Interaction between MicroRNA-7 and its Target Genes in Schizophrenia Patients

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Abstract

Objective: To verify interaction between miRNA-7 and its target genes, and explore its possible role in schizophrenia.

Methods: The hippocampal neuron cells (HT22) in mice transfected by lentiviral vector with microRNA-7 interference and over-expression were cultured, and the microRNA expression profiling of HT22 target genes were measured by quantitative RT-PCR.

Results: The expression of three genes (ERBB4, GABRA6, and GAD1) increased along with the low expression of miRNA-7, and decreased along with the over-expression of microRNA-7. Expression of GRIN2A gene increased along with microRNA-7 over-expression, and decreased when miRNA-7 was interfered.

Conclusion: Expression of miRNA-7 may affect its target genes in schizophrenia patients, leading to alteration of neuronal morphology and function, which may play crucial roles in patho-mechanism of schizophrenia. Different from common working pattern, miRNA-7 promotes rather than inhibits the expression of GRIN2A, and the specific molecular mechanism warrents further study.

Keywords Schizophrenia; miRNA-7; Target genes; Interaction

Introduction

Schizophrenia (SZ) is a disabling clinical syndrome characterized by derangement in thought, mood, perception and so on, which mainly merged for practical reasons [1]. With a broad schizophrenia conception, the prevalence rate was reported ranging from 0.3‰ to 22‰ [2,3]. If it is narrowly defined (i.e. with a nuclear schizophrenic syndrome consisting mainly of first-rank symptoms at onset), the incidence of which is very similar between different cultures [4]. Studies have shown that the combination of genetic and environmental factors play a role in the development of SZ [5,6].

MicroRNAs (miRNA, or miR) are a class of endogenous non-coding small RNAs(~22nt) which are key regulators of gene expression via binding of miRNAs to target mRNAs triggers the cleavage, translational repression, or deadenylation of the targets [7]. It has been shown that miRNAs are ubiquitous expression in many body fluids such as serum, plasma, saliva, urine and so on, the different compositions depend on the fluid type, and this specificity of miRNAs may functionally associated with the surrounding tissues [8].

It was shown that miRNAs regulate more than 60% of genes, and multiple studies have indicated that miRNAs regulate various key biological processes, such as cell proliferation and differentiation [9-12] apoptosis [13,14] and cell adhesion [15-17].

Studies have proved that miRNAs regulate the development and plasticity of neuron. Schatt et al. [18] indicated the brain-specific miR-134 is localized to synapto-dendritic compartment of neurons which regulates the size of dendritic spines through suppressing the expression of Limk1. Some miRNAs are enriched in the dendrite and regulate the expression of genes which are important in synapse plasticity [19].

miR-7 was first described in 2003 [20] and then multiple studies have been focused on the anti-tumorigenic effects of this miRNA [21-24]. It was reported that many genes can be regulated by miR-7, such as Pak1 [25], EGFR [21] and Ack1 [23]. What's the function of this miRNA in neuron, few studies can be found up to now. One paper which reviewed multiple studies in central nervous system diseases (include SZ) indicated that circulating miRNAs maybe a novel class of potential biomarkers for diagnosing and prognosing [26]. Due to previous studies, we hypothesize that miR-7 may have aberrant expression in patients with SZ, which may affect the expression of its

targets in neurons synchronously. To verify if miR-7 is aberrant in SZ, we compared the expression of the miRNA in SZ patients with healthy people used q-PCR. To investigate the targets of miR-7, the expression of four potential genes (predicted by TargetScan and MicroRNA.org) which are involved with the development of synapse was tested when miR-7 was over-expressed and inhibited.

Materials and Methods

Design

This article integrates clinical research with fundamental research, focusing on genetic pathway of microRNA-7 in patients with schizophrenia by investigating the expression of miR-7 in patients with SZ and verifying its targets in neurons.

Ethics statement

This study was conducted according to the principles represented in the Declaration of Helsinki. This study was agreed by the Institutional Review Board of No. 102 Hospital of PLA and informed consents were obtained from all patients and healthy controls.

