Impact of microRNAs in Interaction with Environmental Factors on Autism Spectrum Disorder: An Exploratory Pilot Study

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Research

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Abstract

Background: The aim of this study was to explore the main effects of miRNAs as well as their interaction effects with well-replicated autism spectrum disorder (ASD) environmental risk factors on the risk of ASD.

Methods: 159 ASD children (ASD group) and 159 healthy children (control group), aged 2-6 years, were included in this study. ASD diagnoses were based on DSM-5 criteria. The extensive medical and demographic characterization of the two groups were recorded. And microRNAs (miRNAs) in serum were detected by qRT-PCR.

Results: Comparing to control group, ASD group had significant higher rates of maternal stress during pregnancy (p<0.001), maternal drinking during pregnancy (p=0.006), threatened abortion (p=0.011), pregnancy-induced hypertension (p=0.032), gestational diabetes (p=0.039), maternal anemia during pregnancy (p<0.001), umbilical cord knot (p<0.001), neonatal jaundice (p<0.001), family psychiatric history (p=0.001), and much lower birth weight (p=0.012). Furthermore, ASD group had much lower expression levels of has-miR-181b-5p (p<0.001) and has-miR-320a (p<0.001), and significant higher levels of has-miR-19b-3p (p<0.001). The multivariate logistic regression analysis revealed that the interactions of has-miR-320a and maternal stress during pregnancy (OR=39.42, p<0.001), has-miR-19b-3p and neonatal jaundice (OR=2.44, p<0.001), has-miR-181b-5p and family psychiatric history (OR=8.65, p=0.001) could increase ASD risk.

Conclusions: The dysregulation of has-miR-181b-5p,has-miR-320a and has-miR-19b-3p could interact with environmental factors, such as maternal stress during pregnancy, neonatal jaundice and family psychiatric history, to impact the risk of ASD.

1. Background

Autism spectrum disorder (ASD) is a group of heterogeneous brain-based neurodevelopmental disorders characterized by a continuum of deficits in communication, social interaction, behavior and restricted interests[1, 2]. It has been reported that the worldwide prevalence of ASD is between 1% and 3% of the general population[3]. The prevalence of ASD is sex imbalance, with a distribution of three males to one female[4]. In most cases, ASD symptoms are first recognized in early childhood, with an average diagnosis age of 4 years[5]. ASD often impairs social skills and autonomy. Children with ASD experience are difficult in developing social, speech, and behavioral skills.

The etiological factors of ASD remain largely unknown. But it has been reported that risk factors, such as genetics, environmental factors, prenatal and perinatal factors are involved in the development of ASD[6]. Among these, genetics occupies the main factor. Whole-genome and candidate-gene analyses have shown the complex genetic background of ASD[]. Approximately 10% of ASD patients were reported to have an identifiable genetic cause[8], with estimated genetic contributions accounting for > 50–60% of ASD risk[5]. The characteristics of highly genetic and phenotypic heterogeneity made the pathophysiology

of ASD really elusive[9]. Noncoding RNA is a crucial player involved in regulating chromatin structure and gene expression of ASD patients[10]. MicroRNAs (miRNA) are short non-coding RNA molecules with 18–25 nucleotide. They have functions to influence gene expression and numerous cellular processes, such as proliferation, differentiation, and apoptosis[5]. Previously, miRNAs were known to be essential for normal brain development and function, making them attractive biomarker candidates for the central nervous system disorders[2]. It has been reported that miRNAs are also closely associated with the pathogenesis of ASD[2]. miRNAs, such as miR-146a, miR-19b, miR-181b, miR-107 in brain tissues, serum and/or saliva, could be used as diagnostic biomarkers of ASD[11].

