MicroRNAs as potential biomarkers for diagnosis of attention deficit hyperactivity disorder

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From the Contents

Abstract

Inappropriate levels of hyperactivity, impulsivity, and inattention characterize attention deficit hyperactivity disorder, a common childhood-onset neuropsychiatric disorder. The cognitive function and learning ability of children with attention deficit hyperactivity disorder are affected, and these symptoms may persist to adulthood if they are not treated. The diagnosis of attention deficit hyperactivity disorder is only based on symptoms and objective tests for attention deficit hyperactivity disorder are missing. Treatments for attention deficit hyperactivity disorder in children include medications, behavior therapy, counseling, and education services which can relieve many of the symptoms of attention deficit hyperactivity disorder but cannot cure it. There is a need for a molecular biomarker to distinguish attention deficit hyperactivity disorder from healthy subjects and other neurological conditions, which would allow for an earlier and more accurate diagnosis and appropriate treatment to be initiated. Abnormal expression of microRNAs is connected to brain development and disease and could provide novel biomarkers for the diagnosis and prognosis of attention deficit hyperactivity disorder. The recent studies reviewed had performed microRNA profiling in whole blood, white blood cells, blood plasma, and blood serum of children with attention deficit hyperactivity disorder. A large number of microRNAs were dysregulated when compared to healthy controls and with some overlap between individual studies. From the studies that had included a validation set of patients and controls, potential candidate biomarkers for attention deficit hyperactivity disorder in children could be miR-140-3p, let-7g-5p,-30e-5p,-223-3p,-142-5p,-486-5p, -151a-3p,-151a-5p, and-126-5p in total white blood cells, and miR-4516,-6090,-4763-3p,-4281,-4466, -101-3p,-130a-3p,-138-5p,-195-5p, and-106b-5p in blood serum. Further studies are warranted with children and adults with attention deficit hyperactivity disorder, and consideration should be given to utilizing rat models of attention deficit hyperactivity disorder. Animal studies could be used to confirm microRNA findings in human patients and to test the effects of targeting specific microRNAs on disease progression and behavior.

Key Words: adults; attention deficit hyperactivity disorder; biomarkers; blood plasma; blood serum; children; microRNA; total white blood cells; whole blood

Introduction

Inappropriate levels of hyperactivity, impulsivity, and inattention characterize attention deficit hyperactivity disorder (ADHD), a common childhood-onset neuropsychiatric disorder (Battle, 2013) more frequently identified in young males (Biederman, 2005). The cognitive function and learning ability of children with ADHD are affected, and these symptoms may persist to adulthood if they are not treated (Tarver et al., 2014). Worldwide, ADHD prevalence in children is estimated to be about 5% (Polanczyk et al., 2014). However, at least a further 5% of children have symptoms that are subthreshold (Sayal et al., 2018). Owing to age-based variation in behaviors school-grade children are often misdiagnosed (Layton et al., 2018).

The American Psychiatric Association Diagnostic and Statistical Manual (DSM-5) states that children diagnosed with ADHD must have at least six symptoms of inattention or six hyperactivity/impulsivity symptoms from parent or teacher ratings (Austerman, 2015). The judgement of a threshold for abnormal symptoms may be influenced by individual perception and objective tests for ADHD are missing. Applicable molecular biomarkers can assist in the accurate evaluation of ADHD in clinical practice, especially for children. Several clinical subtypes of ADHD are recognized, including predominantly inattentive (ADHD-AI), predominantly hyperactive-impulsive (ADHD-HI), and combined (ADHD-C).

The heritability of ADHD has been estimated as between 70% and 80%, and its pathogenesis and etiology have been suggested to be linked to complex environmental and genetic factors (Gallo and Posner, 2016) and the structural or functional brain abnormalities that occur in ADHD (Wang et al., 2012). MicroRNAs (miRNAs) are a class of endogenous small (18–24) nucleotides which function in central nervous system development such as cell proliferation and differentiation, synaptogenesis, synaptic plasticity, and apoptosis. Abnormal expression of microRNAs affects the regulatory mechanisms of target genes, leading to alterations in neurodevelopmental processes (Corbin et al., 2009; Srivastav et al., 2018). Dopamine levels in patients with ADHD differ from those without the disorder and are caused by increased levels of dopamine transporter-containing proteins in the neurons of patients with untreated ADHD (Family Medicine Austin, 2022). The involvement of multiple signaling pathways in psychiatric disease complicates both the investigation of the underlying biological causes and efforts to identify effective therapies. Focusing on the role of microRNAs in psychiatric diseases may lead not only to identifying the dysregulation of multiple pathways but also to novel therapies that can target entire gene networks.

Genes associated with ADHD include dopamine D4 receptor (*DRD4*), dopamine D5 receptor (*DRD5*), dopamine transporter 1 (*DA1*), serotonergic receptor (*HTR1B*), dopamine beta-hydroxylase enzyme (*DBH*), serotonin transporter enzyme (*5-HTT*), and synaptosomal-associated protein 25 (*SNAP-25*) (Faraone et al., 2005). Therefore, microRNAs might enable novel biomarkers of ADHD to be identified (Paul et al., 2020; Juvale II and Che Has, 2021; Takahashi et al., 2021). Recognition and treatment of ADHD in children are important so that their long-term outcomes can be improved (Colvin and Stern, 2015; Rostain et al., 2015).