Participants

Inclusion criteria: firstly, the symptoms should meet the criteria of SZ as defined by the Diagnostic and Statistical Manual 4th edition (DSM-IV); secondly, the diagnosis of SZ was confirmed by at least two psychiatrists; thirdly, the patients either have never received any antipsychotic treatment or in the absence of psychotropic medication within 3 months or longer were enrolled. Exclusion criteria: firstly, no history of severe medical diseases and other psychiatric disorders; secondly, no receiving blood transfusion within one month, electroconvulsive treatment in 6 months or had a brain injury history which causing traumatic amnesia longer than 24h were excluded.

Then fifty patients (32 males and 18 females) aged from 18 to 56 (mean 27.22) were including in this study. Fifty healthy controls (32 males and 18 females) aged from 16 to 58 (mean 26.82) without any medical problems now and a history of physical disease. The healthy controls who with any history of blood transfusion, severe traumatic event within a month or any family history of major psychiatric disorders (SZ, bipolar disorder, major depression and anxiety disorder) within the last three generations were excluded. The gender, age and ethnicity were matched between patients and healthy controls.

Prediction of miR-7 targets

TargetScan (http://www.targetscan.org) and MicroRNA.org(http://www.microrna.org) were used to predict the candidate targets of miR-7.

Cell culture and virus infection

The mouse hippocampal HT22 cell line is immortalized with a SV40 antigen but exhibits neuronal properties [27]. HT22 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Life Technologies, Shanghai, China) in a humidified 5% (v/v) CO_2 incubator at 37°C. Cells were plated on a 6-well plate at a density of 5×10^5 cells/ml 16 h prior to infection. Lentiviral infections of HT22 cells were performed at a multiplicity of infection (m.o.i.) of 48hrs.

Lentiviral production

The pLL3.7-miR-7 and pLL3.7-anti-miR-7 vectors were transfected into 293T and virus collected according the protocols provided by the inventor of the system. Titers were approximately 1×10^9 infectious units per ml.

Immunocytochemistry

Forty eight hours after infected of pLL3.7-miR-7 and pLL3.7-anti-miR-7 virus, HT22 cells were fixed with 4% paraformaldehyde (PFA), washed, permeabilized with 0.1%. Triton/phosphate-buffered saline (PBS), blocked for 1 h with 3% bovine serum albumin(BSA) before incubating for 1.5 h with primary antibodies at room temperature in 3% NGS/PBS, then washed in PBS and incubated with secondary antibodies in 3%NGS/PBS. Then, phalloidin was incubated for 1 h in 0.2% BSA/PBS.

Reverse transcription and quantitative real-time PCR

Whole blood (5 mL) was collected from all subjects (SZ patients and healthy controls) using EDTA anticoagulant tube. The plasma was separated by centrifugation and transferred into a 2 mL RNase-free microcentrifuge tube, and stored at -80^0C until use. The miRNeasy Serum/plasma Kit (Cat.NO.217184, Qiagen, CA) was used to isolate total RNA from plasma following the manufacturer's protocol. The total miRNA samples were stored at -80^0C. elegans synthetic miR-39 was used as a spiked-in and normalization control (Cat.NO.219610, Qiagen, Valencia, CA) following the manufacturer's protocol [28,29]. For quantitative detection of miRNA by RT-PCR, purified plasma miRNA was converted to cDNA by reverse transcription reactions using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Inc., Grand Island, NY) and miRNA-specific stem-loop primers were supplied by the TaqMan MicroRNA Assays (Applied Biosystems). The primers of microRNA-7 were UGGAAGACUAGUGAUUUUGUUGU, and the universal primer was Universal real-time PCR RP: GTGCAGGGTCCGAGGT.

The RT reactions was mixed according to the manufacturer's protocol and performed in an Applied Biosystems 9700 PCR instrument using the following conditions: 160^0C for 30 min, 42^0C for 30 min, 850C for 5 min, holding at 4. Real-time PCR reactions were performed in a 5µL reaction mixture containing 2.5µL TaqMan Universal PCR Master Mix II (Applied Biosystems, Inc.), 0.25µL miRNA-specific primer/probe mix (Applied Biosystems, Inc.), and 2.25 µL diluted RT cDNA product. PCR reactions in a 384 well reaction plate were run in a 7900HT real-time PCR system (Applied Biosystems, Inc.) using following cycling parameters: 95^0C for 10 min, followed by 40 cycles of 95^0C for 15 s and 60^0C for 1min. Each reaction was performed in triplicate. Real time PCR data were collected by SDS software and relative levels of the tested miRNA in plasma were calculated based on the following formula using C. elegans synthetic miR-39 as a normalization control [**28-30**].

