITG β1 3′ UTR for miR-320a-3p were cloned together or separately into pmiR-RB-Report carriers. The products were named ITGB1-BS1+2+3-Report (Fragment area: no. 1-715 bp), ITGB1-BS1-Report (no. 1-255 bp), ITGB1-BS2-Report (no. 200-525 bp) or ITGB1-BS3-Report (no. 510-715 bp) after confirmation by gene sequencing. 293 cells (Suer Biological Technology, China) were inoculated in 12-well plates (5×10^4 cells/well) and incubated overnight. Report carriers (>200 ng) and 50 ng *Renilla* carriers were transfected into 293 cells using Lipofectamine 2000. Then, the cells were incubated for 24 hours. The cells were lysed to detect the activity of luciferase in the Report and *Renilla* groups with fluorospectro photometry after washing in PBS. Ratios of Report/*Renilla* were used for comparing the luciferase activity of cells processed by different fragments. All assays were performed in triplicate.

2.9. Western blotting

RIPA pyrolysis liquid was used to lyse the cells, which were transfected with miRNA mimics and inhibitors. The protein concentrations of the cell lysates were detected using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Then, western blot analysis was performed for proteins extracted from the cell lysates. Anti-ITG β1 antibody (1:1000 abcam, Catalog Number ab179471, Cambridge, MA) incubate O/N at 37°C; Anti-beta Actin antibodies (1:2000 abcam, Catalog Number ab189073) incubate 3 hours at room temperature. All assays were performed in triplicate.

2.10. Statistical analyses

All collected data were processed by analysis of variance and the least significant difference test, using GraphPad Prism 4.0 statistics software for statistical analysis and the production of graphs. The difference was statistically significant at $P<.05$.

3. Results

3.1. Down-regulation of miR-320a-3p and miR-320b in patient serum and verification in a large cohort

Microarray analyses of serum miRNA expression profiles (3 schizophrenia patients with no treatment, 3 cured patients and 3 healthy controls; One patient's sample did not pass the quality control checks for microarray analyses) are shown in Figure 1A. Significantly differences were found in the expressions of 6 miRNAs. miR-1281 was up-regulated in SZ group compared with H group, while miR-2861, miR-638 were down-regulated. miR-1469 was down-regulated in the SZ-H group compared with H group. miR-320a-3p and miR-320b expression levels were significantly under-expressed in the serum of patients without treatment and up-regulated in cured patients and healthy controls (Fig. 1B and Table 1). The validation experiments were not performed in the cured patient group as it was difficult to collect specimens from this group. The large cohort, comprising 59 schizophrenic patients without treatment and 60 healthy controls, were included in the miR-320a-3p and miR-320b quantitative PCR analyses. miR-320a-3p and miR-320b expression was decreased in patients without treatment compared with healthy controls ($P=.023<.05$ and $P=.019<.05$; Fig. 1C and Fig. 1D).

3.2. Results of bioinformatics analysis and ELISA arrays

The predicted targets of miR-320a and miR-320b are in Table 2.

One prior study found that integrin family members were highly expressed in the platelets of schizophrenic patients.[25] They play important roles in brain development, the maturation of neural circuits and adult neuroplasticity.[26] ITGB1 was a predicted target for mir-320a-3p in both TargetScan and miranda. Analysis using Targetscan indicated that there were 3 potential targets in the ITG β1 3′ UTR for miR-320a-3p (Fig. 2A). They were named BS1, BS2, and BS3 in our study. We speculated that ITG β1 might be regulated by miR-320a-3p, and this would play important roles in the occurrence of schizophrenia. ELISA tests of serum ITG β1 identified that its concentration in schizophrenic patients was higher than that in healthy controls ($P=.012<.05$; Fig. 2B).

