Association between the methylation of six apoptosis-associated genes with autism spectrum disorder

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Abstract. Excessive apoptosis hinders the process of brain maturation and is regarded as one of the principal risk factors for the development of autism spectrum disorder (ASD). The aim of the present study was to investigate the association between the methylation of six apoptosis-associated genes [*transforming growth factor β 1 (TGFB1)*, *BCL2 associated X, apoptosis regulator*, *insulin like growth factor binding protein 3*, *protein kinase C β 1*, *presenilin 2* and *C-C motif chemokine ligand 2*] and ASD. Using quantitative methylation-specific polymerase chain reaction technology, DNA methylation levels were detected in 42 autistic and 26 control subjects. The logistic regression analysis results demonstrated that of the six genes, only *TGFB1* was significantly hypomethylated in peripheral blood samples from children with autism compared with control samples (mean percentage of methylated reference, 0.011% vs. 0.019%; age-adjusted P=0.028). In addition, *TGFB1* methylation was identified to be positively associated with the interaction ability score from the Autism Behavior Checklist (r=0.452; P=0.035). These data suggested that decreased *TGFB1* methylation may contribute to the development of ASD.

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Introduction

Autism spectrum disorder (ASD) is a disorder of the central nervous system (CNS) that is characterized by impairments in social communication skills, including difficulties with forming and maintaining relationships (1). According to the data of the 2007 and 2011-2012 US National Survey of Children's Health (NSCH), the prevalence of ASD increased between 1.16 and 2.00% (2). At present, the diagnosis of ASD is primarily based on Childhood Autism Rating Scale (CARS) (3) and Autism Behavior Checklist (ABC) (4) scores. Furthermore, there is no cure for ASD, and medical therapy is limited to managing behavioral symptoms (5).

In addition to exposure to environmental toxins at critical periods during brain development, nutritional influences, genetic predisposition and epigenetic modifications contribute to the development of ASD (6). DNA methylation was suggested as a potential mechanism by which environmental factors confer the risk of ASD (7). Evidence additionally suggested that alterations to DNA methylation are common in multiple brain regions of autistic people (8).

Excessive apoptosis impaired brain maturation and mediated autism-like behaviors (9), and results in CNS dysfunction (10). *Transforming growth factor β 1 (TGFB1)* serves an important role in cell proliferation, differentiation, invasion, altering the cellular microenvironment and apoptosis (11). *TGFB1* expression levels were previously observed to be significantly decreased in the plasma of autistic children (12). Apoptosis regulator BAX protein mediates the translocation of cytochrome c between the outer mitochondrial membrane and the cytosol in the apoptotic process (13). *C-C motif chemokine ligand 2 (CCL2)* is important in the protection of human neurons and astrocytes from N-methyl-D-aspartate or human immunodeficiency virus-tat-induced apoptosis (14). *Insulin like growth factor binding protein 3 (IGFBP3)* activates pro-apoptotic factors in various cell lines (15). *Protein kinase C β 1 (PRKCB1)* was demonstrated to serve a pivotal role in ischemia/reperfusion-induced apoptosis (16). *Presenilin 2 (PSEN2)* overexpression was previously reported to be a cause of aberrant apoptosis (17).

At present, to the best of the authors' knowledge, there are no existing studies regarding the DNA methylation status of

the above six apoptosis-associated genes in ASD, the purpose of the present study was to test the association between ASD and the methylation of these genes.

Materials and methods

Subjects. A total of 68 subjects were recruited for the present study. The ASD group included 42 children diagnosed with ASD (35 males, 7 females; mean age, 4.07±2.78 years). All of the children with autism and the control group were recruited from the Children's Psychiatric Clinic of Ningbo Kangning Hospital (Ningbo, China), and their peripheral blood samples were collected (2 ml) between September 2015 and September 2017. They were diagnosed with ASD by at least two psychiatric chief physicians according to the Diagnostic and Statistical Manual-5 diagnostic criteria (DSM-5, American Psychiatric Association, 2013) (18). The CARS and the ABC was additionally used to confirm the accuracy of the diagnosis (19). The tested phenotypes comprised 21 clinical characteristics, including interaction ability, athletic ability and emotional response. The ABC scale score of the 24 participating children with autism that were tested was 30.10±10.30, and the CARS scale score was 78.00±36.15. Patients diagnosed with mental retardation, congenital/genetic disease or severe physical illness were excluded from the present study. The cases included in this study had a CARS score of >30 points, or an ABC scale score of >31 points. A total of 26 children whose physical examination results were completely normal were included in the control group (22 males, 4 females; mean age, 5.85±0.78 years); none of the control subjects had a family or personal medical history of neurological or psychiatric disorders. The general information of the recruited subjects is presented in Table I. All the participants were Han Chinese. The present study was approved by the Bioethics Committees of Ningbo Kangning Hospital and Ningbo University (Ningbo, China). All the parents or guardians of the participating children provided written informed consent.

