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Changes in serum miRNA-let-7 level in children with attention deficit hyperactivity disorder treated by repetitive transcranial magnetic stimulation or atomoxetine: An exploratory trial

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ABSTRACT

We aimed to investigate whether microRNA-let-7d (miRNA-let-7d) and miRNA-107 may serve as diagnostic and therapeutic biomarkers of attention deficit hyperactivity disorder (ADHD). The relative expression level of miRNA-let-7d and miRNA-107 in patients with ADHD and in a healthy control group was detected by real-time polymerase chain reaction. The blood samples were collected at 6 weeks after repetitive transcranial magnetic stimulation (rTMS) or atomoxetine (ATX) in ADHD patients, and the relative expression levels of the two miRNAs before and after treatments were compared. There were significant differences in the expression level of miRNA-let-7d between ADHD patients and healthy children, as well as before and after rTMS or ATX treatment in ADHD patients. However, the expression of miRNA-107 showed no significant difference between ADHD patients and healthy children or before and after rTMS (or ATX treatment). These results suggest that serum miRNA-let-7d may serve as a potential diagnostic and therapeutic biomarker for children with ADHD.

1. Introduction

Although the pathogenesis of attention deficit hyperactivity disorder (ADHD) is still unclear, it is generally considered to be the result of the interaction of physiological, genetic and environmental factors (Thapar and Cooper, 2016). The evidence from molecular genetic studies has shown that ADHD is a complex genetic disease with multiple genetic interactions (Hayman and Fernandez, 2018). Studies on the candidate genes mainly involved dopamine (DA) receptor genes (DRD4, DRD5), noradrenaline (NE) receptor genes (ADRA2A, ADRA2C), serotonin (5-HT) receptor genes (HTR1B, HTR2A), and synapse-associated protein genes (SNAP25) (Banaschewski et al., 2010; Hawi et al., 2015; Hayman and Fernandez, 2018).

MicroRNAs (miRNAs), which are a class of endogenous small non-coding RNAs containing about 22 nucleotides, degrade or inhibit the translation of targeted mRNAs by specific base pairing, thereby regulating gene transcription (Pillai, 2005). In humans, miRNAs are estimated to control the activities of 30% of protein-coding genes (Lewis et al., 2005). Therefore, miRNAs play a very important role in the biological information network (Bartel, 2009).

Previous studies mainly focused on the pathogenesis, diagnosis, and treatment of ADHD. However, there have been few studies on miRNAs in ADHD patients, especially as potential molecular markers for diagnosis and treatment. The therapeutic approaches for ADHD include drug treatments, physical treatments and cognitive behavior interventions. Atomoxetine (ATX), a highly selective noradrenaline reuptake inhibitor, is the first non-stimulant drug approved by Food and Drug Administration (FDA) of America for ADHD treatment, with reliable safety and tolerance (Upadhyaya et al., 2015). Repetitive transcranial magnetic stimulation (rTMS), an established non-invasive

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neurostimulation technique, regulates the function of cerebral cortex by a magnetic coil over a specific cortical area of the brain (Rossini and Rossi, 2007). Recently, a few studies showed that rTMS exerts a significant therapeutic effect on ADHD (Weaver et al., 2012; Zaman, 2016). In addition, high frequency rTMS can significantly improve ADHD symptoms (Bloch et al., 2010; Kim et al., 2016; Weaver et al., 2012). There is evidence that miRNA-1et-7d exhibits a remarkable increase, whereas miRNA-107 displays a significant decrease in ADHD patients (Kandemir et al., 2014; Wu et al., 2010). However, it is unclear whether the two miRNAs may serve as biomarkers for ADHD diagnosis and treatment. In this study, we first detected the relative expression levels of these two miRNAs in the serum of ADHD patients and healthy controls by real-time polymerase chain reaction (PCR), and compared their differences between the two groups. Next, blood samples were respectively collected from patients with ADHD before and after 6 weeks of real or sham rTMS, or before and after the six-week treatment of ATX or placebo. By comparing the relative expression levels of the two miRNAs between before and after treatment, we evaluated whether miRNA-let-7d and miRNA-107 may serve as potential biomarkers for the diagnosis and treatment of ADHD.

