SUPPLEMENTAL DATA

Serum extracellular vesicle microRNA dysregulation and childhood trauma in adolescents with major depressive disorder

SUPPLEMENTARY METHODS

Assessment

All participants were assessed by well-trained interviewers for clinical and sociodemographic data. The study collected the following information from participants: sex, age, weight, height, smoking history, mental disease history, and the history of neurological or physical illness. At the same time, all participants were assessed by two experienced psychiatrists using the following scales.

A combination of examiner-rating and self-rating scales were used to assess the severity of depression in patients. The examiner-rating scales included CDRS-R [1] and HAMD-17 [2]. The self-rating scale was PHQ-9 [3]. In addition, we used GAD-7 [4], a seven-item self-rating scale, to assess symptoms of anxiety. We also used the CGAS [5], a ten-section examiner-rating scale, to assess the overall functioning of children (age 0-18). Subsequently, we used the CTQ-SF [6], a 28-item retrospective self-rating scale to detect early stressors; this is divided into five sub-scales: sexual abuse, emotional abuse, emotional neglect, physical abuse, and physical neglect. Each sub-scale consists of five items, and each item is rated on five levels (1, 2, 3, 4, and 5 corresponding to never, occasionally, sometimes, often, and always, respectively).

EV isolation

The steps about the extracellular vesicles (EVs) isolation from the serum utilizing the Total EV Isolation Kit were as follows: 1 ml of serum was centrifuged at 2000xg for 30 min at

room temperature at 2000×g; 200 μl (0.2 × volume) of serum EV precipitation reagent (the Total EV Precipitation Reagent) was added to the precipitate, mixed well, and allowed to stand at 4 °C for 30 min. The sample was then centrifuged at 10 000xg for 10 min at room temperature and the supernatant was discarded. After further centrifugation at 10 000xg for 30 s, the residual supernatant was carefully discarded. The EVs forming the precipitate were resuspended in 250 μL 1 × PBS.

Transmission electron microscopy

Approximately 10 μl of the EV suspension was loaded onto the fixed carbon grid at room temperature for 20 min, with excess suspension blotted dry with filter paper. Two percent phosphotungstic acid (Ted Pella, Inc.) was added dropwise to the carbon grid for 20 seconds, with excess phosphotungstic acid removed with filter paper. The carbon grids were examined under a transmission electron microscope (JEM-1400, JEOL, Japan).

Western blot

Serum EVs were lysed in RIPA lysis buffer (Beyotime, China) on ice, with the addition of PMSF (Beyotime, China) a few minutes before homogenization, followed by centrifugation at 12 000×g for 15 min at 4 °C. The protein concentration was measured using a BCA kit (Beyotime, P0010S). The EV protein (50 μg) was then separated on 10% SDS-PAGE, followed by transfer to PVDF membranes (Millipore, IPVH00010), according to the standard instructions. The membranes were blocked with 5% nonfat milk at room temperature for 60 min, after which the primary antibodies were incubated at 4 °C overnight. Then, we recovered the primary antibodies and washed the membranes with 20ml TBST for 10 min at room temperature; this step was repeated three times. After washing, the membranes were incubated with the secondary antibody for 2 h at room temperature. The blots were visualized using the LumiGLO chemiluminescent reagent (Cell Signaling Technology, 7003) and the chemiluminescence image analysis system (Tanon4600, China).

The primary antibodies included rabbit anti-CD9 (1:1000, A1703, ABclonal Technology), anti-CD81 (1:1000, 41779, SAB), anti-TSG101 (1:1000, A5789, ABclonal Technology), and anti-Calnexin (1:1000, 12186, SAB). The secondary antibody was Goat anti-rabbit IgG Peroxidase Conjugated (1:5000, AP132P, Merck Millipore).

Nanoparticle tracking analysis

The concentrations and particle sizes of the extracted EV samples were evaluated by the Nanoparticle tracking analysis device (Particle Metrix, Germany, ZetaView PMX 110, Software version: ZetaView 8.04.02 SP2). After cleaning the sample pool with deionized water, the instrument was calibrated with polystyrene microspheres (110 nm). The sample pool was then cleaned with 1x PBS and the sample was diluted in PBS and loaded for testing.