3.3. Expression level changes of ITG β1 induced by miRNA mimics or miRNA inhibitors

The infection efficiency of miR-320a-3p mimics, miR-320b mimics, miR-320a-3p inhibitors and miR-320b inhibitors in SK-

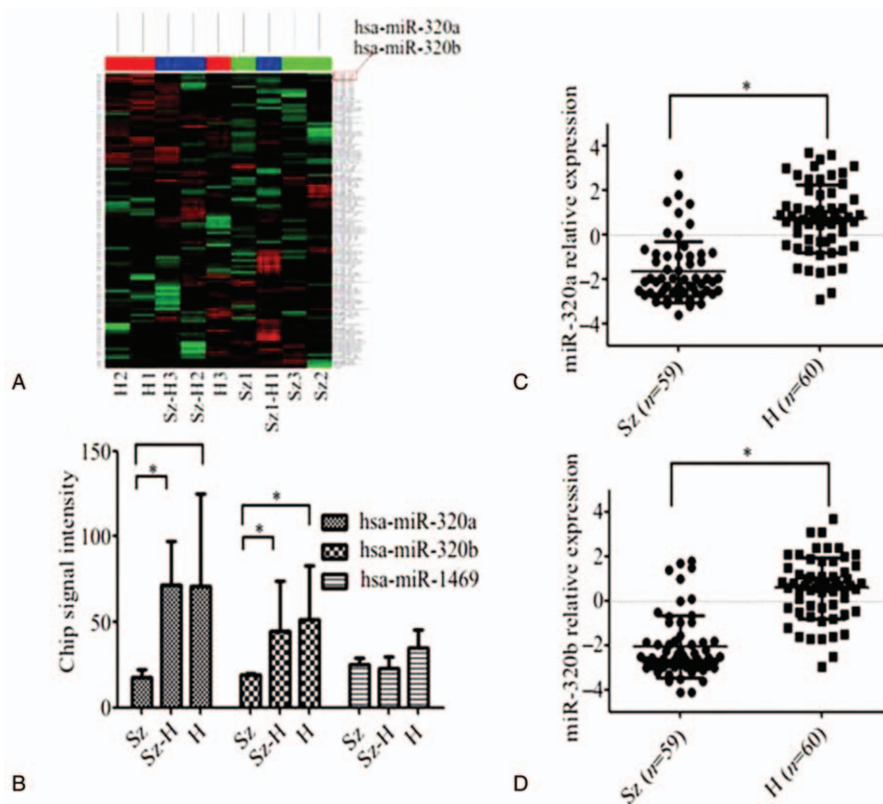


Figure 1. ChIP results and quantitative PCR validation. Notes: A: heatmap of ChIP result; B: part of the ChIP results shown via a bar chart; C: quantitative analysis of miR-320a-3p in Sz and H serum specimens; D: quantitative analysis of miR-320b in Sz and H serum specimens. Sz: schizophrenic patients without treatment; Sz-H: cured schizophrenic patients; H: healthy adults.*$P<.05$.

N-SH cells were estimated at 85%, 80%, 80%, and 75%, respectively through fluorescence microscopy. The ITG β1 expression level was significantly down-regulated or up-regulated by transfection with miR-320a-3p mimics or inhibitors, respectively, while miR-320b mimics and inhibitors had no obvious effects (Fig. 3A). The results suggested that miR-320a-3p decreased ITG β1 mRNA expression by targeting the ITG β1 3′ UTR. The results of the western blot analysis also confirmed that ITG β1 expression was down-regulated or up-regulated by transfecting miR-320a-3p mimics or inhibitors into SK-N-SH cells, respectively (Fig. 3B).