Pharmacotherapy, particularly psychostimulant medications which contain methylphenidate and amphetamine, remains a treatment option for ADHD (Rabito-Akon and Correas-Lauffer, 2014). Methylphenidate (MPH) acts by increasing the levels of dopamine and norepinephrine in the synaptic cleft (Steingard et al., 2019). MPH upregulates the expression of the postsynaptic scaffold protein Homer 1a (Yang et al., 2013). The Food and Drug Administration in the USA has approved three non-stimulant medications to treat ADHD: atomoxetine, guanfacine, and clonidine (Food and Drug Administration, 2016). These provide an alternative for children who do not tolerate stimulants well.

Animal studies have investigated whether microRNA expression on miRNA target genes is related to an ADHD phenotype (Wu et al., 2010, 2017; Hong et al., 2013; Yang et al., 2013; Pietrzykowski and Spijker, 2014). The role of specific microRNAs in learning difficulties and memory deficits may be investigated using lentiviral vectors injected into the lateral ventricles of an ADHD rat model to increase or decrease microRNA levels (e.g., miR-384-5p, Xu et al., 2020). We chose to analyze recent literature on the levels of microRNA expression in ADHD human patients that could serve as diagnostic and therapeutic biomarkers.

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MicroRNAs in Attention Deficit Hyperactivity Disorder

A PubMed search was performed for original research articles published from January 2014 to November 2022 on possible miRNA biomarkers in ADHD patients compared to healthy subjects. The steps involved are shown (**Figure 1**). A total of 13 articles were found for this review of which 12 were studies in children and one was in adults. Of the former, three were performed with whole blood, three with total white blood cells, two with blood plasma, and four with blood serum. Of the latter, peripheral blood mononuclear cells were examined. In the studies, ADHD was diagnosed by use of the DSM-4 or -5 criteria together with various ADHD rating scales and interviews. Unless otherwise stated, the ADHD and control groups were matched in gender distribution and age and had not received any treatment for ADHD.

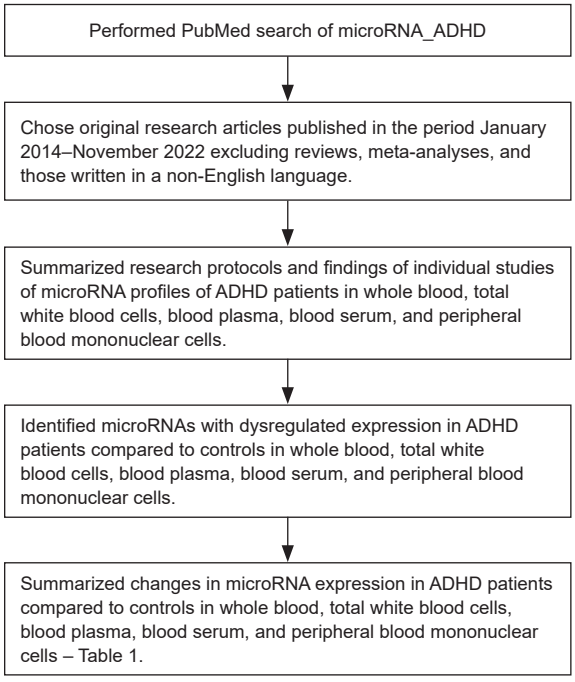

Figure 1 | **Flow diagram showing how the review was performed and its contents.**

In children

Whole blood

Nuzziello et al. (2019) recruited 9 ADHD patients and 20 typically developing children as controls. The ADHD patients were < 18 years of age and not previously treated or under concomitant psychotropic drugs. The ADHD group was comprised wholly of males whereas the control group contained both males and females. Peripheral blood samples with RNA integrity number scores > 7 and with absorbance ratio 260 nm to 280 nm values 1.8–2.2 were analyzed by microfluidic qPCR analysis. Comparisons were performed between the ADHD patients and both the 20 control subjects and the 14 male control subjects, and as the results did not change, those from the first set of data were used for the comparison. Six mature miRNAs were significantly differentially expressed between ADHD and controls (5 upregulated: let 7b-5p fold change (FC)=2.82, miR-942-5p FC=2.46, miR-652-3p FC=1.94, miR-181a-5p FC=2.02, miR-320a FC=2.09; 1 downregulated: miR-148b-3p FC=1.97). After Benjamini-Hochberg correction for multiple testing, only miR-942-5p, -652-3p, and -148b-3p remained significant. By receiver operating curve (ROC) analysis, significant diagnostic values were indicated for all 6 differentially expressed miRNAs with area under the curve (AUC) values > 0.7 for discriminating ADHD patients from controls. The highest AUC values were obtained for miR-320a (0.81) and miR-148-3p (0.88).

Peripheral blood samples were obtained from 30 ADHD patients and 30 healthy controls by Aydin et al. (2019). All of the ADHD subjects were of the combined type and medication naïve. By real time polymerase chain reaction (RT-PCR), the levels of miR-5692b were significantly higher and those of let-7d were significantly lower in the ADHD group compared to controls. There was no significant difference in the levels of miR-124-3p, -4447, and -107 between the two groups. The relative levels of miR-107, -124-3p, -5692b, let-7d, and -4447 in the ADHD group as fold changes compared to the control group were 1.13, 4.81, 3.07, 0.26, and 1.26, respectively.

Using RT-PCR, Kandemir et al. (2014) analysed total RNA extracted from peripheral whole blood samples collected from 52 ADHD patients and 52 controls. All ADHD patients were ≤ 17 years of age and medication naïve. The levels of miR-155a-5p were significantly increased and those of miR-18a-5p, -22-3p, -24-3p, -106b-5p, and -107 were significantly decreased in the ADHD group compared to controls. Decreased levels of miR-125b were found but this was not statistically significant. ROC analysis was performed for miR-107. The positive predictive value and negative predictive value of miR-107 for the cut-off point of 0.4480 were 70% and 86.5%, respectively.