Environmental factors are also important for the etiology of ASD. According to several previous studies, environmental factors including drugs, toxic exposures, parental age, nutrition and fetal environment, make up of 40–50% of variance in ASD liability[12]. For example, it is revealed by a meta-analysis of 27 studies that parental age is associated with the risk of ASD in children, as a 10-year increase in maternal and paternal age resulting in a 20% higher risk of ASD in children[13]. Two meta-analyses have focused on the associations between ASD risk and obstetric factors, they found that umbilical cord complications, injury or trauma at birth, multiple births, maternal hemorrhage, low birth weight, neonatal anemia, genital malformation, ABO or Rh blood group incompatibility and hyperbilirubinemia were associated with the risk of ASD[14, 15].

Recent studies have shown that ASD symptoms can be exacerbated by the interaction of genetic and environmental risk factors, suggesting that gene-environment interaction may be a mechanism underlying the etiology of ASD[16]. At present, few studies have focused on the interaction of miRNAs and environmental risk factors in children with ASD. Thus, in this study, we aimed to explore the main effects of miRNAs as well as their interaction effects with well-replicated ASD environmental risk factors on the risk of ASD.

2. Materials And Methods

2.1 Subjects

This multi-center, cross-sectional study included ASD children and healthy children, aged 2–6 years. The recruitment occurred between June, 2018 and June, 2020 at Tangshan Maternal and Child Health Care Hospital and two Special Training Centers. Children were diagnosed as ASD according to the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders) criteria[17] assessed by two developmental pediatricians with at least 10 years experiences. The inclusion criteria for ASD children (ASD group) were clinical diagnosis of ASD and the absence of other medical, neurological, genetic or metabolic condition such as epilepsy, cytogenetically visible chromosomal abnormalities or single-gene disorders. Healthy children for the same period in Tangshan maternal and child health care hospital child health care department of healthy children without any history of ASD, who suffered from neither chronic neurological, metabolic or genetic diseases nor psychiatric disorders, were enrolled as control group.

This study was approved by the ethical committee of North China University of Science and Technology, and written informed consent for participation and publication was obtained from the parents.

2.2 Subject characterization

For all subjects in the two groups, extensive medical and demographic characterization were recorded, including: sex, age, paternal/maternal age, maternal stress during pregnancy, maternal smoking during pregnancy, maternal drinking during pregnancy, toxic exposure during pregnancy, threatened abortion, premature birth, pregnancy-induced hypertension, gestational diabetes, maternal anemia during pregnancy, multivitamins intake during pregnancy, and family psychiatric history.

2.3 RNA extraction and Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

Blood sample was collected from all children in a non-fasting state. 4 mL of peripheral venous blood was collected in an anticoagulant tube with ethylenediaminetetraacetic acid (EDTA). Subsequently, blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to separate the pellet and serum. The serum was stored at -80 °C until analysis.

Serum total RNA was isolated using the TRIZOL reagent (Invitrogen, USA). The cDNA used to evaluate miR-181b/miR-19b/miR-320a was synthesized using the miRcute miRNA cDNA First-Strand Synthesis Kit (Tiangen, China) according to the manufacturer's instructions. The expression of hsa-miR-181b-5p/hsa-miR-19b-3p/hsa-miR-320a was examined using SYBR® Premix Ex Taq TM II (Takara, Japan), with U6 serving as an internal reference. The PCR primers were as follows: hsa-miR-181b-5p, 5’-AACAUUCAUUGCUGUCGGUGGGU-3’;hsa-miR-19b-3p,5’-UGUGCAAAUCCAUGCAAAACUGA-3’;hsa-miR-320a, 5’- AAAAGCUGGGUUGAGAGGGCGA-3’. The qRT-PCR was run on ABI StepOne Plus (Applied Biosystems, CA, USA) using a two-step PCR protocol with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles with a denaturation step at 95 °C for 2 minutes, and an annealing/extension step at 60 °C for 60 seconds. The qRT-PCR data analysis was performed in StepOne Software v2.1 (Applied Biosystems). The miRNA expression was calculated using the $2^{-\Delta\Delta}$Ct method.