Total RNA of cells was isolated by Trizol Reagent (Invitrogen). miRNA expression analysis was conducted by quantitative PCR using SYBR green dye, with relative changes calculated by the 2-ΔΔCT method. Primers used were as follows: GAPDH_F, 5'-TGCACCACCAACTGCTTAGC-3'; GAPDHR, 5GGCATGGACTGTGGTCATGAG-3; EBRR4_F, 5'GCTGTTGAACTGGTGTGTCC3';EBRR4_R, 5'-TCCCGATGAACAAGCCGTC-3';GABBR2_F, 5'-ACCACCGCCTACGAATGAAG-3';GABBR2_R,

5'-CCCAAGCTGAGGATGTCGTT, 3';GABRA6_F5'-TCTGAGGGGAGGTAAAAACAGG-3';GABRA6_R,5'-AGTTGAGCTTGGGCATTTTCT-3';GAD1_F,5'-TACTCCTGTGACAGAGCCGA-3'; GAD1_R,5'-GTGGTCTTGGGGTCTCTACG-3';GRIN2A_F,5'-AGACCCACTGACTGAGACCT-3';GRIN2A_R, 5'AGGATGTCGATGCAGAACCC-3'.

Statistical analysis

Statistical analysis was performed with SPSS version 17.0, and data were expressed as mean ± standard deviation (SD). Mann-Whitney U test was used to compare the expression levels of miR-7 between schizophrenia and healthy controls. To estimate the effect size, Cohen's d was calculated by the following formula.
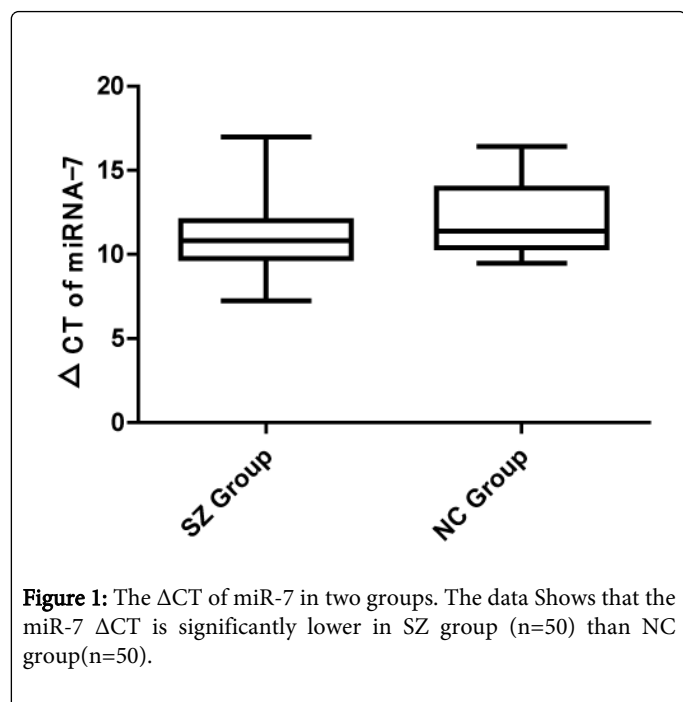
$$\text{Cohen's d} = \left(\overline{x1} - \overline{x2}\right) / \sqrt{\left[(n1-1)S1\wedge2 + (n2-1)S2\wedge2\right]/(n1+n2)}$$

Comparisons between two groups were done with t test and those among multiple groups with one way analysis of variance (ANOVA) followed by LSD test. A value of p<0.05 was considered statistically significant.

Results

The expression of miR-7 was aberrant in SZ patients

As the ΔCT of miR-7 was lower in SZ patients (SZ group) than healthy people (NC group) (Figure 1) miR-7 in SZ patients were significantly over-expressed (P<0.05). The effect size was nearly moderate (Cohen's d=0.45).