Total white blood cells

Wang et al. (2022) recruited 145 ADHD patients and 83 healthy controls. ADHD patients were 6–16 years of age, drug-naïve and of Han Chinese ethnicity. The healthy control subjects were from the same catchment area and were ethnically Han Chinese. There was a higher proportion of males in the ADHD group, who were younger and had a lower intelligence quotient, compared to the control group. Whole blood was collected from each subject followed by red blood cell (RBC) lysis. Total RNA was extracted from the RBC-free pellets. By RT-PCR, the ΔCt values of 12 miRNAs (miR-140-3p, -27a-3p, -101-3p, -150-5p, let-7g-5p, -30e-5p, -223-3p, -142-5p, -486-5p, -151-3p, -151-5p, and -126-5p) were significantly increased in the ADHD group compared to the control group. When the samples were compared based on gender or age, no miRNA was significantly altered. ADHD patients underwent a 12 months of treatment with MPH. 92 of the 145 ADHD patients completed a follow-up assessment at 12 months. A 30% improvement in symptoms compared to the ADHD-Rating Scale scores (Zhang et al., 2005) at baseline was taken to indicate a response to therapy. In addition, the total ADHD-RS scores ≤ 18. Fifty of the 92 patients met the criteria for remission (responder group) while 42 did not achieve remission during the follow-up period (nonresponder group). The responder group was significantly younger than the nonresponder group (mean age 8.2 years in responders and 9.3 years in nonresponders). The average daily dose of MPH in the responders and nonresponders was 27.0 ± 8.0 mg and 26.9 ± 7.9 mg, respectively. In the 92 ADHD patients, the ΔCt values of 8 miRNAs (miR-140-3p, -27a-3p, -101-3p, let-7g-5p, -30e-5p, -486-5p, -151-5p, and -126-5p) significantly decreased with MPH treatment for 12 months. However, the ΔCt value of miR-150-5p significantly increased. Further stratified analyses showed that the ΔCt values of nine miRNAs (miR-140-3p, -27a-3p, -101-3p, let-7g-5p, -30e-5p, -486-5p, -151-3p, -151-5p, and -126-5p) in the responder group significantly decreased. In the nonresponder group, the ΔCt values of miR-101-3p and miR-150-5p significantly decreased and increased, respectively. From 145 ADHD subjects and 83 healthy controls as a training set, a high-performance prediction model was derived using the miRNA ΔCt values with an AUC of 0.966 in ROC analysis.

Whole blood samples were taken from 30 ADHD patients and 25 controls, and treated with a RBC lysis buffer to obtain RBC-free pellets for extraction of total RNAs by Wang et al. (2020). The ADHD patients were of Han Chinese ethnicity and medication-naïve. The control subjects were drug-naïve, ethnically Han Chinese from the same catchment area as the ADHD patients. By qRT-PCR, the ΔCt values of miR-30e-5p, -126-5p, and -140-3p of the ADHD group were significantly higher than those of the control group. A significantly lower gray volume was found in the cingulate gyrus, left middle temporal gyrus, right middle occipital gyrus, and left fusiform gyrus of ADHD patients. Among the 30 patients with ADHD, the gray matter volume of the cingulate gyrus was negatively correlated with the ΔCt values of miR-30e-5p and miR-140-3p. The gray matter volume of the left fusiform gyrus was negatively correlated with the ΔCt values of miR-30e-5p, -126-5p, and -140-3p. ADHD inattention symptoms were correlated with the ΔCt values of miR-140-3p and ADHD hyperactivity/impulsivity symptoms were correlated with the ΔCt values of miR-30e-5p, -126-5p, and -140-3p.

Wang et al. (2018) recruited 68 ADHD patients and 54 controls in a Training Set. ADHD patients were of Han Chinese ethnicity and medication-naïve. The control subjects were without ADHD and ethnically Han Chinese from the same catchment area as the ADHD patients. Whole blood collected from each subject was treated with RBC-lysis buffer, with total RNAs extracted from the RBC-free pellets. Samples with RNA integrity number scores > 8 were examined. One pooled ADHD patient library and one pooled control library were produced by mixing RNA samples from 5 ADHD patients and 5 healthy controls, respectively. By next-generation sequencing, at least 18 potential miRNA candidates with altered expression levels between ADHD patients and controls were identified. From previous findings (Kandemir et al., 2014; Wu et al., 2015), miR-18a-5p and let-7d were also considered potential biomarkers. By qPCR analysis of samples in the Training Set, the expression levels of 9 miRNAs were significantly altered between ADHD patients and controls (ΔCt values were increased in ADHD compared to control for miR-140-3p, let-7g-5p, -30e-5p, -223-3p, -142-5p, -486-5p, -151a-3p, -151a-5p, and -126-5p). In addition, four miRNAs (miR-27a-3p, -101-3p, -150-5p, and -92a-3p) had a *P* value < 0.1. An ADHD biomarker panel was created using the ΔCt values of the 13 potential biomarker miRNAs from the Training Set. An independent cohort of 20 ADHD patients and 20 healthy control subjects was recruited as the Testing Set and the miRNA ΔCt of the 40 individuals was determined. By support vector machine model alignment, 18 of the 20 ADHD samples were correctly classified as ADHD, and 16 of the 20 healthy control samples were classified as not having ADHD, indicating a sensitivity of 90%, a specificity of 80%, and an accuracy of 85% (AUC 0.91). ADHD patients were differentiated from controls in both the older group (> 9 years, AUC 0.93) and younger group (≤ 9 years, AUC 0.91), and also in both males (AUC 0.90) and females (AUC 0.94).