Quantitative methylation-specific (qMSP) polymerase chain reaction (PCR) assay. The tested fragments of the six genes were obtained from the UCSC genome browser (http://genome.ucsc.edu/). The details of genomic DNA extraction and bisulfite conversion were as described in our previous study (20). The details of the qMSP and the validation of the qMSP products were the same as previously described (21-24). The primer sequences for the six apoptosis-associated genes [*TGFB1*, *BCL2 associated X, apoptosis regulator (BAX)*, *IGFBP3*, *PRKC1*, *PSEN2* and *CCL2*] are presented in Table II.

Capillary electrophoresis of qMSP product. To verify that the fragment size matched the theoretical fragment length, the qMSP product was analyzed by the fully automatic capillary electrophoresis apparatus (Qsep100™ Capillary Gel Electrophoresis System; BiOptic, Inc. Taiwan, China). Equipment and reagents used in the present study were self-contained, including a replaceable gel-cartridge kit, 96-well auto-sampler, buffer tank, burette, centrifugal tubes used for Alignment marker, Alignment marker (20 and 1,000 bp), Alignment marker mineral oil, dilution buffer and separation buffer. The gel-cartridges were stored at 4˚C and the Alignment marker was stored at -20˚C. The percentage of gel matrix in the gel-cartridge was suitable for the analysis of DNA samples of 100-500 bp. One gel-cartridge provides 200 sample injections. All operations were conducted according to the manufacturer's protocol.

Table I. General information of the individuals in the present study.

Variables	ASD	Control	χ^2 value	P-value
No.	42	26	-	-
Sex, M/F	35/7	22/4	0.019	0.889
Age, years	4.07±2.78	5.85±0.78	-	$9.5x10^{-6}$

ASD, autism spectrum disorder; F, female; M, male.

Gene expression omnibus (GEO) data-mining study. The mRNA expression data was extracted from the GSE30192 (25) and GSE5230 (26) GEO datasets (www.ncbi.nlm.nih.gov/gds/). 5-Aza-2'-deoxycytidine (5-AZA) was a demethylation agent. The expression values of genes in 5-AZA-treated C2C12 and HepG2 cells were compared with negative controls. Notably, the C2C12 cell line is a mouse myoblast cell line, and the HepG2 cell line was originally identified as a hepatocellular carcinoma cell line; however, has been demonstrated to be derived from hepatoblastoma (27). An independent samples t-test was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) for the comparison of expression values.

Statistical analysis. Data with normal distribution was presented as the mean ± standard deviation; otherwise, it was presented as the median with interquartile range. Each sample was subjected to three experimental replicates and the mean value was calculated to represent final methylation data. An independent samples t-test was applied to compare the gene methylation data between the case and control groups. A Mann-Whitney U test was used to compare the age distribution between the groups. Pearson's χ^2 test was used to compare the sex distribution between the groups. As the age distribution difference was statistically significant between cases and controls, the binary logistic method (28) was used to correct the age difference. The Pearson's correlation test (normal distribution) and Spearman's rank test (abnormal distribution) were used to determine the associations between gene methylation and ABC or CARS scores. An independent samples t-test was applied to compare gene expression between 5-AZA-treated cells and negative controls. Two-tailed $P<0.05$ was considered to indicate statistically significant difference. The statistical analysis was performed by SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). All figures were produced with GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Location of the tested fragments is identified by the qMSP assay. The tested fragments in the qMSP assay were located in the promoter CpG islands of