2. Methods

2.1. Study subjects

This was a random and double-blinded study. A total of 75 eligible ADHD patients were recruited, including the non-drug group ($n = 43$) and the drug group ($n = 32$). According to a random distribution table, the non-drug group and the drug group was randomly divided into the two subgroups, respectively: the real rTMS group ($n = 22$) and the sham rTMS group ($n = 21$), the ATX group ($n = 16$) and the placebo group ($n = 16$). Except for the principal investigators, the patients and their parents/guardians, as well as the research executors did not know the procedure of this study. During treatment, 4 patients in the real rTMS group and 5 patients in the sham rTMS group were lost, but there were no patients dropping out during ATX or placebo administration. The enrollment criteria were as follows: (1) met the diagnostic criteria for ADHD in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) of the American Psychiatric Association (Ustun et al., 2017); (2) no other mental disorders or a history of neurological diseases affecting cognitive functions; (3) no heart, liver, lung, kidney, or other serious somatic diseases; (4) intelligence quotient ≥ 80 by Wechsler Intelligence Scale for Children (WISC). The exclusion criteria were as follows: (1) no other medications or other treatments; (2) recent treatment with TMS or electrical shock (ECT); (3) mood-regulating medications within 14 days; (4) audiovisual impairment, color blindness, or weak or narrow-angle glaucoma; (5) a history of epilepsy, brain trauma, or brain surgery; (6) brain metal implants (such as cochlear implants).

A total of 18 healthy children were included in the control group. The enrollment criteria were as follows: (1) after detailed physical examination, there were no serious heart, liver, lung, kidney diseases or other serious somatic diseases; (2) after detailed neurological and mental examinations, there were no mental disorders such as attention deficit hyperactivity disorder, mental retardation, autism, schizophrenia, or a history of neurological diseases affecting cognitive functions. The exclusion criteria were the same as for the ADHD group.

This study was approved by the Ethics Committee of the People's Liberation Army No.102 Hospital and the Ethics Committee of the Changzhou No.2 People's Hospital. All enrolled participants and their parents or guardians signed informed consent.

2.2. rTMS application and ATX administration

In this study, two groups of ADHD patients (the real rTMS group and the sham rTMS group) were treated with a stimulation for 6 weeks. In this study, the real rTMS group received rTMS by a commercially available Magneuro100 magnetic stimulator (VCB001 Type, Nanjing Weisi Medical Technology Co., Ltd.) equipped with an "8" shaped coil. The rTMS treatment parameters were as follows: stimulation frequency, 10 Hz; stimulation intensity, 100% motor threshold (MT); 4 s stimulation time followed by 26 s interval; a total of 30 min per session with 2400 pulses; one session/per day from Monday to Friday, two weekend day interval; a treatment course of 6 weeks. The stimulation site was the right dorsolateral prefrontal cortex (rDLPFC). After the coil was positioned over the cortical representative area of the left abductor pollicisbrevis muscle where the rest motor threshold was detected, and then moved forward 5 cm to reach the stimulation site (rDLPFC). For the sham rTMS group, the coil was simply placed perpendicular to the scalp of the stimulation site. The stimulation parameters were the same as those of the real rTMS.

ATX Hydrochloride (Eli Lilly Ci. Ltd) was initially administrated with a dose of 0.5 mg/kg.d, three days later, and then increased to 1.2 mg/kg.d (Arnold et al., 2018; Greenhill et al., 2007; Spencer et al., 2008). ATX or placbo was administrated for 6 weeks after breakfast. Parents or guardians of the patients monitored their medications and kept a diary.

2.3. Clinical symptom assessment for ADHD

SNAP-IV scale (Association, 2013) questionnaires were used to assess clinical symptoms before treatment and 6 weeks after treatment. In order to maintain the consistency of the evaluation criteria, the explanation or analysis of the scale was completed by the same experienced researcher before and after treatment.

The SNAP-IV scale, based on the diagnostic criteria for ADHD in the DSM-IV, is composed of 26 items containing three aspects of ADHD: attention deficit, hyperactivity impulse, and oppositional defiance. According to the severity of the symptoms, the items are scored on a scale from 0 to 3. In our study, the parents scored the items according to their general impression. Attention deficit and hyperactivity impulse were classified by the mean scores, with ≤ 1 being normal or remission and ≥ 2 being abnormal. For oppositional defiance, more than 4 items with a score of 2 or 3 was considered abnormal.