2.4 Statistical analyses

Analyses were performed using SPSS version 22.0 (IBM Corp., NY, USA). Demographic characteristics were compared between ASD and control groups using t-test for continuous variables or $\chi 2$ test for categorical variables where appropriate. Multi-variable logistic regression was used to assess possible risk factors for ASD. *P* values < 0.05 were considered statistically significant.

3. Result

3.1 Demographic characteristics

A total of 318 children aged 2–6 years were recruited to this study. Of them, 159 children with ASD were assigned to ASD group, and the remaining 159 healthy children were enrolled as controls. The demographic characteristics of the ASD and control groups are shown in Table 1. Comparing to control group, ASD group had significant higher rates of maternal stress during pregnancy ($p < 0.001$), maternal drinking during pregnancy ($p = 0.006$), threatened abortion ($p = 0.011$), pregnancy-induced hypertension ($p = 0.032$), gestational diabetes ($p = 0.039$), maternal anemia during pregnancy ($p < 0.001$), umbilical cord knot ($p < 0.001$), neonatal jaundice ($p < 0.001$), family psychiatric history ($p = 0.001$), and much lower birth weight ($p = 0.012$).

Table 1
Comparison of demographic characteristics between ASD children and healthy controls

Characteristics	Control group (n = 159)	ASD group (n = 159)	P value
Age (years)	3.30 ± 0.78	3.13 ± 0.91	0.077[a]
Males/females	137/22	142/17	0.393[b]
Paternal age (years)	33.10 ± 6.62	33.18 ± 5.73	0.913[a]
Maternal age (years)	31.92 ± 4.87	31.49 ± 5.24	0.452[a]
Maternal stress during pregnancy [n (%)]	2 (1.26)	48 (30.19)	< 0.001[b]
Maternal smoking during pregnancy [n (%)]	2 (1.26)	8 (5.03)	0.446[b]
Maternal drinking during pregnancy [n (%)]	2 (1.26)	12 (7.55)	0.006[b]
Toxic exposure during pregnancy [n (%)]	5 (3.14)	12 (7.55)	0.081[b]
Threatened abortion [n (%)]	6 (3.77)	18 (11.32)	0.011[b]
Premature birth [n (%)]	14 (8.81)	8 (5.03)	0.185[b]
Pregnancy-induced hypertension [n (%)]	2 (1.26)	9 (5.66)	0.032[b]
Gestational diabetes [n (%)]	0	6 (3.77)	0.039[b]
Maternal anemia during pregnancy [n (%)]	12 (7.55)	44 (27.67)	< 0.001[b]
Multivitamins intake during pregnancy [n (%)]	25 (15.72)	33 (20.75)	0.245 [b]
Caesarean delivery [n (%)]	101 (63.52)	96 (60.38)	0.564 [b]
Birth weight (kg)	3.49 ± 0.51	3.35 ± 0.48	0.012[a]
Umbilical cord knot [n (%)]	20 (12.58)	48 (30.77)	< 0.001[b]
Neonatal jaundice [n (%)]	31 (19.50)	81 (50.94)	< 0.001[b]
Family psychiatric history [n (%)]	4 (2.52)	19 (11.95)	0.001[b]

Note: [a] Student t-test; [b] Chi-square tests.

3.2 The comparion of miRNAs expression between the two groups

As shown in Fig. 1, ASD group had much lower expression levels of has-miR-181b-5p (0.774 ± 0.131 vs. 0.991 ± 0.037, $p < 0.001$) and has-miR-320a (0.805 ± 0.121 vs. 1.010 ± 0.043, $p < 0.001$), and significant higher level of has-miR-19b-3p (1.502 ± 0.413 vs. 1.004 ± 0.044, $p < 0.001$), comparing to the control group.