Table 1

miRNA signal from chip analysis.

groups	No.	Sex	age	miR-320a-3p	miR-320b	miR-1469
Sz	Sz1	M	19	23.69	18.90	19.72
	Sz2	M	27	18.91	20.39	27.81
	Sz3	F	17	14.67	19.60	27.80
	Sz4	F	24	14.46	18.99	26.29
Sz-H	Sz-H1	M	36	84.19	31.69	19.35
	Sz-H2	F	27	88.52	78.07	30.77
	Sz-H3	F	28	41.98	23.95	18.09
H	H1	F	33	67.09	54.58	35.76
	H2	M	46	126.46	81.32	45.04
	H3	M	32	18.94	17.79	25.02

F = female, H = healthy adults, M = male, Sz = schizophrenic patients without treatment, Sz-H = cured schizophrenic patients.

3.4. Regulatory effect of miRNA mimics and miRNA inhibitors on the ITGβ1 3′ UTR

Fragments of 3 recognition sites in the ITG β1 3′ UTR (BS1, BS2, and BS3) targeted by miR-320a-3p were cloned together or separately into pmiR-RB-Report carriers. 293 cells were co-transfected with miR-320a-3p mimics or miR-320a-3p inhibitors and the clone carriers. The experiments showed that miR-320a-3p mimics had obvious inhibitory effects on the luciferase activity of ITGB1-BS1+2+3-Report, a lesser effect on ITGB1-BS1-Report and ITGB1-BS2-Report, and no clear effect on ITGB1-BS3-Report. Similar effects were observed with the miR-320a-3p inhibitor transfection (Fig. 4).

4. Discussion

The extracellular matrix (ECM), as an important component of the extracellular microenvironment, has a prominent role in brain development, the maturation of neural circuits and adult neuroplasticity.[26] Mature obstacles to neural circuits, such as an unstable connection between neurons, are the main

Table 2

The predicted targets of hsa-miR-320a and hsa-miR-320b.

MiRNAs	miRanda predicted targets	TargetScan predicted targets	cross predicted targets
miR-320a	4943	347	78
miR-320b	5234	120	65

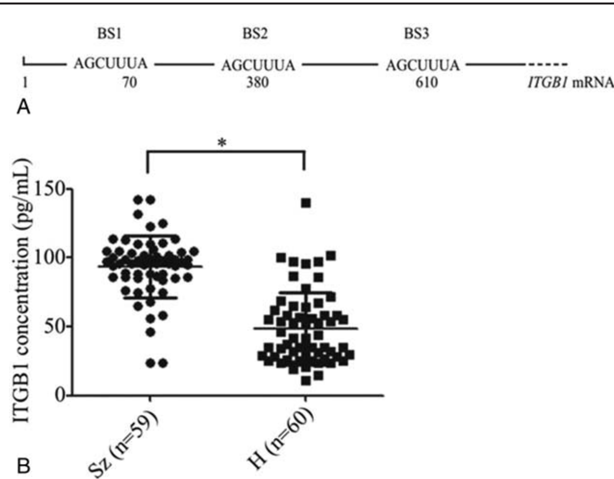


Figure 2. miR-320a-3p binding sites and ITG β1 concentrations in the different groups. Notes: A: diagram of miR-320a-3p binding sites in the ITG β1 3′ UTR; B: ITG β1 concentrations in Sz and H serum specimens.

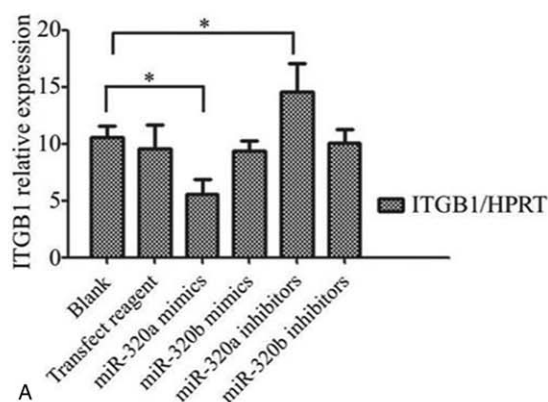
pathophysiological mechanisms of schizophrenia. Perineuronal net (PNN) and Reelin proteins are important ECM components.[27] PNN can make stable the contacts between synapses by maintaining the balance between excitation and inhibition of transmission. The expression level of PNN was found to be decreased markedly in specimens from postmortem brains from schizophrenic patients. Proteoglycans, pluripotent chitosan, and matrix metalloproteinases (MMPs) are particularly abnormally expressed.[28] Reelin is under-expressed in patient brain tissues, suggesting that it may be related to glutamate metabolism.[29]