2.4. Detection of serum miRNAs

2.4.1. Instruments

PCR amplification instrument (9700, Applied Biosystems, USA), real-time fluorescence quantitative PCR system (7900HT, Applied Biosystems, USA), high-speed refrigerated centrifuge (CT14RD, Shanghai Tianmei), micro-whirlpool mixer (WH-2, Shanghai Huxi).

2.4.2. Reagents

RNA extraction kit: miRNeasy Serum/Plasma Kit (217184, Qiagen, USA); reverse transcription kit: TaqMan MicroRNA Reverse Transcription Kit (4366596, ABI, USA); real-time quantitative PCR reagents: TaqMan Universal Master Mix II, no UNG (4440040, ABI, USA); External reference reagents: cel-miR-39 (000200, ABI, USA), hsa-miR-107 (000443, ABI, USA), and hsa-let-7d (002283, ABI, USA).

2.4.3. Specimen collection and storage

4 ml of venous blood was collected at baseline in the ADHD children and the healthy controls, and after the six-week of treatment in the ADHD participants. After collection, the blood sample was left to stand for 1 h at room temperature and was then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant (serum) was transferred into a sterile test tube and then stored in a −80 °C freezer until use.

2.4.4. Serum miRNA extraction

The serum miRNeasy kit was used for serum miRNA extraction, and the exogenous reference product cel-miR-39 was added at the time of

extraction.

2.4.5. MiRNA reverse transcription

miRNA reverse transcription was performed using a miRNA reverse transcription kit and specific primers in the miRNA assay.

2.4.6. Fluorescent quantitative PCR reaction

The fluorescence quantitative PCR reaction was performed using the TaqMan Universal Master Mix II, and the exogenous cel-miR-39 was used as an internal reference.

2.5. Data analysis

Data analysis was performed using SDS 2.2 and Data Assistant 3.0 software. The data were expressed in fold change (FC) for relative expression levels of miRNA, $FC = 2^{-\Delta Ct}$, where ΔCt represents the threshold cycle (Ct) difference between miRNA and exogenous reference cel-miR-39, i.e., $\Delta Ct = Ct_{miRNA} - Ct_{cel\text{-}miR\text{-}39}$. Data were analyzed using GraphPad Prism 5.0 software and the SPSS 19.0 software package. Comparisons were made by using the independent sample non-parametric test method (Mann-Whitney *U* test) or Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. General demographic information

A total of 75 ADHD patients, including 46 males (61.33%) and 29 females (38.67%), were divided into two groups: the magnetic stimulation group ($n = 43$) and the drug group ($n = 32$). The stimulation group was further randomly divided into two subgroups: the real rTMS group ($n = 22$) and the sham rTMS group ($n = 21$). The drug group was further randomly divided into two subgroups: the ATX group ($n = 16$) and the placebo group ($n = 16$). With informed consent, blood samples were collected before treatments, and then treatment was started. Four patients in the real rTMS group and five patients in the sham rTMS group terminated treatment before this study was complete. No patients dropped out in the drug groups. The details of these cases are shown in Table 1. The healthy control group had 18 subjects, including 12 males (66.67%) and 6 females (33.33%), with a gender ratio of 2:1. There was no significant difference in age ($P = 0.337$) or gender ($P = 0.165$) between the ADHD group and the healthy control group (HC) (Table 2). During this study, only two ADHD patients reported mild mouth dryness after 4-week medication in the ATX group, and drunk some water to relieve the symptom. In the real rTMS group, one patient reported headache at the end of two-week rTMS, which was eliminated by decreasing the stimulation intensity. No other adverse effects were reported.

3.2. rTMS or ATX effectively improves the symptoms of ADHD patients

As seen in Fig. 1A and B, following six-week of rTMS treatment or ATX administration, a significant improvement in attention deficit (AD) ($U = 106.76$, $P = 0.000$ for rTMS; $U = 96.28$, $P = 0.000$ for ATX), hyperactivity impulse (HI) ($U = 76.46$, $P = 0.000$ for rTMS; $U = 87.32$,

Table 2
General information of participants.