Table II. Primer sequences of the six apoptotic genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product, bp	Tm, ˚C
TGFB1	TTGTAGGTGGATAGTTTC	CTACTACCGCTACTACTA	80	58
BAX	GAAGGTATTAGAGTTGCGATT	CCAATAAACATCTCCCGATAA	78	58
IGFBP3	GGTTGTTTAGGGCGAAGTAC	GAAACTATAAAATCCAAACAAAAAACG	209	58
PRKCB	CGGCGTGTTTGATGTTATGAT	GCAACCATCCAACCAACTC	113	58
PSEN2	GGTAGGGTCGTAGGTTTA	ACTTCTAACTATCTCCTCACTA	98	58
CCL2	TGTATTGTTAGGGAGTCGGTTA	TCGCTACCAACTTACCTTCA	89	56

TGFB1, transforming growth factor β 1; BAX, BCL2 associated X, apoptosis regulator; IGFBP3, insulin like growth factor binding protein 3; PRKCB, protein kinase C β 1; PSEN2, presenilin 2; CCL2, C-C motif chemokine ligand 2; Tm, melting temperature.

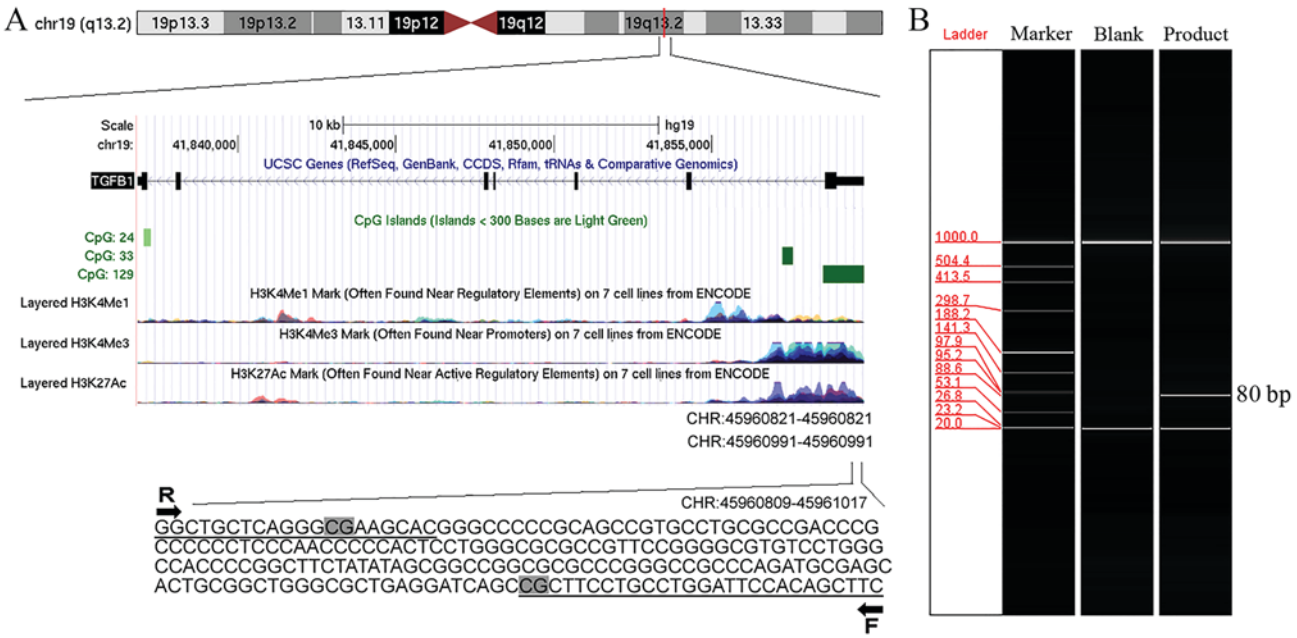

Figure 1. Location and sequence information of *TGFB1* with the PCR product verification. (A) Genomic position and functional annotations of *TGFB1* were obtained from UCSC genome browser, according to human 2013 (GRCh38/hg38) assembly. The quantitative methylation-specific primers are underlined and the two 'CG' dinucleotides are in grey. (B) Capillary electrophoresis demonstrated that the PCR amplified fragment was 80 bp, which was consistent with the prediction. *TGFB1, transforming growth factor β 1*; PCR, polymerase chain reaction; UCSC, University of California, Santa Cruz; F, forward; R, reverse; chr, chromosome.