Integrins are a type of transmembrane receptor protein and an important component of the ECM that conduct cellular signals inside and outside cells. Many features of tumor cells are associated with integrins, such as invasion, metastasis, drug resistance, and autophagy.[30] Research about the function of integrins in the central nervous system has been carried out. Studies have found that integrins play an important and therapeutically significant role in moderating cellular responses to ischemic brain injury.[31] The correlation between integrins and other nervous system diseases has been widely reported.[26] However, studies on integrin function in schizophrenia are fewer in number. One study in 2002 found that the expression of integrin α (IIb) β (IIIa) in the peripheral blood of schizophrenic patients was increased significantly, which was associated with the high disease in schizophrenic patients.[25] The mechanism of this is not yet clear. Our research found some similar results, in that the serum concentration of ITG β1 in patients with schizophrenia was higher than that in healthy controls ($P=.001 < .05$). It was interesting to find that the expression of miR-320a-3p and miR-320b changed significantly, according to the results of the microarray analyses in our work. Through biological information retrieval, we speculated that ITG β1 expression would be regulated by miR-320a-3p. This would make these miRs potential auxiliary biomarkers for the clinical diagnosis of schizophrenia. Their expression changes during the course of treatment may be used to evaluate the effects of treatment.

MiR-320a-3p played important role in the apoptosis of cells, signal pathways, synaptic function and the growth of nerve cells.[32] Wang et al found that miR-320a-3p was significantly down-regulated in depressed patients and in early Alzheimer's disease.[33] This is the first study at the cellular level to confirm that miR-320 regulates ITG β1 expression by targeting the 3 'UTR of ITG β1 mRNA. Experiments involving luciferase reporter gene assays have found that the gene loci at 70 bp (BS1) and 380 bp (BS2) play critical roles in the process and that at 610 bp (BS3) is affected to a lesser degree. This is due to the fact that miRNA identification of target sequences requires a certain contact structure. The gene sequence we cloned of the locus at 610 bp (BS3) in our research was shorter, rendering it more difficult to recognize. Microarray analyses demonstrated that miR-320b was up-regulated in the serum of schizophrenic patients. However, ITG β1 was not obviously regulated by miR-320b mimics and inhibitors at the cellular level. We did not find specific binding sites for miR-320b in the ITG β1 mRNA 3′ UTR through the bioinformatics retrieval, which was consistent with the experimental results. From another point of view, the negative results for miR-320b have proven the high specificity of the regulatory effect of miR-320a-3p on ITG β1.

4.1. Limitations section

There are some limitations in this study. First, with our limited resource, we chose the number of subjects enough to locate the differential expression of miRNA between patients of schizophrenia and controls. So a limited number of subjects were included in the discovery sample. Second, the significance of miR-320 in neural development, inflammation, and synaptic trans-



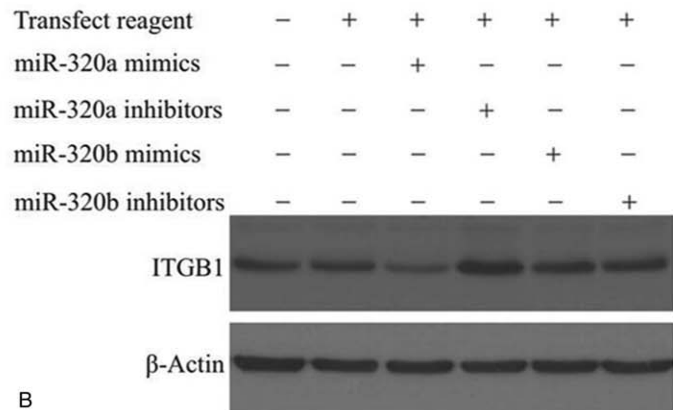


Figure 3. Functional study of the miRNAs of interest. Notes: A: ITG β1 mRNA levels in cells after treatment; B: western blot results of cells after treatment. $^{*}P < .05$.