Small RNA library construction

The miRNA library was built using the NEBNext Small RNA Library Prep kit (NEB, Ipswich, MA, USA) with total RNA (10 ng) as input, following the manufacturers' protocol, except that half quantities of the 3' adaptor, RT primer, and the 5' adaptor were employed. The RNA 3' adaptor was specifically linked to the miRNA with the extra adaptor eliminated through hybridization. The 5' adaptor was linked to the 5' ends of miRNA, and reverse-transcribed to transform the ligated small RNA into cDNA. Then, specific indices were introduced via PCR to produce the sequencing library. Libraries of 147 bp were cut using 6% polyacrylamide gel electrophoresis and quantified with the Kapa Library Quantification kit (Kapa Biosystems, Wilmington, MA, USA) using an ABI 9700HT Fast Real-Time PCR system (Thermo Fisher Scientific).

Bioinformatics analysis

The raw data obtained by sequencing were screened using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). To evaluate the preliminary sequencing results, the sequences were subjected to quality control and were compared with mature miRNA

sequences in the corresponding species miRBase (http://www.mirbase.org/) database. Sequences were also aligned against the Rfam database (http://rfam.xfam.org/) and reference genome.

Real-time PCR quality control and preprocessing

Both the individual samples and the investigator performing the PCR were blinded. We chose one sample from a normal adolescent as the reference sample and divided it into three parts. Each part was carried out with technical repeats and amplified in 3 separate 384-well plates.

We used box and whisker plots based on interquartile ranges to preprocess the outlier data. The box and whisker plot of the interquartile range is an effective and widely used method to identify outliers [7]. Values falling outside the minimum observation of the box and whisker plot (the bottom of the plot = 25th percentile -1.5 × [75th percentile -25th percentile]) and maximum observation of the plot (the top of the plot = 75th percentile + 1.5 × [75th percentile - 25th percentile]) are outliers [8]. We eliminated seven samples as outliers (five depressed adolescents and two healthy adolescents). The average CT values of cel-miR-39 of the samples were located outside the ends of the whiskers (four cases and one control above the top of the whisker, one case and one control below the bottom.) (See Supplementary Figure 4).

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 1. The sequences of the gene primers

MiRNA name	Primer sequence (5' to 3')
cel-miR-39-3p-F	GCTCACCGGGTGTAAATCAGCTTG
hsa-miR-450a-2-3p-F	GCATTGGGGACATTTTGCATTCAT
hsa-miR-615-3p-F	CGAGCCTGGGTCTCCCTCTT
hsa-miR-5100-F	CAGATCCCAGCGGTGCCTCT
hsa-miR-3691-5p-F	AGTGGATGATGGAGACTCGGTAC
hsa-miR-556-3p-F	ATATTACCATTAGCTCATCTTT
hsa-miR-4448-F	GGCTCCTTGGTCTAGGGGTA
hsa-miR-2115-3p-F	CATCAGAATTCATGGAGGCTAG
hsa-miR-4772-5p-F	GTGATCAGGCAAAATTGCAGACT
cel-miR-39-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCT
hsa-miR-450a-2-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGAAT
hsa-miR-615-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGAGG
hsa-miR-5100-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAGGC
hsa-miR-3691-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTACCG
hsa-miR-556-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAGAT
hsa-miR-4448-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACCCC
hsa-miR-2115-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTAGCC
hsa-miR-4772-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCTG

SUPPLEMENTARY TABLE 2. Differentially expressed serum EV miRNAs in nine adolescents with MDD and eight controls shown by high-throughput sequencing