TGFB1 [chromosome (chr)19:45960809-45961017; Fig. 1A], *BAX* (chr19:49457911-49457988; data not shown), *IGFBP3* (chr7:45960809-45961017; data not shown), *PRKC1* (chr3:169940046-169940158; data not shown), *PSEN2* (chr1:227058897-227058994; data not shown) and *CCL2* (chr17:32581869-32581947; data not shown). The PCR products were verified by capillary electrophoresis (Fig. 1B).

Rate of TGFB1 methylation is significantly decreased in children with ASD. The present analysis demonstrated that the sex distribution was not different between the ASD and control groups (χ^2=0.019; P=0.889; Table I); whereas, age distribution differed significantly between the patients with ASD and the controls (4.07±2.78 vs. 5.85±0.78; P<0.0001; Table I). A logistic regression analysis was subsequently conducted to adjust for the difference in age in the analysis of the association of the six apoptosis-associated genes with ASD. The results suggested that the rate of *TGFB1* methylation in the blood samples from the children with ASD was significantly decreased compared with the control blood samples (mean percentage of methylated reference, 0.011% vs. 0.019%; adjusted P=0.028; Table III and Fig. 2). There were no significant associations of the remaining five genes with ASD subsequent to age adjustment (Table III).

TGFB1 methylation is positively correlated with the interaction ability of the ASD group. In the present study, 24 participants with autism were included, and the CARS and ABC were used to assess ASD clinical phenotypes. Using the Spearman's rank correlation coefficient test, it was identified that *TGFB1* methylation was positively correlated with the interaction ability of the ASD group (r=0.452; P=0.035; Fig. 3). There was no correlation between *TGFB1* methylation and other clinical characteristics (P>0.05; data not shown).

Table III. Comparison of apoptotic gene methylation between patients with ASD and the controls.

Gene	PMR of ASD, %	PMR of controls, %	P-value	P-value[a]
TGFB1	0.011±0.011	0.019±0.016	0.007	0.028
BAX	1.104±0.868	1.238 (0.033, 3.859)	0.595	0.644
IGFBP3	0.032 (0.018, 0.048)	0.021 (0.011, 0.030)	0.003	0.483
CCL2	3.123 (2.747, 3.848)	2.552 (2.064, 3.102)	$2x10^{-4}$	0.995
PSEN2	0.716±0.396	0.894 (0.376, 1.592)	0.107	0.095
PRKCB	1.366±0.725	0.519 (0.365, 0.814)	$3x10^{-14}$	0.281

PMR values are presented as the median (lower quartile, upper quartile) or the mean ± standard deviation. P-values were calculated using the independent samples t-test, and [a]adjusted to correct the difference in age using the binary logistic method. ASD, autism spectrum disorder; PMR, percentage of methylated reference; TGFB1, *transforming growth factor β 1*; *BAX, BCL2 associated X, apoptosis regulator; IGFBP3, insulin like growth factor binding protein 3; PRKCB, protein kinase C β 1; PSEN2, presenilin 2; CCL2, C-C motif chemokine ligand* 2.

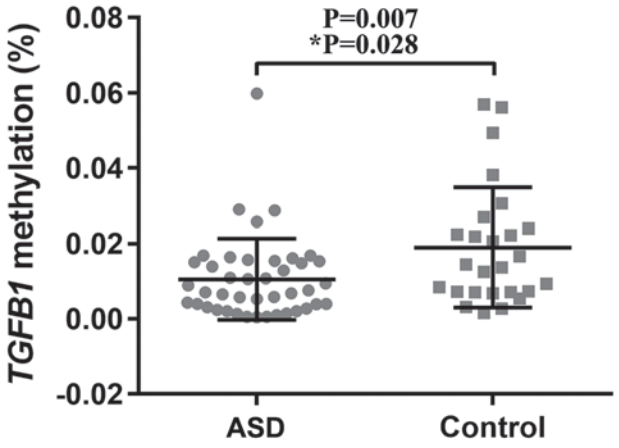

Figure 2. Comparisons of *TGFB1* methylation levels between ASD cases and normal controls. *TGFB1* methylation was significantly decreased in children with ASD compared with the control children (mean percentage of methylated reference, 0.010% vs. 0.018%; P=0.007). *The binary logistic method was used to correct for the age difference (P=0.028). The data are presented as the mean ± standard deviation. *TGFB1, transforming growth factor β 1*; ASD, autism spectrum disorder.