Blood plasma

Coskun et al. (2021) recruited 50 ADHD-C patients and 48 healthy controls. ADHD patients had not been treated for ADHD. Plasma was obtained from whole blood samples of subjects and total RNA was extracted. By qRT-PCR, Ct

values for miR-132-3p were significantly lower in the ADHD group compared to the control group, while miR-942-5p measurements were not significantly different between the groups. MiR-132-3p expression increased 2.12 times and miR-942-5p expression increased 1.3 times in the ADHD-C group. There was no significant correlation between the age and miR-132-3p and miR-942-5p measurements of ADHD patients.

Blood plasma was obtained from 50 ADHD patients and 44 control subjects by Karadag et al. (2019). ADHD patients were medication-naïve. By qRT-PCR, miR-142-3p and miR-378 measurements were not significantly different in the ADHD group compared to the control group. However, both miR-142-3p and miR-378 were significantly lower in those with a psychiatric disease among the immediate relatives. In the correlation analysis, the presence of any psychiatric disease in the immediate relatives of the ADHD group reduced miR-142-3p and miR-378 levels in the ADHD group compared to the control group.

Blood serum

252 ADHD patients and 430 healthy controls were recruited by Zhu et al. (2022). All ADHD patients were newly diagnosed and medication-naïve. The healthy control subjects were without medication treatment within the previous two weeks. No family members of subjects enrolled in the study had a history of mental illness. Serum samples were obtained from all the subjects and total miRNA was isolated. Microarray analysis was performed of serum samples from 40 ADHD patients and 120 paired healthy controls. 104 miRNAs had significantly altered expression between the ADHD group and the control group. 25 miRNAs had a fold-change > 3 and 9 miRNAs with the most significant differences in expression level were selected for further study (miR-3960, -4281, -4516, -6869-5p, -320c, -16-5p, -4466, -4763-3p, and -6090). Compared to the healthy controls, the expression levels of 8 miRNAs were significantly lower in ADHD patients (miR-4516, -6090, -3960, -4281, -6869-5p, -4763-3p, -320c, and -16-5p) and 1 miRNA had significantly higher expression levels in ADHD patients (miR-4466). These 9 miRNAs were further measured and verified by qPCR in a screening cohort of 40 ADHD patients and 120 healthy controls. The expression levels of miR-4516, -6090, -4763-3p, and -4281 in the ADHD group were significantly lower, while miR-4466 was significantly higher than in the control group. MiR-16-5p could not be detected by qPCR, and the expression level of miR-320 was not significantly different between the ADHD group and the control group. The expression profiles of miR-6869-5p and miR-3960 were not confirmed by qPCR. Based on the screening data, miR-4516, -6090, -4763-3p, -4281, and -4466 were selected for further measurement in an independent cohort of 80 ADHD patients and 80 healthy controls. The expression levels of miR-4516, -6090, -4763-3p, and -4281 were decreased in the ADHD group while that of miR-4466 was increased compared to the control group. To test the specificity, two cohorts were randomly selected of 30 subjects with bronchopneumonia and 30 subjects with vitamin D deficiency matching in gender, age, and height with ADHD patients. The expression levels of miR-4516, -6090, -4763-3p, and -4281 in ADHD were significantly lower and that of miR-4466 was significantly higher in ADHD than those with bronchopneumonia or vitamin D deficiency. To explore whether the three ADHD subtypes could be diagnosed, 99 ADHD-AI, 38 ADHD-HI, and 15 ADHD-C patients were further analyzed. The AUC for ADHD-AI was 0.915, sensitivity 84.0%, and specificity 86.9%. The AUC for ADHD-HI was 0.847, sensitivity 76.3%, and specificity 81.6%. The AUC for ADHD-C was 0.949, sensitivity 96.5%, and specificity 78.3% in comparison with healthy controls. This indicated that the diagnostic performance of the miRNA panel was significant in all ADHD subtypes. 37 ADHD patients were followed-up for 3 months and 9 for 6 months. They were treated with methylphenidate: in the first 6 weeks, the dose was initially 0.3 mg/kg/day, which was increased by 0.3 mg/kg/day every 10 days to a target dose of 1.2 mg/kg/day. Then, treatment with the target dose was maintained until the 10th week. At the 12th week, as the symptoms improved, the patients were evaluated by the Swanson, Nolan and Pelham Version 4 Rating Scale. The score decreased significantly following treatment. Compared with the values before treatment, the expression levels of miR-4516, -6090, -4763-3p, and -4281 in the ADHD patients increased significantly while miR-4466 decreased. Nine patients were followed-up for 6 months after treatment. After treatment, the expression level of serum miRNAs in the ADHD patients became close to that of healthy controls. There was no significant difference between the AUC 0.617 of the 3-month follow-up group and the AUC 0.543 of the 6-month follow-up group.