3.3 The impact of miRNAs and environmental factors interaction on the risk of ASD

Based on the results of univariate analysis, risk factors including maternal stress during pregnancy, maternal drinking during pregnancy, threatened abortion, pregnancy-induced hypertension, gestational diabetes, maternal anemia during pregnancy, umbilical cord knot, neonatal jaundice, family psychiatric history and their interactions with has-miR-181b-5p, has-miR-320a or has-miR-19b-3p were included in the multi-variable logistic regression model. As shown in Table 2, the interactions of has-miR-320a and maternal stress during pregnancy (OR = 39.42, 95% CI: 6.07-255.84; $p < 0.001$), has-miR-19b-3p and neonatal jaundice (OR = 2.44, 95% CI: 1.55–3.84; $p < 0.001$), has-miR-181b-5p and family psychiatric history (OR = 8.65, 95% CI: 2.23–33.58; $p = 0.001$) could increase ASD risk.

Table 2
Final models of stepwise regression for environment risks and miRNAs interaction effects

	β-estimate	SE	*p*-value	OR	95% CI for OR
has-miR-181b-5p	-6.41	1.08	< 0.001	0.002	0-0.014
has-miR-320a	-7.50	1.12	< 0.001	0.001	0-0.005
Family psychiatric history × has-miR-181b-5p	2.16	0.69	0.002	8.65	2.23–33.58
Neonatal jaundice × has-miR-19b-3p	0.89	0.23	< 0.001	2.44	1.55–3.83
Maternal stress during pregnancy × has-miR-320a	3.67	0.95	< 0.001	39.42	6.07-255.84

4. Discussion

ASD is a neurodevelopmental disorder characterized by social communication deficits and repetitive behaviors. Given the multifactorial genetic and environmental risk facts that have been identified in ASD, it is possible that environment and gene interaction might play a role in ASD pathogenesis[16]. In this study, we enrolled 318 ASD and healthy children to explore the main effects of miRNAs as well as their

interaction effects with well-replicated ASD environmental risk factors on the risk of ASD. And we found that the dysregulation of has-miR-181b-5p, has-miR-320a and has-miR-19b-3p could interact with environmental factors to impact the risk of ASD.

A large number of studies demonstrate that ASD is a heritable disorder involving multiple gene networks[5]. miRNAs could influence gene expression, playing important roles in neurodevelopment. It has been reported that miRNAs could influence neurogenesis and synaptogenesis, and participate in the ASD pathogenesis, serving as the biomakers of ASD[5]. miRNAs in salivary or serum showed high accuracies to differentiatiate control and ASD subjects[18, 19]. Several miRNAs including miR-181b-5p, miR-320a, miR-19b-3p, miR-106b, miR-140 and miR-199b are regarded as candidates to identify ASD[5]. In the study conducted by Mundalil et al, miR-181b-5p and miR-320a were downregulated, while miR-19b-3p was upregulated in ASD individuals, comparing to the controls[19]. Our results are in line with Mundalil's findings. We found that the serum levels of has-miR-181b-5p and has-miR-320a in ASD children were much lower than those in healthy controls, while the serum levels of has-miR-19b-3p in ASD children were much higher. The molecular mechanisms underlying miRNA upregulation or downregulation in ASD are still being explored. A review published previously has revealed that the location of specific miRNAs at copy number variant (CNV) loci in ASD may lead to their dysregulation[5]. Another possible mechanism is that individual miRNAs sequences are altered in children with ASD[20].

Investigated biological environmental risk factors in ASD include maternal and paternal age, fetal environment (e.g., sex steroids, maternal infections/immune activation, diabetes, hypertension, or ultrasound examinations), perinatal and obstetric events (e.g., hypoxia), smoking and alcohol use, nutrition and toxic exposures[21]. In this study, we found that ASD group had significant higher rates of maternal stress during pregnancy, maternal drinking during pregnancy, threatened abortion ($p = 0.011$), pregnancy-induced hypertension, gestational diabetes, maternal anemia during pregnancy, umbilical cord knot, neonatal jaundice, family psychiatric history, and much lower birth weight, comparing to the control group. Parental or maternal age is a well-established risk factor for ASD[21]. Unfortunately, in the present study were not significantly different between the ASD and control groups. Lacking of enough sample size may contribute this result.

