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Identification of miR-22-3p, miR-92a-3p, and miR-137 in peripheral blood as biomarker for schizophrenia

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ABSTRACT

MicroRNAs (miRNAs) are a class of endogenous and non-coding single-stranded RNAs with length of about 22 nucleotides, and many are evolutionarily conserved. Although postmortem brain samples provide direct evidence of miRNA dysregulation within the brain, peripheral tissue samples can be obtained from living subjects and have the potential to yield biomarkers that could be used as diagnostic tools. To verify and detect additional miRNAs differentially expressed in peripheral blood and further explore their diagnostic value and function for schizophrenia, we performed a next-generation sequencing approach in combination with a literature search to select appropriate miRNAs. We then used real-time quantitative polymerase chain reaction (RT-qPCR) to identify miRNAs expressed aberrantly in schizophrenia. Binary regression analysis identified miR-22-3p, miR-92a-3p, and miR-137. Analysis of receiver operating characteristics (ROC) indicated that these three miRNAs could be used in combination as a biomarker for schizophrenia. Bioinformatic analyses of these genes and gene ontology (GO) enrichment revealed that the combination of miR-22-3p, miR-92a-3p, and miR-137 was closely associated with synaptic structure and function, which play important roles in the etiology and pathophysiology of schizophrenia.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous and non-coding single-stranded RNAs with length of about 22 nucleotides (Bartel, 2004), and many are evolutionarily conserved (Farh et al., 2005). These small RNAs are the specificity factors for a large multi-protein effector complex known as the RNA induced silencing complex (RISC), which promotes the degradation and/or repression of messenger RNA (mRNA) through sequence-specific interactions with the 3′ untranslated regions (UTRs) of specific mRNA targets (Calin and Croce, 2006; Gebauer and Hentze, 2004). MiRNAs are widely expressed in the brain, affecting numerous gene expression and functional pathways, and thus have important implications for neuropsychiatric disorders such as schizophrenia (Cao et al., 2006). Schizophrenia is a chronic, severe and disabling mental disorder with a lifetime risk of about 1%. The condition is characterized by a diverse range of symptoms and cognitive impairments including hallucinations and delusions (Cardno and Gottesman, 2000; Sullivan et al., 2003). Expression profiling studies in samples of postmortem gray matter and dorsolateral prefrontal cortex from patients with schizophrenia have implicated numerous miRNAs in the disease.

While postmortem brain samples provide direct evidence of miRNA dysregulation within the brain, peripheral tissue samples can be obtained from living subjects and have the potential to yield biomarkers that could be used as diagnostic tools. In recent research, Sullivan et al. showed that gene expression is similar in whole blood and brain tissue (Sullivan et al., 2006). Harris et al. investigated schizophrenia through studies of blood-based biomarkers; changes in peripheral biomarkers may mirror pathological processes in the brain (Harris et al., 2012). Because brain tissue is not readily accessible, investigations into the pathophysiology of schizophrenia and potential biomarkers for the condition have increasingly relied upon blood-based expression

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profiling of miRNAs (Lai et al., 2011; Yu et al., 2015; Zhang et al., 2014).

The first miRNA found to be associated with schizophrenia by genome-wide association study was miR-137 (Ripke et al., 2011). *MIR137* is one of the genes associated most strongly with schizophrenia; its product, miR-137, regulates numerous schizophrenia-related genes (Ripke et al., 2014; Ripke et al., 2013; Ripke et al., 2011). MiR-137 is enriched in the dentate gyrus, a subregion of the hippocampal formation, and implicated in regulating adult neurogenesis and neuronal maturation (Silber et al., 2008; Smrt et al., 2010). We measured the expression of miR-137 in peripheral blood of first-onset schizophrenia patients and healthy controls. The results showed that miR-137 was upregulated in schizophrenia patients, showing that the gene has certain diagnostic value (Wu et al., 2016).

Aberrant expression of various other miRNAs (e.g., miR-181b-5p, miR-195, miR-30e, miR-497) in schizophrenia has been reported (Banigan et al., 2013; Beveridge and Cairns, 2012; Sun et al., 2015). To identify additional miRNAs expressed differentially in peripheral blood and further explore their diagnostic value for schizophrenia, we performed a Solexa sequencing approach in combination with a literature search. We used real-time quantitative PCR (RT-qPCR) to validate the aberrant expression of miRNAs selected using these approaches in schizophrenia. Regression analysis showed that miR-22-3p, miR-92a-3p, and miR-137 have important effects in the context of schizophrenia. Analysis of receiver operating characteristics (ROC) indicated that the combination of these three miRNAs could be used as a potential biomarker for schizophrenia. To elucidate the function of these miRNAs as well as the associated regulatory network, we used online software, TargetScan v6.2 and miRanda, to predict the genes targeted by these three miRNAs and performed gene ontology (GO) analysis. GO enrichment analysis yielded postsynaptic density (PSD), synapse and synaptic transmission, all terms associated with synaptic function.

2. Materials and methods

2.1. Clinical subjects and assessment

The study was approved by the medical ethics committee of Xi'an Jiaotong University Health Science Center, in accordance with the Declaration of Helsinki, and informed consent was obtained from all subjects. All patients included in the study were first-onset patients who had been independently diagnosed by at least two experienced psychiatrists according to the Diagnosis and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV) criteria for schizophrenia. Controls were drawn from unrelated volunteers and blood donors. Any individuals with current or past evidence of mental illness or a relative with mental illness were excluded. All subjects were from Northwest China, of *Han* Chinese ethnicity.

