Heliyon 9 (2023) e12635

Contents lists available at ScienceDirect

Heliyon

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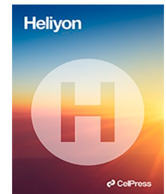

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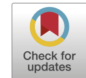

Hypermethylation of the serotonin transporter gene and paternal parenting styles in untreated anorexia nervosa patients: A pilot study

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A R T I C L E I N F O

Keywords:
Anorexia nervosa
SLC6A4
DNA methylation
5-HTTLPR
Epigenetics
Parenting styles

A B S T R A C T

Purpose: It has been reported that serotonergic systems and parenting styles are involved in the pathogenesis of anorexia nervosa (AN). The present study made attempts to examine the DNA methylation profiles in the promoter region of serotonin transporter (5-HTT) encoding gene SLC6A4, and explore the association between the methylation level and severity of symptoms, 5-HTT linked polymorphic region (5-HTTLPR) genotypes and parenting styles in untreated Chinese Han AN patients.
Methods: Ninety-one untreated female AN patients (ANs) and eighty-seven matched healthy controls (HCs) were analyzed for DNA methylation status at CpG islands in the promoter region of SLC6A4 using MassARRY EpiTYPER, and genotypes of 5-HTTLPR using PCR-RFLP. The severity of eating disorder (ED) symptoms was evaluated by body mass index (BMI) and Questionnaire Version of the Eating Disorders Examination (EDE-Q 6.0), and part of participants were assessed parenting styles using the short Chinese Egna Minnen av Barndoms Uppfostra (s-EMBU-C).
Results: ANs had greater methylation levels at CpG26.27.28, CpG 31.32, and CpG 37 than HCs (P = 0.039, 0.042, 0.018 respectively). A positive association of methylation level at CpG26.27.28 with ED symptoms detected by EDEQ-6.0 was discovered in AN group ($r = 0.216$, $P = 0.047$). Methylation level at CpG26.27.28 was showed to be or tend to be positively correlated with the parenting styles of paternal rejection ($r = 0.425$, $P = 0.038$) and paternal overprotection ($r = 0.362$, $P = 0.062$) in ANs. No significant differences were found in SLC6A4 promoter region methylation levels among 5-HTTLPR genotypes in our samples ($P > 0.05$) and no interaction effect between 5-HTTLPR genotypes and parenting styles on SLC6A4 promoter region methylation was observed ($P > 0.05$).
Conclusions: This study suggested that hypermethylation of SLC6A4 promoter region may be implicated in the pathological mechanisms of untreated Chinese Han female ANs, which is possibly associated with poor parenting styles. This finding may provide a direction for the epigenetic and family treatments for ANs and further investigation with larger samples is warranted.

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https://doi.org/10.1016/j.heliyon.2022.e12635
Received 10 July 2022; Received in revised form 28 October 2022; Accepted 19 December 2022
Available online 12 January 2023
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1. Introduction

Anorexia nervosa (AN) is a complex, chronic and refractory eating disorders (EDs) characterized by severe dietary restriction, misperceptions of body shape and weight, combined with some emotional problems, primarily affecting young women [1-3]. AN is divided into two subtypes of restricting type (AN-R) and binge/purging type (AN-BP), in which ANBP additionally experience recurrent symptoms of binge-eating and/or purging behaviors [4,5]. In recent years, the incidence and prevalence of AN patients (ANs) in China have been rising continuously, and has reached the level of other countries in the world [6]. The pathogenesis of AN has been the major awareness for many researchers.