MiRNA expression in non-hemolyzed blood serum samples collected from 60 ADHD patients and 60 control subjects was examined by Zadehbagheri et al. (2019). All subjects were of the same race and ethnicity and from the same region in Iran. None of the ADHD patients received drug treatment. In the preliminary array screening, four samples from ADHD patients and four healthy controls were randomly selected. Altered expression of 10 miRNAs in the ADHD samples was observed in comparison to controls: five miRNAs (miR-101-3p, -130a-3p, -138-5p, -195-5p, and -19b-3p) were upregulated and five miRNAs (let-7d-5p, miR-105-5p, -106b-5p, -181a-5p, and -320a) were downregulated. In the validation phase of differentially expressed miRNAs, 56 ADHD patients and 56 healthy controls were examined. Using SYBR Green qPCR, four miRNAs (miR-101-3p, -130a-3p, -138-5p, and -195-5p) were upregulated and one miRNA (miR-106b-5p) was downregulated. ROC analysis was performed with the data from the validation cohort of 56 ADHD patients and 56 controls. Significant diagnostic values were shown for all the differentially expressed miRNAs for ADHD. Higher predictive values of AUC (0.959, 0.942, 0.833, and 0.856), sensitivity and specificity were observed for four miRNAs (miR-101-3p, -106b-5p, -130a-3p, and -138-5p). To test the correlation between the expression of validated miRNAs and clinical characteristics of ADHD patients, their expression levels were tested against different neuropsychiatric parameters including intelligence quotient score, impulsive-hyperactive score, hyperactive score and total score. There was only a poor correlation between the total score and the expression level of miR-138-5p. No correlation was found between the expression levels of other miRNAs and these parameters.

Cao et al. (2019) recruited 75 ADHD patients and 18 healthy controls, with the ADHD patients comprising a non-drug group (n = 43) and a drug group (n=32). The non-drug group and drug group were randomly divided into two subgroups, respectively, the real repetitive transcranial magnetic stimulation (rTMS) group (n = 22) and sham rTMS group (n = 21), the atomoxetine (ATX) group (n = 16) and placebo group (n = 16). Four patients in the real rTMS group and five patients in the sham rTMS group were lost, but none were lost during ATX or placebo administration. There was no recent treatment with TMS or electrical shock therapy or mood-regulating medications within 14 days. The rTMS parameters were stimulation frequency 10 Hz; stimulation intensity, 100% motor threshold; 4-second stimulation time followed by 26-second interval; a total of 30 minutes/session with 2400 pulses; one session/day from Monday to Friday, a two-day weekend interval; treatment course of 6 weeks. The right dorsolateral prefrontal cortex was the stimulation site. For the sham rTMS group, the coil was placed perpendicular to the scalp of the stimulation site. The stimulation parameters were the same as those of the real rTMS. ATX HCl was initially administered with a dose of 0.5 mg/kg/day, 3 days later, and then increased to 1.2 mg/kg/day. ATX or placebo was administered for 6 weeks after breakfast. Venous blood was collected at baseline in ADHD children and controls and after the 6-week treatment in ADHD patients. Serum was obtained by centrifugation of the blood. Following 6-week of rTMS or ATX treatment, ADHD patients showed a significant improvement in attention deficit, hyperactivity impulse, and oppositional defiance. Sham rTMS or placebo did not cause any obvious improvements. By qPCR, the expression level of serum let-7d was significantly increased in the ADHD group compared with the healthy control group, but no significant difference was found in the expression level of miR-107 between the two groups. Compared with pre-rTMS or pre-ATX treatment in ADHD patients, the serum let-7d expression level was decreased at post-rTMS or at post-ATX treatment, but there was no significant difference in the expression level of miR-107 between pre-rTMS and post-rTMS or between pre-ATX ad post-ATX. No significant changes were seen in the let-7d level of the sham rTMS or placebo group,

Serum was obtained from 35 ADHD patients and 35 control subjects by Wu et al. (2015). All of the ADHD patients were newly diagnosed and without treatment. In their family, no patient suffered from epilepsy or mental disorders. By RT-qPCR, the expression levels of miR-15b, -337-5p, and -503 were not significantly different between ADHD and controls. Serum let-7d levels were significantly higher in ADHD patients than in controls. The ADHD group comprised 9 ADHD-AI, 5 ADHD-HI, and 21 ADHD-C subtypes, and let-7d was especially increased in ADHD-C. A Ct value ≥ 30 in a 0.5 μg RNA sample was taken as a threshold to differentiate miRNA expression levels among subjects according to a previous report (Ai et al., 2010). Using this cut-off, only 16 (46%) of 35 control subjects had let-7d Ct value < 30, while 69% of the ADHD subjects had Ct value < 30. In 1-year follow-up, a significantly higher rate of clinical improvement was seen in ADHD subjects with low level of serum let-7d than those with high level of serum let-7d.

In adults

Peripheral blood mononuclear cells

A discovery set and follow-up set were recruited by Sanchez-Mora et al. (2019). The discovery set contained 59 ADHD patients who were medication-naïve and 69 healthy controls, while the follow-up set comprised 44 ADHD patients who were medication-naïve and 46 healthy controls. All subjects were of European ancestry and from a restricted geographic area. Peripheral blood mononuclear cells were separated by the Ficoll density gradient method immediately after blood extraction, and total miRNA was isolated. RNA integrity and concentration of samples were assessed. After normalization and background correction, using next-generation sequencing a total of 573 miRNAs were detected in peripheral blood mononuclear cells of at least 45% of individuals from the discovery set. A total of 79 miRNAs were differentially expressed in ADHD after multiple-testing correction, with 55 upregulated and 24 downregulated. The upregulated miRNAs were enriched for the cluster families miR-92a-19a-17-19b, miR-191-425, and let-7a-7b. The predictive performance of the 79 differentially expressed miRNAs was assessed in the follow-up set consisting of 44 ADHD patients and 46 controls. Four miRNAs were significantly downregulated (miR-221-5p, -873-3p, -28-3p, and -191-5p) and 11 were significantly upregulated (miR-199a-3p, -33a-5p, -185b-5p, -26b-5p, -140-3p, -425-3p, -497-5p, -545-5p, -186-3p, -589-3p, and -144-5p) in ADHD subjects compared to controls. Substantial variation in the predictive capacity was observed with AUC values ranging from 0.47 to 0.78. The highest AUC values were found for miR-191-5p (AUC 0.78), miR-26b-5p (AUC 0.72), and miR-185b-5p (AUC 0.74).