Predictor	ADHD ($n = 75$)	NC ($n = 18$)	*P* value
Age (years)	8.83 ± 2.53	9.17 ± 2.26	0.337
Sex (male/female)	46/29	12/6	0.165

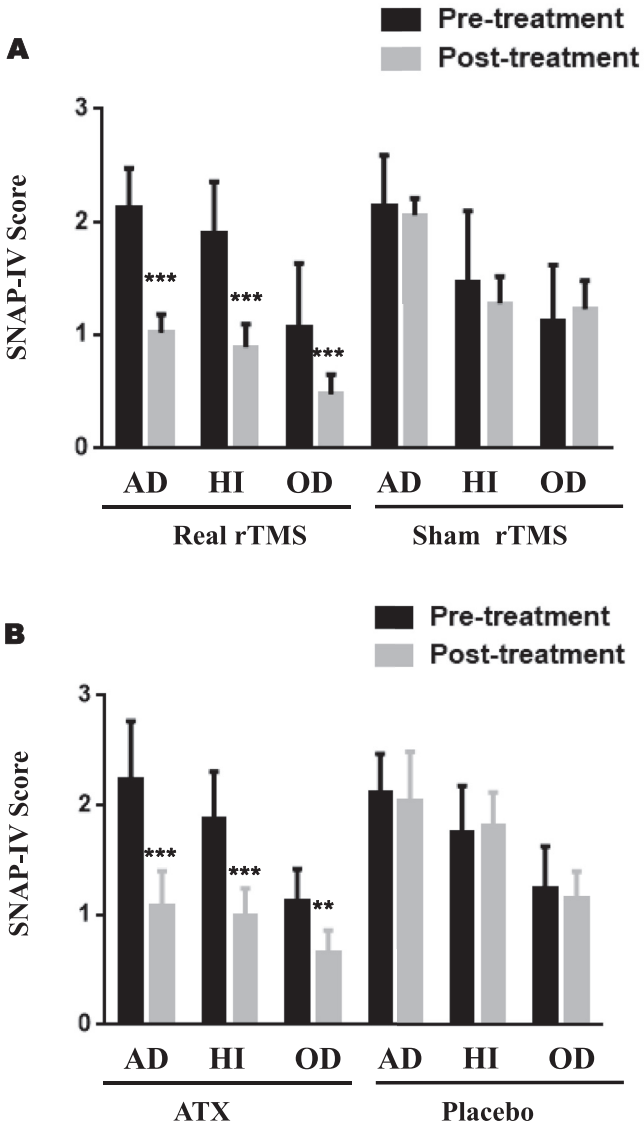

Fig. 1. SNAP-IV score at pre- and post- treatment. (A) SNAP-IV score pre- and post- rTMS or Sham treatment; (B) SNAP-IV score at pre- and post- ATX or Placebo treatment; $^{***}P < 0.001$, $^{**}P < 0.01$, compared to pre-treatment, Mann–Whitney *U* test. rTMS, repetitive transcranial magnetic stimulation; ATX, atomoxetine; AD, attention defect; HI, hyperactivity impulse; OD, oppositional defiance.

Table 1
The enrollment and group of subjects.

Group	subgroup	Number of enrolled participants	Number of participants withdrew treatments	Eventually eligible participants
ADHD	real rTMS	22	4	18
	sham rTMS	21	5	16
	ATX	16	0	16
	Placebo	16	0	16
Healthy controls	18	0	18	
Total	93	9	84	

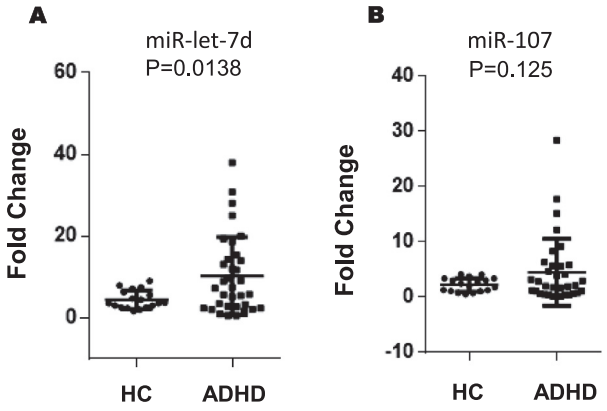

Fig. 2. The level of serum miR-let-7d and miR-107 in ADHD patients and healthy controls. (A) The level of serum miR-let-7d in ADHD patients and healthy controls; (B) The level of serum miR-107 in ADHD patients and healthy controls. HC, healthy controls; ADHD, attention defect hyperactivity disorder. *P* values are shown at the top of each graph, Student's *t*-test.