ID-miRNA	MDD	HC	log2FoldChange	p-value	type
*hsa-miR-450a-2-3p	43.06773082	17.18801977	1.321630193	0.001184741	Up
*hsa-miR-615-3p	115.9505545	253.5728074	-1.129488112	0.000511543	Down
*hsa-miR-5100	77.08405756	156.1581592	-1.016076344	0.023910548	Down
*hsa-miR-3691-5p	30.01403496	11.98972807	1.325533968	0.010818114	Up
*hsa-miR-556-3p	59.6038487	28.70436321	1.053239022	0.013458894	Up
*hsa-miR-4448	35.98610219	15.33678182	1.228828163	0.02280119	Up
*hsa-miR-2115-3p	57.14715168	28.54289742	1.000424884	0.036938944	Up
*hsa-miR-4772-5p	21.96770822	9.906155328	1.155478845	0.042470329	Up
hsa-miR-365a-5p	3.534920114	14.2644581	-2.023070793	0.022894253	Down
hsa-miR-3620-5p	5.726584913	15.93185811	-1.488815092	0.026469642	Down
hsa-miR-5701	3.262203798	0	4.048614984	0.02745404	Up
hsa-miR-944	0.096386185	3.372885392	-4.025388316	0.029210614	Down
hsa-miR-6751-5p	5.757888153	0.564873862	3.381786962	0.034517463	Up
hsa-miR-6818-5p	14.40667303	4.653492459	1.620028377	0.03657465	Up
hsa-miR-6775-3p	1.539107226	6.697626875	-2.110459239	0.039200982	Down
hsa-miR-887-5p	0.436588202	5.624936497	-3.595352694	0.039917358	Down
hsa-miR-5697	17.18099635	7.086610623	1.274064144	0.04270391	Up
hsa-miR-4784	4.139131049	0.270289826	3.668718169	0.044217325	Up
hsa-miR-16-1-3p	16.74987041	8.08002852	1.046582202	0.045081806	Up
hsa-miR-3194-3p	5.54504992	16.52183142	-1.566839313	0.046553801	Down
hsa-miR-3196	3.521231426	0.391823002	3.075655267	0.046571731	Up
hsa-miR-6515-3p	0	2.891748957	-4.122726321	0.048900542	Down
hsa-miR-616-5p	11.10246131	0.886778617	3.705664933	0.000986115	Up
hsa-miR-3160-3p	0.174635281	5.509574263	-4.412668264	0.00131914	Down
hsa-miR-4672	12.74549793	2.487456663	2.3448328	0.004444901	Up
hsa-miR-6730-3p	0	4.183893648	-4.65698911	0.006655234	Down
hsa-miR-29b-1-5p	5.872978199	16.05350384	-1.435869715	0.009763602	Down
hsa-miR-455-3p	11.15187437	2.052880234	2.448281771	0.013057946	Up
hsa-miR-6748-5p	4.680204826	0.262013785	3.851026247	0.0171825	Up
hsa-miR-182-3p	1.09955188	10.90811655	-3.288102968	0.018362311	Down
hsa-miR-4659a-5p	1.168298657	6.681926324	-2.545185035	0.019364634	Down
hsa-miR-371b-5p	18.39046576	4.489070247	2.032342293	0.020776968	Up

* For subsequent qRT-PCR verification. MDD: The mean expression level in the major depressive disorder group. HC: The mean expression level in the healthy adolescent control group.

SUPPLEMENTARY TABLE 3. Logistic regression analysis of the expression of three miRNAs and MDD in adolescents

MiRNA	β	SE	p-value	OR	95% CI
miR-450a-2-3p	1.124	0.330	*<0.001*	3.078	1.612-5.877
miR-556-3p	0.791	0.285	*<0.01*	2.206	1.261-3.860
miR-2115-3p	0.883	0.289	*<0.01*	2.419	1.372-4.265

Adjusted for age, gender, body mass index, and smoking status. The expression levels of the miRNAs were carried into the regression model using standardized variables. SE: standard error; OR: odds ratio; CI: confidence interval.

SUPPLEMENTARY TABLE 4. Spearman correlation matrix for variables

	miR-450a-2-3p		miR-556-3p	
	r	p-value	r	p-value
miR-556-3p	0.913	<0.001	-	-
miR-2115-3p	0.964	<0.001	0.921	<0.001