Figure 1: The ΔCT of miR-7 in two groups. The data Shows that the miR-7 ΔCT is significantly lower in SZ group (n=50) than NC group(n=50).

Identification of HT22

Three days after culture, cells were harvested and immunocytochemistry was done for NSE. The NSE positive cells were counted. Results showed the proportion of NSE cells was 100%. It was therefore deemed as neurons culture. (Figure. 2A, 2B and 2C).

The expression of four genes was affected by miR-7 in neurons at mRNA level

To determine whether miR7 regulates the expression of its target ge nes, we assessed ERBB4, GABRA6, GAD1 and GRIN2A mRNA levels by RTqPCR, Significant difference(P<0.05) in ERBB4, GABRA6 and G AD1 mRNA expression among HT22 cells transfected with hsa-miR-7, anti-miR-7 or miRNC precursor molecules, but miRNA7 promotes rather t han inhibits the expression of GRIN2A (Figure 3).

Discussion

While the neuropathology of SZ is relatively subtle and inconsistent at the anatomical, cellular and molecular levels, developments in neural imaging and histological techniques are refining our understanding of the spectrum of changes in gross anatomy, neural circuitry and cyto-architecture. The neurodevelopmental mode of SZ remains poorly understood till this day.

MiRNAs play a role as regulator of protein-coding genes, single miRNA regulates the expression of hundreds genes at posttranscriptional level. It was reported that miR-7 was significantly up-regulated in the dorsolateral prefrontal cortex (DLPFC) which region is associated with neuropathology of SZ [31]. Kim et al. [32] investigated hundreds of miRNAs in postmortem brain tissue of SZ patients, found that Seven miRNAs including miR-7 were differentially expressed in SZ group. This study found the expression of miR-7 was aberrant in serum of patients with SZ, and the results (Figure 3) indicated that miR-7 has an influence on the mRNA level of four genes (ERBB4, GABRA6, GAD1 and GRIN2A). It is easier to obtain blood samples than brain tissues, and Bekris et al. [33] suggested that both the miRNAs in CSF and plasma can be a feasible marker of neuropathologic changes of AD. Investigate the expression of miRNAs and verify its targets in neurons may be a way to uncover the possible molecular mechanism of SZ. Further studies are needed to identify the disturbance of miRNAs and its targets in brain tissues of SZ patients and animal model.

Several studies proposed that NRG1 and its receptor ErbB4 as susceptibility genes of SZ [34-36]. The mouse with ERBB4 knockout presented a reduction of dendritic spines in hippocampus and cortex, and the over expression of this gene caused the number of dendritic spines to increase [37,38]. When hippocampal slices was treated with NRG, the induction of long-term potentiation (LTP) was suppressed [39]. These studies indicated ERBB4 and NRG1 play a crucial role in maintaining the normal morphology and electric activity of neurons.
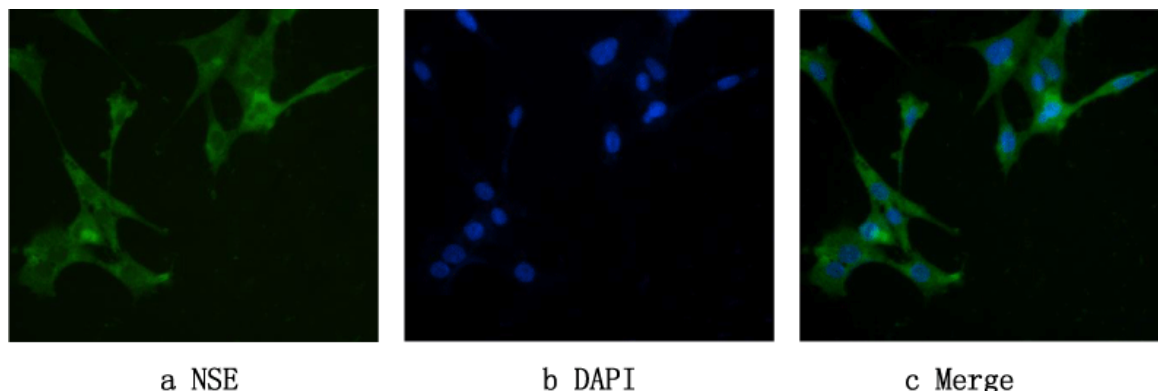
a NSE   b DAPI   c Merge