The miRNAs having altered expression in whole blood, total white blood cells, blood plasma, and blood serum in children with ADHD and in peripheral blood mononuclear cells in adults with ADHD are summarized in **Table 1**. The group sizes, gender distribution, and mean ages are also shown.

Table 1 | **Alterations of miRNA expression in whole blood, total white blood cells, blood plasma, and blood serum of ADHD children and in peripheral blood mononuclear cells of ADHD adults**

Author	Analysis method	Comparison; number, gender and mean age of subjects	Altered miRNA expression
In children			
Whole blood			
Nuzziello et al., 2019	qPCR	ADHD *vs.* control 9M/0F 14M/6F 9.8±2.6 yr 8.8±3.3 yr	Upregulated: miR-942-5p,-652-3p Downregulated: miR-148b-3p
Aydin et al., 2019	RT-PCR	ADHD-C *vs*. control 23M/7F 16M/14F 9.0±2.3 yr 10.4±3.6 yr	Upregulated: miR-5692b Downregulated: let-7d
Kandemir et al., 2014	RT-PCR	ADHD *vs.* control 52 52 10.1±2.4 yr 10.9±3.0 yr	Upregulated: miR-155a-5p Downregulated: miR-18a-5p,-22-3p,-24-3p,-106b-5p,-107
Total white blood cells			
Wang et al., 2022	qRT-PCR	ADHD *vs.* control 111M/34F 47M/36F 8.9±2.2 yr 9.9±2.6 yr	Downregulated: miR-140-3p,-27a-3p,-101-3p,-150-5p, let-7g-5p,-30e-5p,-223-3p,-142-5p,-486-5p,-151a-3p,-151-5p, 126-5p,
Wang et al., 2022	qRT-PCR	ADHD MPH-responder 50 *vs*. before treatment 8.2 yr	Upregulated: miR-140-3p,-27a-3p,-101-3p, let-7g-5p,-30e-5p,-486-5p,-151-3p,-151-5p,-126-5p Downregulated: miR-150-5p
Wang et al., 2022	qRT-PCR	ADHD MPH-non-responder 42 *vs*. before treatment 9.3 yr	Upregulated: miR-101-3p Downregulated: miR-150-5p
Wang et al., 2020	qRT-PCR	ADHD *vs.* control 19M/11F 12M/13F 10.6±2.1 yr 10.6±3.1 yr	Downregulated: miR-30e-5p,-126-5p,-140-3p
Wang et al., 2018	qPCR	ADHD *vs.* control 57M/11F 31M/23F 9.1±2.2 yr 10.0±2.7 yr	Downregulated: miR-140-3p, let-7g-5p,-30e-5p,-223-3p,-142-5p, -486-5p,-151a-3p,-151a-5p,-126-5p
Blood plasma			
Coskun et al., 2021	qRT-PCR	ADHD-C *vs.* control 37M/13F 33M/15F 8.52.3 yr 9.5±2.8 yr	Upregulated: miR-132-3p
Karadag et al., 2019		ADHD *vs.* control 40M/10F 33M/11F 8.2±2.2 yr 8.5±2.8 yr	Downregulated: miR-142-3p,-378 in those ADHD patients with a psychiatric disease among their immediate relatives
Blood serum			
Zhu et al., 2022	qPCR	ADHD *vs.* control 80 80	Upregulated: miR-4466 Downregulated: miR-4516,-6090,-4763-3p,--4281
Zhu et al., 2022	qPCR	ADHD MPH treated 37 *vs*. before treatment	Upregulated: miR-4516,-6090,-4763-3p,-4281 Downregulated: miR-4466
Zadehbagheri et al., 2019	qPCR	ADHD *vs.* control 56 56	Upregulated: miR-101-3p,-130a-3p,-138-5p,-195-5p Downregulated: miR-106b-5p
Cao et al., 2019	qPCR	ADHD *vs.* control 46M/29F 12M/6F 8.8±2.5 yr 9.2±2.3 yr	Upregulated: let-7d
Cao et al., 2019	qPCR	ADHD rTMS treated 22 *vs*. sham rTMS 21	Downregulated: let-7d
Cao et al., 2019	qPCR	ADHD ATX treated 16 *vs*. placebo 16	Downregulated: let-7d
Wu et al., 2015	qRT-PCR	ADHD *vs.* control 30M/5F 30M/5F 8.8±0.4 yr 8.8±0.4 yr	Upregulated: let-7d
In adults			
Peripheral blood mononuclear cells			
Sánchez-Mora et al. 2019	NGS	ADHD *vs.* control 44 46 30.7±10.8 yr 36.3±9.0 yr	Upregulated: miR-199a-3p,-33a-5p,-185b-5p,-26b-5p,-140-3p, -425-3p,-497-5p,-545-5p,-186-3p,-589-3p,-144-5p Downregulated: miR-221-5p,-873-3p,-28-3p,-191-5p

Ages are given as means (± SD in most cases). All the miRNAs listed are human miRNAs (hsa-miRs). ADHD: Attention deficit hyperactivity disorder; ADHD-C: attention deficit hyperactivity disorder combined type; ATX: atomoxetine; F: female; M: male; MPH: methylphenidate; NGS: next generation sequencing; rTMS: repetitive transcranial magnetic stimulation; RT-PCR: real time polymerase chain reaction; yr: years.