AN is a complex genetic disorder that is thought to be the result of the interaction of gene and environment, both of which are closely linked to the pathogenesis of AN [7,8]. Multiple research groups, including our group, have confirmed the important role of the serotonin (5-HT) system in the pathological mechanism of AN [9-11]. The serotonin transporter (5-HTT) could terminate serotonergic neurotransmission by mediating presynaptic re-uptake of serotonin, coded by the human solute carrier family 6 member 4 (SLC6A4), locating at chromosome 17q11.1-q12 [12]. The 5-HTT could modulate eating behaviors by increasing hunger and promoting a sense of fullness, and is largely affected by nutritional intake [13]. Some animal studies have also identified a role for 5-HT in inhibiting eating behaviors by promoting a sense of fullness and producing anorexic effects [14,15]. Serotonin Transporter Linked-Polymorphic Region (5-HTTLPR) is a functional polymorphism, locating at the promoter region of the SLC6A4 gene and consisting of two common alleles corresponding to insertion (L allele) or delete (S allele) of 44 base pairs. S-allele decreases the 5-HTT transcription activity, resulting in diminished 5-HTT expression and 5-HT reuptake [16,17]. There were some evidences that the 5-HTTLPR genetic variations confer susceptibility for AN [11,18], while few studies have been performed its effect on DNA methylation in ANs.

The family environment and parenting styles were reported to be important factors in the development of EDs [19]. Some prior observations indicated that negative parenting and some adverse childhood experiences may leave imprints on peripheral DNA methylation, which in turn contribute to maladaptive stress response patterns in their later life [20,21], and thus may heighten susceptibility to ED. Limited studies showed that eating disorders were closely associated with the parenting styles of paternal overprotection and authoritarian [22,23], however, scarce studies were conducted to explore the impact of parenting styles on DNA methylation.

DNA methylation is the most extensive epigenetic study, caused by a combination of genetic and environmental factors [24], whose changes can affect gene expression associated with the stress regulatory systems that has been noted to greatly contribute to the pathogenesis of mental disorders [25]. The process of DNA methylation is mediated by environmental influences on gene expression without changing DNA sequences [26], with the addition of a methyl group to nucleotide base pairs cytosine beside a guanine at specific dinucleotide units called CpG islands (CGIs) [27], which are CpG-rich regions approximately 1 kb base pairs long with increased G + C base composition and are exceptionally hypomethylated [28]. SLC6A4 gene hypermethylation is widely studied, as an epigenetic stress marker, resulting in reduced 5-HTT gene expression and decreased 5-HTT production, and therefore a greater buildup of serotonin in the synaptic cleft [29]. Altered function and expression of 5-HTT are closely related to abnormal eating behaviors such as food restriction and food avoidance in the previous study [30].

A meta-analysis showed that SLC6A4 gene methylation was closely related to childhood maltreatment and stressful life events in depression [31], while scarce study was performed to explore the relationship between SLC6A4 gene methylation and parenting styles in ANs. Evidence suggests that adolescents exposed to a negative family environment have a high probability of methylation in their DNA [32]. Some observations have linked later behavioral and mental health problems in adolescents to epigenetic changes resulting from early life stress exposure [32,33]. 5-HTTLPR hypermethylation increased vulnerability of S-allele carriers to psychiatric disorders when exposed to environmental stressors [29]. A significant interaction was displayed between the parenting styles and 5-HTTLPR genotype that people with S/S genotype had increased the susceptibility to AN when facing parenting problems [34].

To our knowledge, few study has so far explored the potential role of the parenting styles and the 5-HTTLPR genotypes together in bringing about the change of SLC6A4 DNA methylation, and thereby increasing susceptibility to AN. Thus, in the present study we made an attempt to examine the SLC6A4 DNA methylation profiles and explore the association between the methylation level and severity of symptoms, 5-HTTLPR genotypes and parenting styles.