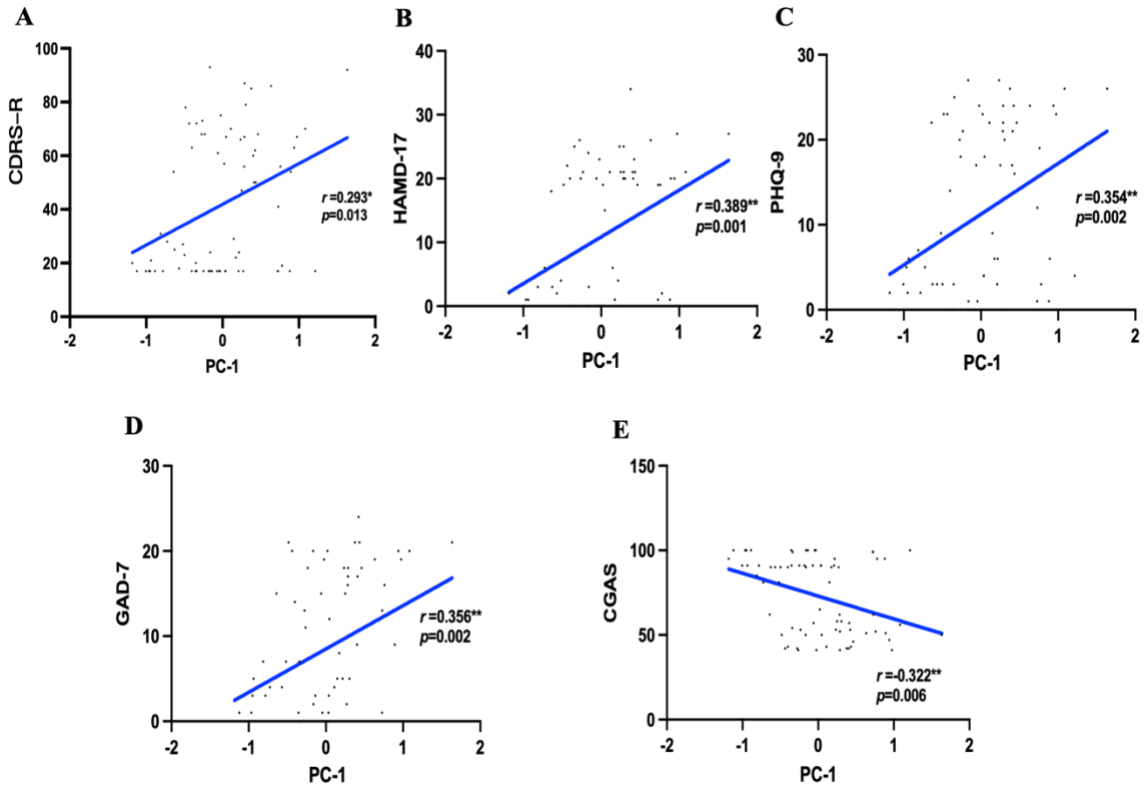
CDRS-R: Children's Depression Rating Scale-Revised; HAMD-17: 17-Item Hamilton Rating Scale for Depression; PHQ-9: Patient Health Questionnaire-9; GAD-7: Generalized Anxiety Disorder; CGAS: Children's Global Assessment Scale.

SUPPLEMENTARY TABLE 5. The prediction of miR-450a-2-3p target genes

miRTarBase ID	miRNA	Species (miRNA)	Target Gene	Target Gene (Entrez ID)	Experiments	Support Type	References (PMID)
MIRT485474	miR-450a-2-3p	Homo sapiens	IGF1R	3480	PAR-CLIP	Functional MTI (Weak)	23592263
MIRT485474	miR-450a-2-3p	Homo sapiens	IGF1R	3480	PAR-CLIP	Functional MTI (Weak)	24398324
MIRT485474	miR-450a-2-3p	Homo sapiens	IGF1R	3480	PAR-CLIP	Functional MTI (Weak)	23446348
MIRT485474	miR-450a-2-3p	Homo sapiens	IGF1R	3480	PAR-CLIP	Functional MTI (Weak)	21572407
MIRT222455	miR-450a-2-3p	Homo sapiens	RAC1	5879	PAR-CLIP	Functional MTI (Weak)	23592263
MIRT506635	miR-450a-2-3p	Homo sapiens	MAPK1	5594	PAR-CLIP	Functional MTI (Weak)	23446348
MIRT506635	miR-450a-2-3p	Homo sapiens	MAPK1	5594	PAR-CLIP	Functional MTI (Weak)	21572407