$P = 0.000$ for ATX), and oppositional defiance (OD) ($U = 112.45$, $P = 0.000$ for rTMS; $U = 136.18$, $P = 0.004$ for ATX) was observed in ADHD patients. In contrast, sham rTMS or placebo failed to cause any obvious improvements.

3.3. The level of serum miRNA-let-7d and miRNA-107 in ADHD patients before and after treatments

As shown in Fig. 2, the expression level of serum miRNA-let-7d was upregulated in the ADHD group compared with the healthy control group (Fig. 2A, $t = 2.55$, $P = 0.0138$), but no significant difference was found in the expression level of miRNA-107 between the ADHD group and the healthy control group (Fig. 2B, $t = 1.56$, $P = 0.125$). Compared with pre-rTMS or pre-ATX treatment in ADHD patients, the serum miRNA-let-7d expression level was downregulated at post- rTMS (Fig. 3A, $t = 2.38$, $P = 0.017$) or at post-ATX treatment (Fig. 4A, $t = 2.96$, $P = 0.006$), but there was no significant difference in the expression level of miRNA-107 between pre-rTMS and post-rTMS (Fig. 3B, $t = 1.78$, $P = 0.0778$) or between pre-ATX and post-ATX (Fig. 4B, $t = 0.72$, $P = 0.624$). In the sham rTMS group or the placebo group, no significant changes were in the miRNA-let-7d level (Fig. 3C for sham rTMS, $t = 1.28$, $P = 0.183$; Fig. 4C for placebo, $t = 0.48$, $P = 0.881$) and the miRNA-107 level (Fig. 3D for sham rTMS, $t = 0.67$, $P = 0.638$; Fig. 4D for placebo, $t = 0.57$, $P = 0.783$).

4. Discussion

MiRNAs, as endogenous non-coding single-stranded RNAs binding to specific miRNA targets, regulate protein translation processes, and thereby regulate gene expression. They play an important role in various cellular physiological processes, such as cell metabolism, proliferation, and apoptosis (Hawkins and Morris, 2008). The abnormal expression of miRNAs will lead to the disorder of human physiological functions. There is evidence that various miRNAs have been found to participate in the pathogenesis and progression of psychiatric diseases through different signaling pathways (Alural et al., 2017). In this study, the expression levels of miRNA-let-7d and miRNA-107 were detected by fluorescence quantitative PCR technology to determine whether these two miRNAs might be used as biomarkers for ADHD.

MiRNA-let-7 is a family of genes first discovered in *Caenorhabditis elegans* and highly conserved among different species. There are 13 different coding genes in humans, including let-7a, 7b, 7c, and 7d (Lee et al., 2016). Investigators have conducted in-depth research on the miRNA-let-7 family, especially in the field of cancer. They are expressed in various tumors and tumor cell lines, and play a vital role in