2. Materials and methods

2.1. Participants

Ninety-one ANs and eighty-seven healthy controls (HCs) whose age ranged from 13 to 30 years were enrolled from the Eating Disorder Treatment Center, Shanghai Mental Health Center (SMHC, Shanghai, China). HCs were enrolled among students and workers through advertisements during the same period. The inclusion criteria for the two studies were the same. Blood samples and symptom assessment were obtained from all participants. Only 41 ANs and 40 HCs completed the parenting styles assessment because of privacy or time. Inclusion criteria for AN patients were: (1) the first current diagnosis meeting the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria for AN; (2) Chinese Han descent, female; (3) junior high school or above, able to complete the assessment; (4) absence of any medication or psychological therapy for at least three months prior to the enrollment; (5) no evidence of serious organic disease and other psychiatric disorder history; (6) no adverse personal histories such as smoking, drinking or drug use; (7) signed the informed consent. All ANs were classified into restricting (AN-R) subtype (n = 44) and binge/purging (AN-BP) subtype (n = 47). The inclusion criteria for HCs were in line with those of ANs, additional criteria were (1) without diagnosis of DSM-5 mental disorder, (2) no history of psychiatric medication or substance abuse, (3) having a score of <20 on the Eating Attitudes Test (EAT-26),

(4) having a score of <19 on the Beck depression inventory, Version 2 (BDI-II), and (5) having a score of <45 on and Beck anxiety inventory (BAI).

All procedures performed in studies involving human participants were in accordance with the ethical standards and it was approved by the Ethics Committee of Shanghai Metal Health Center (Ethical Approval No.2018–28). A consent form was signed by participants and the parents of those below 18 years old.

2.2. *Clinical assessment*

Demographic and clinical characteristics including onset age, duration of the illness (months), body mass index (calculated by current weight and height), age, education level were collected. Depression and anxiety symptoms were measured for all subjects using BDI-II and BAI scales respectively. The severity of ED symptoms and parenting styles were measured using the following instruments.

2.2.1. *Questionnaire Version of the Eating Disorders Examination (EDE-Q 6.0)*

The scale is widely used to assess the psychopathology of ED patients that has twenty-eight items. Four subscales of restraint, eating concern, shape concern, and weight concern were included to assess the corresponding symptoms. Total score of the EDE-Q6.0 was used to evaluate the severity of ED symptoms. Our previous study has confirmed the application of the scale in mainland China with good reliability and validity [35].

2.2.2. *Short Chinese Egna Minnen av Barndoms Uppfostra (s-EMBU-C)*

The scale was used to evaluate parenting styles with two parts of paternal parenting and maternal parenting. Each part has twenty-three questions extracted from standard EMBU scale with three dimensions: rejection, emotional warmth and overprotection. Total numbers of the three dimensions were 7, 7 and 9 respectively. These items were scored adopting 0–4 point. The reliability and validity were good and the Cronbach's α coefficient was 0.84 in the Chinese college students [36].

2.3. *5-HTT promoter methylation and 5-HTTLPR genotyping*

EDTA tubes were used to collect the venous blood samples from all participants. A DNA extraction kit (Tiangen, China) was used to extract DNA and then a EZ DNA Methylation Kit™ protocol (Zymo Research, USA) was used to purify and convert DNA. The target was the promoter-associated CpG island in our study, whose primers were used to amply DNA. According to the promoter sequence of the SLC6A4 gene published in NCBI database (https://www.ncbi.nlm.nih.gov/gene/6532), EpiDesigner online software (http://www.epidesigner.com/) was used to design the methylation primer of SLC6A4, and the length of the amplification fragment was 467bp, covering the promoter region between −506 and −39 upstream (Ensembl Gene ID ENSG00000108576, Transcript ID ENST00000394821) of SLC6A4 transcription start site. The primers were as follows: forward 5′-aggaaga-gagTTGTTAGGTTTTAGGAAGAAAGAGAGA-3′ and reverse 5′-cagtaatacgactcactatagggagaaggctCCCTCACATAATCTAATCTCTAAA-TAACC-3′, attaching the T7-promoter tag to the reverse primer for the following PCR amplification. PCR reaction conditions were as follows: 94∘C for 4min, followed by 45 cycles at 94∘C for 20sec, 56∘C for 30sec, 72∘C for 1min, then 72∘C for 3min. Shrimp alkaline phosphatase (SAP) were used to treat PCR products, uracil-specific cleavage reaction was then performed using Mass CLEAVE™ Reagent Kit (Agena, USA). The promoter region of the SLC6A4 gene methylation levels were analyzed using the Agena Mass ARRAY platform. Our previous study has published the genotyping protocols of the 5-HTTLPR (long (L) and short (S) allele). The following primers were used to amplify the SLC6A4 gene promoter region variants: 5′-GGCGTTGCCGCTCTGAATGC-3′(forward) and 5′-GAGGGACTGAGCTGGACAACCAC-3' (reverse). The PCR products were verified by agarose gel electrophoresis, ethidium bromide staining and UV display.