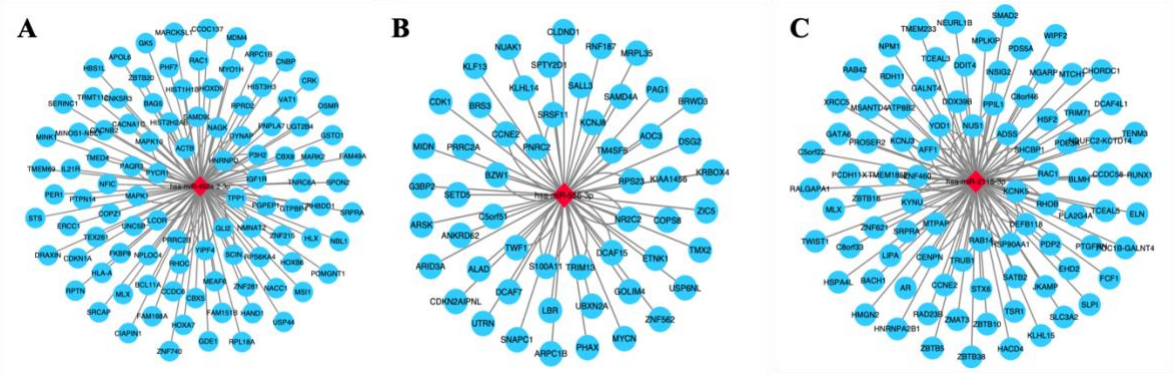
MTI: miRNA-target interaction

SUPPLEMENTARY FIGURES

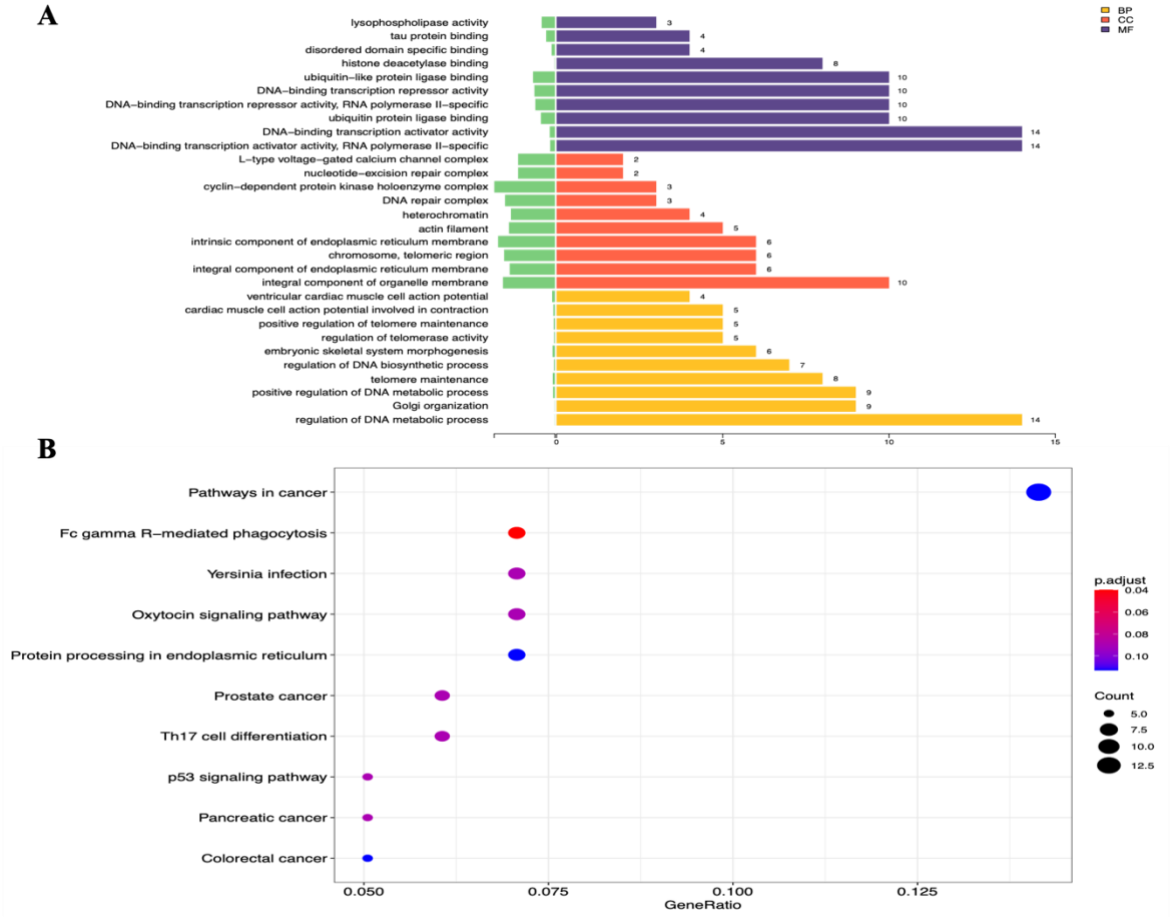


SUPPLEMENTARY FIGURE 1. Correlation analysis of the PC-1 and clinical features. **(A)** A positive correlation between PC-1 and CDRS-R; **(B)** A positive correlation between PC-1 and HAMD-17; **(C)** A positive correlation between PC-1 and PHQ-9; **(D)** A positive correlation between PC-1 and GAD-7; **(E)** A negative correlation between PC-1 and CGAS. * $P < 0.05$. ** $P < 0.001$. PC-1: After the principal component analysis of three differential miRNAs, PC-1 can explained 95.62% of the variance and it was used for correlation analysis.

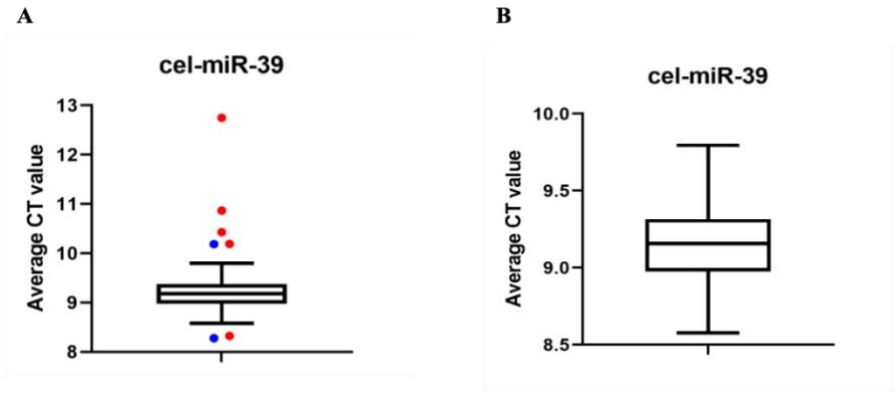
CDRS-R: Children's Depression Rating Scale-Revised; HAMD-17: 17-Item Hamilton Rating Scale for Depression; PHQ-9: Patient Health Questionnaire-9; GAD-7: Generalized Anxiety Disorder; CGAS: Children's Global Assessment Scale.



SUPPLEMENTARY FIGURE 2. Network diagram of the target genes of the four differentially expressed miRNAs. **(A)** miR-450a-2-3p; **(B)** miR-556-3p; **(C)** miR-2115-3p.



SUPPLEMENTARY FIGURE 3. Enrichment analysis of the target genes of the three differentially expressed EV miRNAs. **(A)** Bar plots of Gene Ontology (GO) enrichment analysis. The top 10 enriched GO terms in the three GO categories (molecular function, cell component, and biological process) are displayed. The length of the bar reflects the number of enriched genes. Purple represents molecular function, orange represents cell component, and yellow represents biological process. **(B)** Scatter diagrams of the top 10 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The KEGG enrichment levels were assessed by the P-value, gene concentration in the enriched pathway, and GeneRatio.



SUPPLEMENTARY FIGURE 4. Box and whisker plots of the average CT values of the exogenous control gene cel-miR-39. **(A)** Before removal of outliers. The red dots represent depressed adolescents, and the blue dots represent healthy adolescents. **(B)** After removal of outliers.

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