Figure 2: The expression of NSE in HT22 cells. The neurons marker NSE(A) and nuclear marker DAPI(B), merge NSE and DAPI (C) showed the proportion of NSE cells was 100%
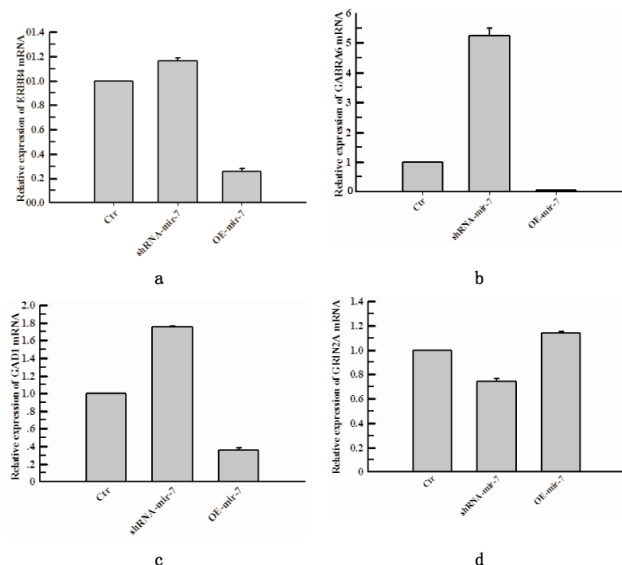

Figure 3: The interaction between miR-7 and its target genes. Four genes mRNA level were regulated by miR-7, relative amount of ERBB4, GABRA6, GAD1 and GRIN2A mRNA 48h after inhibit or transfection and its negative control. The data are the average of three measurements expressed as means ± standard error (SEM).

GABA-A receptors are chloride permeable ligand-gated ion channels, they are receptors ofγ-aminobutyric acid (GABA) which is an important inhibiting neurotransmitter in mammalian brain. Benzodiazepines can regulate the chloride conductance through the interaction with these receptors. As a subtype of GABA-A receptors, it was shown that the SNPs of GABRA6 has linked with the risk of SZ [40]. Exclusively within mature cerebellar granule neurons GABRA6 is highly expressed [41] and correlates with tonic inhibition [42,43].

The gene GAD1 encodes one of several forms of glutamic acid decarboxylase. Guidotti et al. [44] found that the expression of GAD67 (which is encoded by GAD1) but not GAD65 (which is encoded by GAD2) mRNA and protein were significantly decreased in prefrontal cortex of patients with SZ and bipolar disorder, and it is in accord with the hypothesis that these changes are susceptible factors in psychosis.

The encoded protein of GRIN2A is an N-methyl-D-aspartate (NMDA) receptor subunit, it has been associated with SZ, Huntington, Parkinson disease, autism and neurodevelopmental phenotypes [45-49].

In this study, the up-regulated expression of miR-7 in SZ group in is consistent with our study before [50]. Combined the results that the overexpression or low expression of miR-7 had a significant influence on the mRNA level of the four genes (ERBB4, GABRA6, GAD1 and GRIN2A) with the aberrant expression of miR-7 in SZ patients, these data indicates that these changes in neurons may affect their morphology and function through these genes, and this may have an influence on physiopathologic process of SZ.

It was also found in this study that GRIN2A was different from the other three genes, the mRNA of this gene increased along with the overexpression of miR-7 and decreased when miR-7 was suppressed. MicroRNAs usually negative regulating the expression of their targets, further study needs to uncover the mechanism of miR-7 promoting the expression of GRIN2A and how it affects the physiopathologic process of SZ.

Conclusion

These data show that SZ has disturbance in miR-7 expression, and this exception may have an impact on the pathologic process of the disease through its targets (such as ERBB4, GABRA6, GAD1 and GRIN2A). As SZ is a complex syndrome, the disturbance in expression of miRNAs and its targets may occur in one subtype or patients with special symptoms, which warrants further study in future.

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