All participants completed the detection of SLC6A4 DNA methylation successfully and only ANs were genotyped for 5-HTTLPR, of which eighty patients were detected successfully.

2.4. *Statistical analysis*

All statistical analysis were conducted using SPSS software (version 25.0; IBM SPSS, Chicago, IL, USA). T test or one-way analysis of variance (ANOVA) were used to analyze the differences of continuous variables that were normally distributed. Mann-whitney *U* test or Kruskal-Wallis H test were used to analyze the differences of the variables that were not normally distributed. Apart from the methylation level at CpG31.32 in SLC6A4, other CpG units were all normally distributed. The association between SLC6A4 promoter region methylation level, parenting styles and clinical features was analyzed by pearson correlation analysis (normally distributed variables) and spearman correlation analysis (non-normally distributed variables). Interaction effect between 5-HTTLPR genotypes and parenting styles on SLC6A4 methylation was analyzed by multiple linear regression. Multivariate linear model was performed adjusted for age, BMI, age of onset, and duration of illness as cofounders to explore the effect of SLC6A4 methylation. All tests were two-tailed and *P*-values <0.05 were considered to be statistically significant.

3. Results

3.1. Demographic and clinical characteristics of ANs and HCs

The demographic and clinical characteristics of all participants and participants who completed the parenting evaluation were shown in Table 1 and supplementary materials (Table S1) respectively. There were no significant differences between the two groups in age ($P = 0.068$, $P = 0.110$) and education level ($P = 0.062$, $P = 0.201$) in both parenting completers and non-completers. As expected, the current BMI of ANs were significantly lower than that of HCs ($P < 0.05$), and the EDE-Q6.0 total score of ANs were significantly higher than that of HCs ($P < 0.05$) in both parenting completers and non-completers. In addition, the total score of BDI and BAI scales was higher for ANs than for the HCs ($P > 0.05$).

3.2. Group differences in the promoter region of the SLC6A4 promoter region methylation levels

In this study, methylation levels of 40 CpG units in the promoter region of −506 ~ −39 upstream of SLC6A4 transcription start site were detected. The CpG units were adapted based on the UCSC genome browser website, the appropriate detection fragments, the methylation enrichment sites reported in the previous studies [37,38]. The relationship of the selected splicing fragments in the present study and the CpG units of the SLC6A4 gene was given in Table 2.

As some fragments have too large or too small molecular weight (>7000 Da or & <1500 Da), and some peaks of methylation mass spectrometry overlapped and could not be analyzed, eight CpG islands were analyzed in this study. Over 50% of the subjects failed to detect methylation levels at CpG11 units, so seven CpG units were included in the final analysis. The correlation of each CpG units in the AN group were presented in the supplementary materials (Table S2). Methylation levels of CpG26.27.28, CpG31.32, and CpG37 in the AN group was found to be significantly greater than those in the HC group ($P = 0.039$, 0.042, 0.018 respectively). See Table 3.

ANs were further divided into AN-R (n = 44) and AN-BP (n = 47). It showed significant differences in methylation level of CpG31.32 among AN-R, AN-BP and HC groups ($P = 0.036$). Post-hoc analysis found no significant differences in methylation levels of CpG islands between AN-R and AN-BP group ($P > 0.05$). See Table 3.