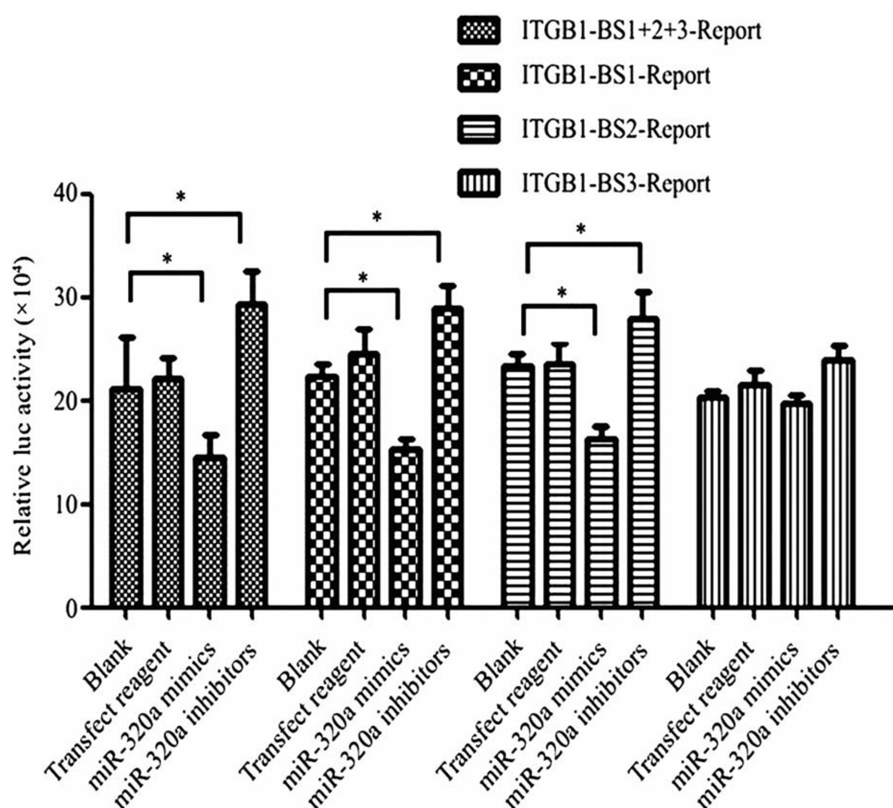


Figure 4. Luciferase activity assay.

mission were not discussed in detail in this article, which has been done in another article (under review) of our group. Finally, Stage of disease was not included in the study, which would be done in our further study.

5. Conclusions

This study confirmed that the expression level of miR-320a-3p in the serum of schizophrenic patients changed significantly, which may regulate the expression of target genes by combining with the ITG β1 mRNA 3′ UTR. This may provide some new ideas for the study of the pathogenesis of schizophrenia. Our results demonstrated that miR-320a-3p, by regulating its target ITG β1, might play an important role in schizophrenia pathogenesis, which could be a potential pathway for schizophrenia diagnosis and therapy. The mechanisms about ITG β1 related to schizophrenia's signaling pathways will be described precisely in our further research.

Author contributions

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Software: Ying Peng.
Supervision: Kaizheng Wang, Kezhen Bai.
Validation: Yuanshuai Huang.
Visualization: Yuanshuai Huang.
Writing – original draft: Yuhan Wang.
Writing – review & editing: Yuanshuai Huang.

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