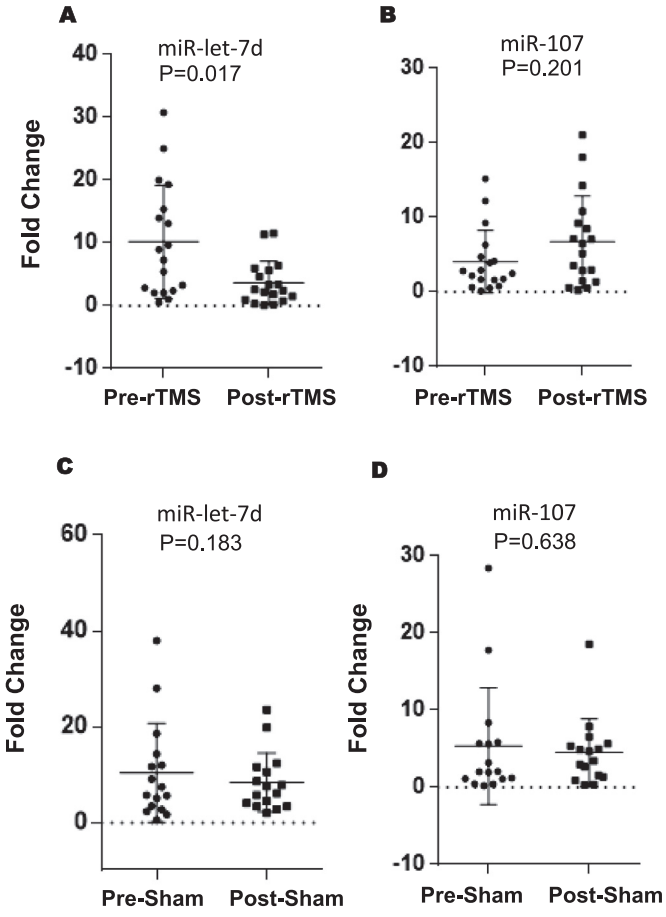

Fig. 3. Fold changes of the level of serum miR-let-7d and miR-107 at pre- and post-rTMS or sham treatment. (A) Fold change of the level of serum miR-let-7d at pre- and post-rTMS; (B) Fold change of the level of serum miR-107 at pre- and post-rTMS; (C) Fold change of the level of serum miR-let-7d at pre- and post-Sham treatment; (D) Fold change of the level of serum miR-107 at pre- and post-Sham treatment. *P* values are displayed at the top of each graph, paired *t*-test.

inhibiting tumor cell activity (Masood et al., 2018). However, the mechanisms of miRNA let-7 family in psychiatric diseases received less attention. MiRNA-let-7d is mainly involved in cell differentiation and pluripotency, neurogenesis, maintenance of pluripotency, somatic reprogramming, tissue regeneration, and plasticity of neural stem cells (Wu et al., 2015). Treatment with retinoic acid induced miRNA-let-7d expression in PC12L differentiated cells, implicating its possible neuronal plasticity potentials (Andolfo et al., 2010; Wong et al., 2012). Extracellular miRNA-let-7d which is widely present in the central nervous system, plays a role in the activation of toll-like receptors and thus causing neuronal degeneration (Wong et al., 2012; Wu et al., 2015). In ADHD animal model, researchers found that increased miRNA-let-7d expression targeted galectin-3 and down-regulated tyrosine hydroxylase gene expression which are involved in dopamine metabolism in the rat prefrontal cortex, suggesting that miRNA-let-7d may be a molecule associated with ADHD (Weaver et al., 2012; Wu et al., 2010). Wu et al. (2015) conducted a controlled study with 35 newly diagnosed ADHD children and 35 healthy children and found that the level of serum miRNA-let-7d in ADHD children was remarkably higher than that in healthy children, suggesting a significant association between miRNA-let-7d and ADHD. During one-year follow-up, it was also found that patients with the lower level of serum miRNA-let-7d showed more pronounced clinical improvement than those with the higher level. All above result suggested that miRNA-let-7d may serve as a specific