3.3. Relationship of the SLC6A4 promoter region methylation levels with clinical symptoms in an patients

According to the correlation analysis, positive correlations were found between CpG26.27.28 methylation level and EDE-Q6.0 total scores ($r = 0.216$, $P = 0.047$). However, it did not find the significant relationship between DNA methylation levels at other CpG units and ED symptoms($P > 0.05$).

3.4. Impact of parenting styles and the 5-HTTLPR genotypes on SLC6A4 DNA methylation in an patients

Through the variance test, it found the scores of paternal rejection and overprotection were significantly higher in AN group than that in HC group ($P = 0.036$, 0.026 respectively), as shown in Table 1. According to the correlation analysis between these poor parenting styles and the SLC6A4 methylation, paternal rejection was found to show a positive correlation with CpG26.27.28 methylation levels ($r = 0.425$, $P = 0.038$). Paternal overprotection tended to have a positive correlation with CpG26.27.28 methylation

Table 1
Demographic and clinical characteristics of the participants.

	AN (n = 91)	HC (n = 87)	*T or Z*	*P*
Demographic characteristics				
Age (years)	18.96 ± 4.14	19.95 ± 3.74	−1.850	0.068
Education level (years)	12.18 ± 2.94	12.99 ± 2.67	−1.876	0.062
Current BMI (kg/m^2)	15.22 ± 2.08	20.02 ± 3.20	−13.13	**<0.001**
Age of Onset (Years)	16.48 ± 2.64			
Duration of illness (months)	29.73 ± 30.99			
Clinical characteristics				
EDE-Q 6.0 total scale	2.33 ± 1.47	0.74 ± 0.74	9.984	**<0.001**
BDI total scale	19.52 ± 12.24	3.08 ± 3.67	11.56	**<0.001**
BAI total scale	11.25 ± 7.69	2.82 ± 5.03	5.28	**<0.001**
	AN(n=41)	**HC(n=40)**	***t***	***P***
s-EMBU-C				
Paternal rejection	10.45 ± 5.57	8.10 ± 2.12	2.182	**0.036**
Paternal emotional warmth	56.72 ± 9.82	57.48 ± 9.34	−0.340	0.735
Paternal overprotection	11.03 ± 4.25	9.32 ± 2.46	2.276	**0.026**
Maternal rejection	13.17 ± 5.57	11.76 ± 2.99	1.265	0.214
Maternal emotional warmth	56.72 ± 9.82	57.48 ± 9.34	−0.340	0.735
Maternal overprotection	35.93 ± 7.96	32.70 ± 6.17	1.883	0.066

AN, anorexia nervosa; HC, healthy control; BMI, body mass index; EDE-Q 6.0, Questionnaire Version of the Eating Disorders Examination; s-EMBU-C, Short Chinese Egna Minnen av Barndoms Uppfostra.

Table 2
The details of the CpG sites in the SLC6A4 promoter region.

CpG ID	CPG Position	SLC6A4_2F_10F	SLC6A4_2F_T7R	base pair	Chr coordinates
SLC6A4_2F_CpG_1	46	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CG	Chr17:28563185
SLC6A4_2F_CpG_3	72	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CG	Chr17:28563159
SLC6A4_2F_CpG_26.27.28	327:329:343	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CGCGGCCCCTCCCTGGCG	Chr17:28562888–28562905
SLC6A4_2F_CpG_31.32	368:370	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CGCG	Chr17:28562861–28562864
SLC6A4_2F_CpG_33.34.35.36	376:378:382:384	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CGCGGCCGCG	Chr17:28562847–28562856
SLC6A4_2F_CpG_37	405	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CG	Chr17:28562826
SLC6A4_2F_CpG_39.40	445:448	aggaagagagTTGTTAGGTTTTAGGAAGAAAGAGAGA	cagtaatacgactcactatagggagaaggct CCCTCACATAATCTAATCTCTAAATAACC	CGGCG	Chr17:28562783–28562787

Table 3
Group differences in the methylation levels of the SLC6A4 promoter region.