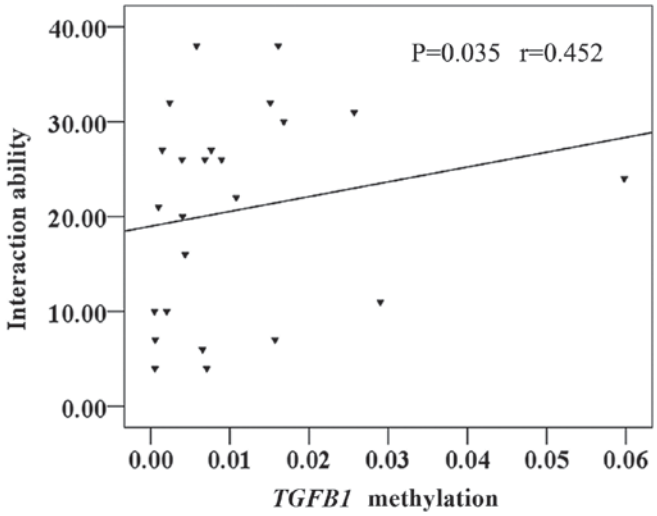

Figure 3. *TGFB1* methylation is positively associated with interaction ability. Spearman's rank correlation coefficient test demonstrated that there was a significant correlation between TGFB1 methylation and interaction ability (P=0.035; r=0.452). *TGFB1, transforming growth factor β 1.*

TGFB1 mRNA expression levels are increased in 5-AZA-treated cell lines. Due to the lack of *TGFB1* expression data for the study participants, it was not possible to assess the effect of *TGFB1* methylation on *TGFB1* expression. Therefore, a GEO dataset was analyzed to identify that the *TGFB1* mRNA expression levels in 5-AZA-treated cell lines were significantly increased compared with the negative controls (C2C12, P=0.022, fold change >1.24; HepG2, P=0.032, fold change >1.96; Fig. 4). These results suggested that *TGFB1* expression may be upregulated by *TGFB1* hypomethylation, although the cell lines were not CNS-specific.

Discussion

In the present study, it was identified that *TGFB1* hypomethylation was significantly associated with ASD. Additionally, aberrant *TGFB1* methylation was positively correlated with the interaction ability score of the subjects. These data suggested epigenetic dysregulation as a potential mechanism for the development of ASD.

Previous studies have identified that alterations in *TGFB1* methylation are associated with the occurrence of CNS disease. Impairment of the TGFB1 signaling pathway is associated with Alzheimer's disease (AD), supporting a role for an alteration in the DNA methylation of *TGFB1* in AD pathogenesis (29). As a complex CNS condition, ASD was previously associated with *TGFB1*-associated apoptotic activity in a number of previous studies (12,30,31). In the present study, it was reported for the first time, to the best of the authors' knowledge, that the hypomethylation of *TGFB1* may be associated with ASD. Additionally, it was observed that the hypomethylation of *TGFB1* is associated with a decreased interaction ability score, which is an important element of social communication ability.

DNA methylation levels of protein-coding genes are generally negatively correlated with expression levels (32,33). To confirm that the *TGFB1* gene methylation identified in the present study affected *TGFB1* expression, the association between *TGFB1* methylation and *TGFB1* expression was examined by GEO data-mining. The present analysis demonstrated that *TGFB1* expression was increased in cells