Discussion

ADHD is controversial to diagnose since it is based on the identification of behavioral signs from parent and teacher ratings and the reports of patients or family members. The reliability and validity of ADHD diagnosis are affected by both rater bias and reporting bias of behavioral signs. ADHD is underrecognized and underdiagnosed in most countries, especially in girls and older children (Sayal et al., 2018). There is an urgent need for objective tests of ADHD and identifying biomarkers, particularly ones that could be measured *in vivo* with minimally invasive methods, which may facilitate the diagnosis of ADHD in clinical practice, especially for children, as well as the development of new therapeutic strategies (Faraone et al., 2014).

MiRNA biomarkers have been identified for many neurological diseases and disorders (Taguchi and Wang, 2018) and are promising drug targets for neurological diseases (Wen, 2016; Titze-de-Almeida et al., 2020). The studies reviewed here had performed miRNA profiling in whole blood (three studies), total white blood cells (three studies), blood plasma (two studies),

blood serum (four studies) in children with ADHD, and peripheral blood mononuclear cells (one study) in adults with ADHD (**Table 1**). Similar findings were found in two or more separate studies for some miRNAs. With regard to findings in children with ADHD, miR-106b-5p was downregulated in whole blood (Kandemir et al., 2014) and blood plasma (Zadehbagheri et al., 2019). In addition, let-7d was upregulated in blood serum (Wu et al.., 2015; Cao et al., 2019). In three separate studies with total white blood cells, Wang et al. (2018, 2020, 2022) showed downregulation of miR-140-3p, let-7g-5p, -30e-5p, -223-3p, -142-5p, -486-5p, -151a-3p, -151a-5p, and -126-5p. However, some inconsistencies were observed in the findings. For example, miR-101-3p was downregulated in total white blood cells (Wang et al., 2022) but was upregulated in blood serum (Zadehbagheri et al., 2019). Also, let-7d was downregulated in whole blood (Aydin et al., 2019), which differed from the previously mentioned findings for blood serum (Wu et al.., 2015; Cao et al., 2019). These inconsistencies need to be investigated further. Two of the studies in children with ADHD had examined miRNA expression in response to methylphenidate treatment. Responders to methylphenidate had a reversal of miRNA expression in total white blood cells (upregulated miR-140-3p, -27a-3p, -101-3p, let-7g-5p, -30e-5p, -486-5p, -151-3p, -151-5p, and -126-5p) and were distinguished from nonresponders (who had upregulated miR-101-3p and downregulated miR-150-5p) (Wang et al., 2022). A reversal of miRNA expression in blood serum in children with ADHD was also shown with the administration of methylphenidate (Zhu et al., 2022). Similarly, the treatment of children with ADHD using repetitive transcranial magnetic stimulation or atomoxetine led to a reversal of let-7d expression in blood serum (Cao et al., 2019). It was noted that the presence of any psychiatric disease in the immediate relatives of the ADHD group reduced the levels of the chosen miRNAs (Karadag et al., 2019) and could lead to significant differences when compared to controls. It was planned initially to examine for similarities in the miRNA findings for children and adults with ADHD; however, there was only one study for the latter and in which miR-140-3p was reported to be upregulated in peripheral blood mononuclear cells of adults with ADHD (Sanchez-Mora et al., 2019) but was downregulated in total white blood cells of children with ADHD (Wang et al., 2018, 2020, 2022). No other comparisons of miRNAs could be made.

Receiver operating curve analysis indicated that several miRNAs had good capability to distinguish ADHD patients from control subjects. Included among these in the whole blood of children were miR-320a (AUC 0.81), miR-148-3p (AUC 0.88) (Nuzziello et al., 2019), and miR-107 (Kandemir et al., 2014). In total white blood cells, a panel from 12 selected miRNAs (miR-140-3p, -27a-3p, -101-3p, -150-5p, let-7g-5p, miR-30e-5p, -223-3p, -142-5p, -486-5p, -151-3p, -151-5p, and -126-5p) gave a high-performance prediction model (AUC 0.966) (Wang et al., 2022). An ADHD biomarker panel was created from 13 miRNAs in total white blood cells (miR-140-3p, let-7g-5p, -30e-5p, -223-3p, -142-5p, -486-5p, -151a-3p, -151a-5p, -126-5p, -27a-3p, -101-3p, -150-5p, and -92a-3p) (AUC 0.94) and could effectively differentiate ADHD patients from controls in both the older group (> 9 years, AUC 0.93) and younger group (≤ 9 years, AUC 0.91). It could significantly differentiate ADHD patients and controls both in males (AUC 0.90) and in females (AUC 0.94) (Wang et al., 2018). A 5-miRNA panel in blood serum (miR-4466, -4516, -6090, -4763-3p, and -4281) had a good discriminatory ability to distinguish ADHD patients and controls (AUC 0.927) and all three ADHD subtypes from controls (ADHD-AI, AUC 0.915; ADHD-HI, AUC 0.847; ADHD-C, AUC 0.949) (Zhu et al., 2022). Four miRNAs in blood serum had high predictive values of AUC (0.959, 0.942, 0.833, 0.85 for miR-101-3p, -106b-5p, -130a-3p, -138-5p, respectively) to discriminate ADHD patients from controls (Zadehbagheri et al., 2019). Each of three miRNAs in peripheral blood mononuclear cells had a fair capability to distinguish adult ADHD patients from controls (miR-191-5p, AUC 0.78; miR-26b-5p, AUC 0.72; miR-185b-5p, AUC 0.74) (Sanchez-Mora et al., 2019).