Previous studies have suggested that the environmental factors may interact with the genetic factors to increase the risk of ASD[22]. Gene-environment interaction is an emerging hypothesis to expound the increased incidence of ASD. miRNAs could possibly be one of those factors which explain this link between genetics and the environment. Hicks et al. found that salivary miRNAs are "altered" in children with ASD, and associated with environmental factors[2]. Nakata et al. identified that miR-6126 was down-regulated in ASD and correlated with the severity of social deficits[23]. In the present study, we found that the dysregulation of has-miR-181b-5p, has-miR-320a and has-miR-19b-3p could interact with environmental factors, such as maternal stress during pregnancy, neonatal jaundice and family psychiatric history, to impact the risk of ASD.

Maternal stress during pregnancy susceptibility appears to affect offspring neurodevelopment[24]. The extent of this risk for ASD has been explored in a number of studies[25]. Previously, dysregulation of miRNAs in offspring brain was found due to maternal stress exposure in rats[26]. This result indicated that miRNAs might be associated with the maternal stress exposure, contributing to the ASD risk. As expected, we found that has-miR-320a could interact with maternal stress exposure to affect ASD risk in this study. And the OR of this interaction was as high as 39.42. It has been reported that total serum/plasma bilirubin levels can exceed an infant's neuroprotective defenses, resulting in neuronal injury[27]. A recent published meta-analysis showed that neonatal jaundice may be associated with ASD[28]. Neonatal jaundice may increase the risk of ASD among children. In this study, we confirmed that neonatal jaundice was a potential risk factor for ASD, and the interaction of neonatal jaundice and has-miR-19b-3p might increase the risk of ASD. Children with a family psychiatric history were more likely to be diagnosed with ASD. Interestingly, in this study, we found that family psychiatric history could interact with has-miR-181b-5p, playing a role on increasing ASD risk.

Several limitations to our findings should be carefully considered. First, the sample size is relatively small. Second, patients consisted of only Chinese individuals from a single hospital. Finally, in this study, the target genes of has-miR-181b-5p, has-miR-320a and has-miR-19b-3p were predicted based on previous studies[5]. The role of other miRNAs should be also investigated.

5. Conclusion

In the present study, we found that the serum levels of has-miR-181b-5p and has-miR-320a in ASD children were much lower than those in healthy controls, while the serum levels of has-miR-19b-3p in ASD children were much higher. The dysregulation of has-miR-181b-5p, has-miR-320a and has-miR-19b-3p could interact with environmental factors, such as maternal stress during pregnancy, neonatal jaundice and family psychiatric history, to impact the risk of ASD.

Abbreviations

ASD:autism spectrum disorder

miRNAs:microRNAs

DSM-5:Diagnostic and Statistical Manual of Mental Disorders

qRT-PCR:Quantitative Real-Time Reverse Transcription PCR

CNV:copy number variant

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the declaration of Helsinki.This study was conducted with approval from the Ethics Committee of North China University of Science and Technology .Written informed consent was obtained from all participants.

The ethics committee's reference number:17168

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Cui LH and Du WR have made substantial contributions to conception and design, Xu N and Dong JY acquisition of data, analysis and interpretation of data;Xia BJ and Ma JY have been involved in drafting the manuscript and revising it critically for important intellectual content;Yan RT,Wang LY and Feng FM have given final approval of the version to be published.