2.2. Peripheral blood collection for miRNA sequencing

A total of 20 unrelated individuals were recruited from northwest China: 10 first-onset schizophrenia patients (5 males, mean age = 27.70 ± 1.71 year; 5 females, mean age = 28.10 ± 1.69 year) with schizophrenia and 10 healthy controls (5 males, mean age = 29.22 ± 2.22 year; 5 females, mean age = 29.06 ± 1.93 year). Peripheral blood samples from the participants were collected in PAXgenet Blood RNA tubes (Hombrechtikon, Switzerland). PAXgenet Blood RNA Tubes were stored at 18 °C to 25 °C prior to use. Immediately after blood collection, the PAXgene Blood RNA Tube was gently inverted, 8–10 times. Then the PAXgene Blood RNA Tube was placed upright at room temperature (18 °C to 25 °C) for a minimum of 2 hours and a maximum of 72 hours before processing or transferring to a freezer for storage at −20 °C for 24 hours. Finally, the PAXgene Blood RNA Tube was transferred to −70 °C or −80 °C until total RNA was extracted.

2.3. RNA extraction and miRNA sequencing

Blood samples were sent to LC-BIO (Hangzhou, China) for RNA extraction and RNA sequencing. Total RNA quantity and purity were analyzed using a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, USA) with RIN number > 7.0. Approximately 1 μg of total RNA was used to prepare a small RNA library, according to the protocol that accompanies TruSeq Small RNA Sample Prep Kits (Illumina, USA). Single-end sequencing (36 bp) was performed on an Illumina Hiseq2500 at the LC-BIO, according to the vendor's recommended protocol. The raw reads were subjected to the Illumina pipeline filter (Solexa 0.3), and the data set was further processed with ACGT101-miR (LC Sciences, USA). Subsequently, unique sequences with length of 18–32 nt were mapped to specific species precursors with miRBase 20.0 by BLAST search. All procedures were carried out by LC Sciences.

2.4. RT-qPCR for further verification

Independent peripheral blood samples from 44 schizophrenia patients and 44 healthy controls were collected in ethylenediaminetetraacetic acid (EDTA) tubes and placed on ice. Demographic information for all participants was presented elsewhere (Wu et al., 2016). Within 2 hours, total RNA was harvested using TRIzol® (Invitrogen, USA) according to the manufacturer's instructions and quantified using a NanoDrop 2000 (Thermo, USA). Total RNA (1 μg) from each sample was reverse-transcribed using the Mir-X miRNA First-Strand Synthesis Kit (Clontech, USA), according to the manufacturer's protocol. RT-qPCR was performed with a FastStart Universal SYBR Green Master (Rox) (Roche, USA) to quantify miRNA expression. The first strand of each mRNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Canada). The expression of *U6* small nuclear RNA (snRNA) was used as internal reference to normalize miRNA expression. Each sample was tested three times, and all data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer information for U6 and the miRNAs is shown in Table 1.

2.5. Statistical analyses

Data were analyzed using SPSS software version 18.0 (New York, USA) and GraphPad Prism version 5.0 (California, USA). Data (presented as mean ± SEM) were analyzed using the Mann-Whitney *U test*. *P*-values < 0.05 were considered as statistically significant. Binary

Table 1
Forward primers of miRNAs and primers of genes for RT-qPCR.

Name	Accession number	Sequence (from 5′to 3′)
hsa-miR-22-3p	MIMAT0004495	AAGCTGCCAGTTGAAGAACTGT
hsa-miR-92a-3p	MIMAT0000092	TATTGCACTTGTCCCGGCCTGT
hsa-miR-30d-5p	MIMAT0000245	TGTAAACATCCCCGACTGGAAG
hsa-miR-30e-5p	MIMAT0000692	TGTAAACATCCTTGACTGGAAG
hsa-miR-181a-3p	MIMAT0000270	ACCATCGACCGTTGATTGTACC
hsa-miR-181a-5p	MIMAT0000256	AACATTCAACGCTGTCGGTGAGT
hsa-miR-181b-5p	MIMAT0000257	AACATTCATTGCTGTCGGTGGGT
hsa-miR-148b-5p	MIMAT0004699	AAGTTCTGTTATACACTCAGGC
hsa-miR-199b-5p	MIMAT0000263	CCCAGTGTTTAGACTATCTGTTC
hsa-miR-195-5p	MIMAT0000461	TAGCAGCACAGAAATATTGGC
hsa-miR-497-5p	MIMAT0002820	CGCCAGCAGCACACTGTGG
U6 snRNA (Forward)	NR_004394	CTCGCTTCGGCAGCACA
U6 snRNA (Reverse)		AACGCTTCACGAATTTGCGT
PSD3 (Forward)	NM_015310	ACAGGAATGACGCTGGATCA
PSD3 (Reverse)		TTGTGGCCATGTAGATCGGT
SCAMP1 (Forward)	NM_004866	GGGGCAATTGTGGTTGGATT
SCAMP1 (Reverse)		ATGCTGTGAAAAGTGCTGCT
SLC6A1 (Forward)	NM_001348250	AACTCCTTCACCACGACACT
SLC6A1 (Reverse)		CGCTGGTCATGTTGGTAGTG
GAPDH (Forward)	NM_002046	CCAAGGTCATCCATGACAACT
GAPDH (Reverse)		CAGGGATGATGTTCTGGAGAG