Alterations in the expression levels of miRNAs that were confirmed in a validation set of children with ADHD compared with control subjects were miR-140-3p, let-7g-5p, -30e-5p, -223-3p, -142-5p, -486-5p, -151a-3p, -151a-5p, and -126-5p in total white blood cells (Wang et al., 2018), miR-4516, -6090, 4763-3p, -4281, and -4466 in blood serum (Zhu et al., 2022), and miR-101-3p, -130a-3p, -138-5p, -195-5p, and -106b-5p in blood serum (Zadehbagheri et al., 2019). These miRNAs can be considered as potential candidate biomarkers for ADHD in children. A recent study using an *in vitro* cell model showed that miR-140-3p and miR-126-5p both promoted the differentiation of human cortical neuronal cells and this was achieved by repressing apoptosis and/or necrosis (Wang et al., 2022). A downregulation of miR-140-3p and miR-126-5p in ADHD patients (Wang et al., 2018, 2020, 2022) may suggest that the differentiation of neuronal cells is affected by apoptosis and/or necrosis. MiR-126-5p was found to regulate H9c2 cell viability and apoptosis by targeting interleukin-17A under hypoxic conditions (Ren et al., 2021) and overexpressing miR-140-3p promoted cytoprotection in PC12 cells by reducing inflammation, oxidative stress, and cell apoptosis in an oxygen-glucose deprivation model (Yi et al., 2020). Subcortical volume differences were associated with ADHD and selective neuronal vulnerability is possibly involved in these volumetric losses. Gene expression profiles for three pathways apoptosis, oxidative stress, and autophagy were significantly correlated with ADHD-associated volumetric decreases especially in children with ADHD (Hess et al., 2017). Using magnetic resonance imaging, the gray matter volume of the cingulate gyrus, left middle temporal gyrus, right middle occipital gyrus, and left fusiform gyrus was significantly decreased in children with ADHD, and with significantly higher ΔCt values of miR-126-5p, miR-140-3p, and miR-30e-5p, compared to controls. In ADHD patients, the gray matter volume of the cingulate gyrus and left fusiform gyrus was negatively correlated with the ΔCt values of miR-140-3p and miR-30e-5p (Wang et al, 2020).

The pathways enriched by the miRNAs' predicted target genes for let-7g-5p, -30e-5p, -223-3p included Wnt signaling pathway, PI3K-Akt signaling pathway, axon guidance, MAPK signaling pathway, calcium signaling pathway, and p53 signaling pathway (Wang et al., 2018). More than 200 mRNA targets were found to be common to the downregulated miRNAs miR-6090, -4763-3p, and -4281. These mRNAs were suggested to be largely involved in exocytosis, cellular response to electrical stimulus, regulation of neuron apoptotic process, and neuron differentiation. Downstream analysis indicated that gene targets directly associated with the nervous system were mainly enriched in ion channels, receptor signaling, and synapse functions (Zhu et al., 2022). The neurological pathways enriched by the miRNAs' predicted target genes for miR-101-3p, -130a-3p, -138-5p, -195-5p, and -106b-5p were regulation of dendrite development, neuron recognition, regulation of axon regeneration, central nervous system neuron axonogenesis, regulation of synapse structure or activity, central nervous system neuron development, cellular senescence, Wnt signaling pathway, and cell cycle G1/S phase transition (Zadehbagheri et al., 2019).

Several limitations were identified in many of the reviewed studies: (1) there was a marked difference in the sizes of the ADHD and control groups in some studies (Cao et al., 2019; Nuzziello et al., 2019; Wang et al., 2022) and none of the studies had included a power calculation to estimate the sizes of the groups needed to avoid a type I or type II error (Jones et al., 2003); (2) a gender disproportion between the ADHD and control groups was present in two of the studies (Nuzziello et al., 2019; Wang et al., 2022) and gender was not reported in two studies (Kandemir et al. 2014; Sanchez-Mora et al., 2019); (3) normalization of miRNA expression levels was not reported in two studies (Karadag et al., 2019; Coskun et al., 2021); (4) the absence or presence of any psychiatric disease in the family or immediate relatives of the ADHD patients was only reported in three studies (Wu et al., 2015; Karadag et al., 2019; Zhu et al., 2022); (5) most of the studies measuring miRNA expression levels should best be regarded as discovery studies as no validation study was subsequently performed to confirm the preliminary findings.

In summary, some progress has been made in identifying possible candidate miRNA biomarkers of ADHD in children in biological samples obtained by a minimally invasive method. Further studies are warranted in children and adults with ADHD, paying particular attention to the limitations and weaknesses identified in the studies reviewed. Animal studies would also be helpful and the spontaneously hypertensive rat has been used as a model of experimental ADHD. The differential expression of miRNAs in selected brain tissues of spontaneously hypertensive rat rats could be evaluated by comparison to Wistar Kyoto rats as controls. The influence of specific miRNAs on behavioral parameters may be investigated by intracerebral injection of lentiviral vectors to overexpress or inhibit miRNA expression (Xu et al., 2020). Also, intracisternal injection of 6-hydroxydopamine in neonatal rats has been used to generate an ADHD animal model with desipramine hydrochloride given prior to 6-hydroxydopamine injection to protect noradrenergic neurons (Fan et al., 2020).

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