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Figures

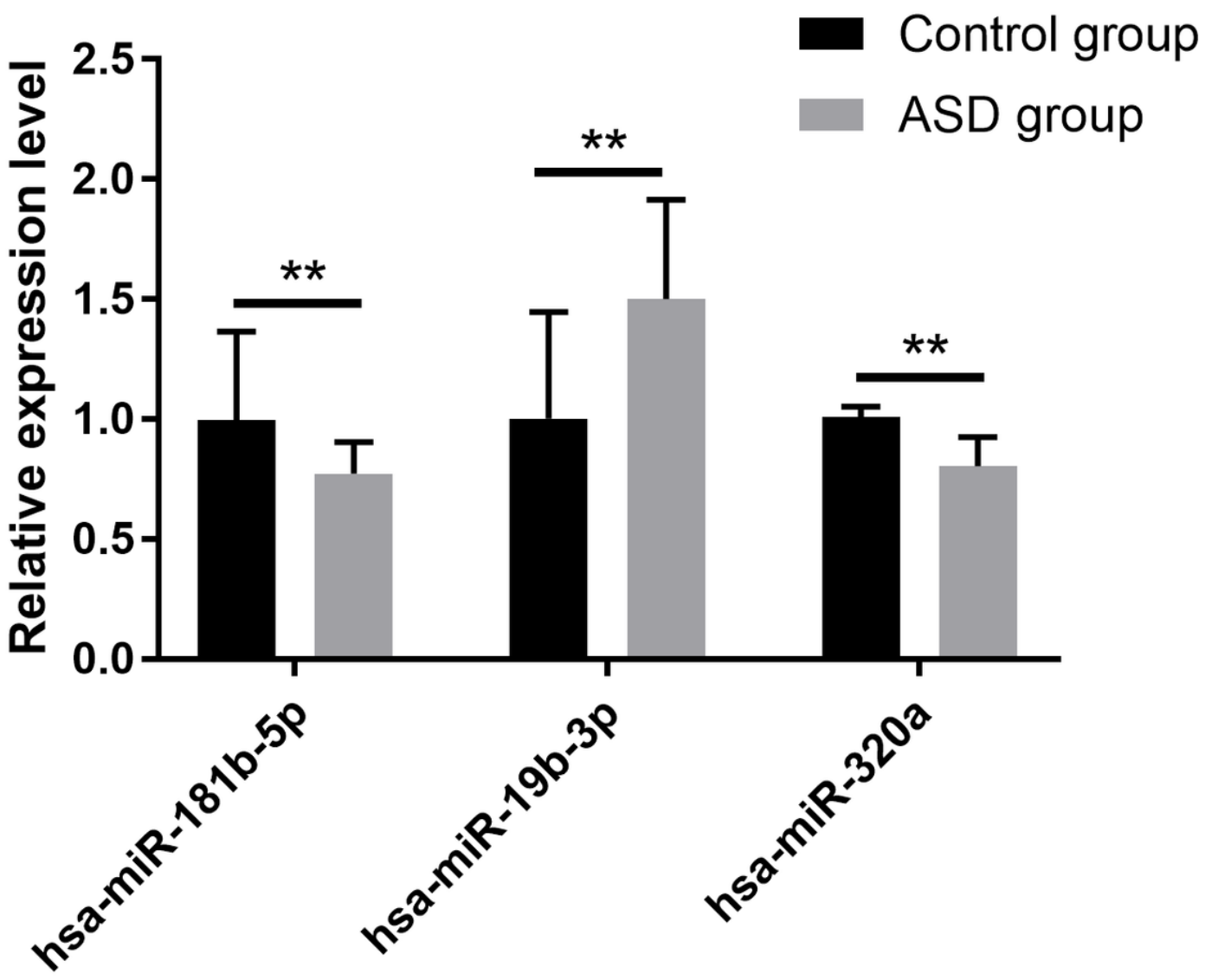

Figure 1

The expressions of miRNAs in two groups. Comparing to the control group, ASD group had much lower expression levels of has-miR-181b-5p and has-miR-320a, and significant higher level of has-miR-19b-3p. **p<0.001 vs. control group.