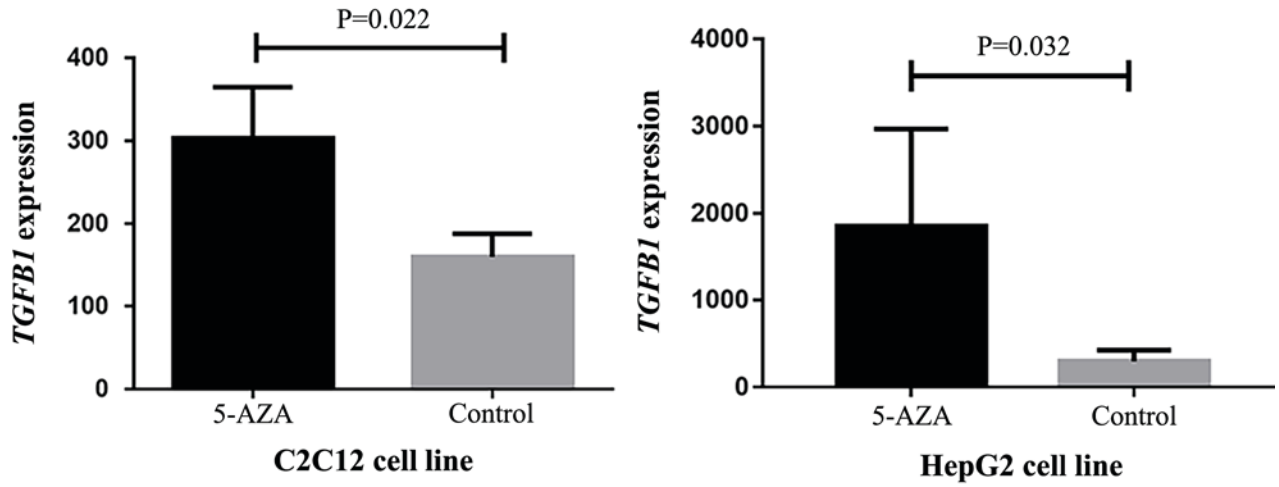

Figure 4. TGFB1 mRNA expression levels are increased in 5-AZA-treated C2C12 and HepG2 cell lines. *TGFB1, transforming growth factor β 1*; 5-AZA, 5-aza-2'-deoxycytidine.

following treatment with the demethylation agent 5-AZA. Although the cell lines were not CNS specific, it is sufficient to demonstrate the negative correlation between methylation and expression of the *TGFB1* gene. Therefore, it was hypothesized that *TGFB1* hypomethylation causes TGFB1 overexpression, leading to subsequent disturbances in apoptosis, inducing cerebral dysplasia and eventually contributing to the development of ASD.

Additionally, other apoptosis-associated genes were considered in the present study that encode proteins previously described to be associated with apoptosis in the brain or ASD. Psychiatric symptoms were accompanied with altered BAX expression and increased neuronal apoptosis in the medial prefrontal cortex in a rat model (34). Children with autism demonstrated increased deficits in cognitive functions, in addition to altered expression levels of immunological markers, including CCL2 (35) and significantly increased blood IGFBP-3 expression levels (36), compared with children without autism. Functional variants of *PRKCB1* were identified to be associated with an increased likelihood of ASD (37). PSEN2 overexpression was observed to induce excessive apoptosis (17). Although the present study was unable to identify any association between the methylation of these genes and ASD, further studies with larger cohorts are required to clarify their roles in ASD.

There were specific limitations to the present study. The verification of the regulatory mechanism of *TGFB1* hypomethylation was based on the GEO data analysis, and the cell lines analyzed were not CNS-specific. Validation with clinical samples is required to confirm the association between *TGFB1* gene methylation and expression. In addition, as the present results were based on a relatively small sample size, larger samples are required to confirm the present results. Furthermore, as obtaining brain tissue from study subjects is not possible, the DNA methylation levels of the six genes were only tested in peripheral blood samples. Further studies are required to examine whether gene methylation may be detected in brain tissue, similar to peripheral blood.

In conclusion, the present data suggested that *TGFB1* hypomethylation may be associated with ASD. The correlation between *TGFB1* hypomethylation and interaction ability may provide novel molecular insight for the understanding of the development of ASD.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SD, ZH and YZ contributed to the conception and design of the study, and the final approval of the manuscript. CZ, HanY, WZ, FC, HaiY, DZ, BL, JL, JD, JZ, MC, TH and RP performed the data analyses and conducted the experiments. YZ and CZ created the figures and tables, and wrote the paper. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Bioethics Committees in Ningbo Kangning Hospital (Ningbo, China) and Ningbo University (Ningbo, China). All the parents or guardians of the participating children provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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