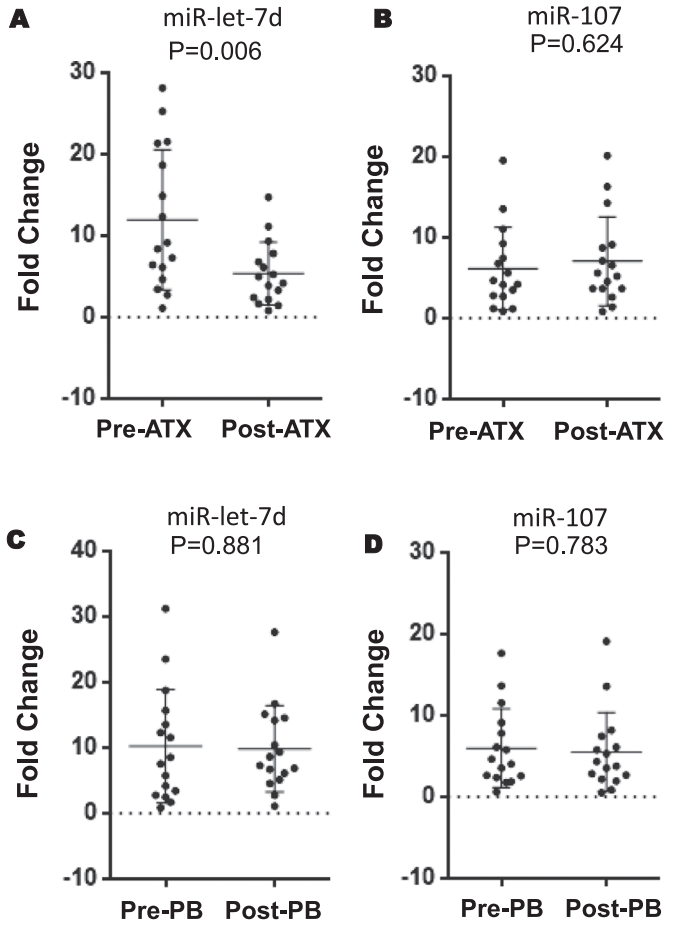

Fig. 4. Fold changes of the level of serum miR-let-7d and miR-107 at pre- and post-ATX or placebo treatment. (A) Fold change of the level of serum miR-let-7d at pre- and post-ATX; (B) Fold change of the level of serum miR-107 at pre- and post-ATX treatment; (C) Fold change of the level of serum miR-let-7d at pre- and post- Placebo treatment; (D) Fold change of the level of serum miR-107 at pre- and post- Placebo treatment. ATX, atomoxetine; PB, placebo. *P* values are displayed at the top of each graph, paired *t*-test.

molecular marker for ADHD diagnosis (Sharma et al., 2017; Wu et al., 2015). Consistently, our results also showed that the level of serum miRNA-let-7d was significantly higher in ADHD patients than in healthy children. These results suggest that miRNA-let-7d may be involved in the pathogenesis of ADHD. Therefore, we also examined the differences in the level of serum miRNA-let-7d before and after treatment in ADHD patients by using two different therapeutic approaches (rTMS and ATX). Interestingly, the results consistently showed the reduced level of serum miRNA-let-7d in patients with ADHD after rTMS treatment compared to the baseline. Thus, the level of serum miRNA-let-7d may be used to assess the therapeutic effects of ADHD patients as a potential biomarker.

Previous studies showed that miRNA-107 is mainly associated with tumors (Sharma et al., 2017). To determine the role of miRNA-107 in ADHD, Kandemir et al. (2014) conducted a controlled study with 52 ADHD patients and 52 healthy volunteers and found that the level of miRNA-107 in blood samples from patients with ADHD patients was significantly lower than that in healthy subjects, with a cut-off point of 0.448 between positive predictive value (70%) and negative predictive value (86.5%). Their findings suggested that abnormal regulation of miRNA-107 levels may be closely related to the pathogenesis of ADHD, perhaps serving as a candidate biomarker for ADHD. In contrast, we failed to observe obvious differences in the level of serum miRNA-107 between the ADHD group and the healthy group, or before and after two different treatments (rTMS or ATX) in the ADHD patients. We speculated that the possible reasons for the difference were from subject heterogeneity and ethnicity.

Although ATX and rTMS were two completely different treatments, they both improved the symptoms of ADHD, and reduced the expression of serum miRNA-let-7d after treatment. Therefore, it was more likely to suggest that it may be used as a biomarker for diagnosis and treatment evaluation. The mechanisms by which the two methods affected the serum level of miRNA-let-7d remained unclear. We speculated that the two treatments may inhibit the formation of miRNA-let-7d in the prefrontal cortex by common mechanisms, such as increasing the level of norepinephrine.

5. Conclusion

Our results showed that the level of serum miRNA-let-7d in ADHD patients is significantly higher as compared to healthy children, and the increased level was downregulated after two different treatments for ADHD: physical therapy (rTMS) or drug treatment (ATX). In contrast, there was no significant difference in the level of serum miRNA-107 between ADHD patients and healthy controls or before and after rTMS or drug treatment. Our findings suggested that serum miRNA-let-7d may serve as a potential biomarker for clinical diagnosis and therapeutic assessment of ADHD.

Conflict of interest

The authors declared no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.02.037.

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