CpG units	All AN(n = 91)	AN-R(n = 44)	AN-BP(n = 47)	HC(n = 87)	AN vs. HC *t/Z* (*P*)	AN-R vs. AN-BP vs. HC *F/H* (*P*)
CpG1	9.32 ± 3.16	10.14 ± 3.03	8.55 ± 3.14	9.03 ± 3.60	0.960 (0.337)	5.141 (0.076)
CpG3	4.29 ± 2.54	4.12 ± 2.62	4.41 ± 2.51	4.99 ± 2.62	−1.754 (0.081)	1.743 (0.178)
CpG26.27.28	5.08 ± 2.45	4.86 ± 2.16	5.80 ± 3.81	4.32 ± 2.57	2.065 (**0.039**)	4.810 (0.090)
CpG31.32	2.52 ± 2.99	1.81 ± 2.19	3.08 ± 3.46	1.71 ± 2.42	2.038 (**0.042**)[a]	6.676 (**0.036**)[a]
CpG33.34.35.36	5.51 ± 2.08	5.17 ± 2.00	5.76 ± 2.16	5.21 ± 1.61	1.113 (0.266)	2.004 (0.367)
CpG37	6.51 ± 2.94	6.69 ± 2.66	6.29 ± 3.17	5.57 ± 2.92	2.358 (**0.018**)	5.175 (0.075)
CpG39.40	4.68 ± 2.28	4.20 ± 2.00	4.94 ± 2.44	4.26 ± 2.20	1.255 (0.210)	2.854 (0.240)

AN: anorexia nervosa; AN-R: anorexia nervosa restricting subtype; AN-BP: anorexia nervosa binge/purging subtype; HC: healthy control.
[a] Methylation level of the CpG31.32 was non-normally distributed, the Mann-whitney *U* test and Kruskal-Wallis H test were used; methylation levels of other CpG units were normally distributed, T test and ANOVA test were used.

Table 4
Comparison of the SLC6A4 methylation levels among different 5-HTTLPR genotypes in AN group.

CpG units	Genotype			*F*	*P*
	S/S(n = 51)	S/L(n = 24)	L/L(n = 5)		
CpG1	9.43 ± 2.69	9.71 ± 3.10	6.60 ± 4.45	2.395	0.098
CpG3	4.27 ± 2.52	4.13 ± 2.76	4.80 ± 4.03	0.132	0.877
CpG26.27.28	5.57 ± 3.76	5.25 ± 2.13	3.20 ± 1.48	1.209	0.304
CpG31.32	2.67 ± 2.98	1.96 ± 2.53	0.40 ± 0.55	1.803	0.172
CpG33.34.35.36	5.41 ± 1.86	5.42 ± 2.04	6.60 ± 3.58	0.796	0.455
CpG3ss l7	6.59 ± 2.84	6.58 ± 3.02	6.60 ± 2.86	0.013	0.987
CpG39.40	4.82 ± 2.43	4.13 ± 1.78	4.40 ± 1.34	0.836	0.437

level (r = 0.362, *P* = 0.062).

The distribution and comparison of DNA methylation levels among 5-HTTLPR genotypes was shown in Table 4, while no significant effect of 5-HTTLPR genotypes was found in predicting DNA methylation ($P > 0.05$).

Multivariate linear regression analysis was performed with each CpG unit methylation level as the dependent variable, parenting styles, 5-HTTLPR genotypes as the independent variables, and age, education level, age of onset, duration of illness, BMI, AN subtypes as covariates respectively and it showed that only the CpG33.34.35.36 methylation levels as the dependent variable was significant (F = 3.311, P = 0.035, R2 = 0.707, adjusted R2 = 0.493). See Table S3 in supplementary materials.

3.5. Interaction effect analysis between 5-HTTLPR genotypes and parenting styles on SLC6A4 methylation

Multiple linear regression was performed with CpG26.27.28 methylation level as the dependent variable and 5-HTTLPR genotypes and parenting styles of paternal rejection, overprotection and their interactions as the independent variables. However, no interaction effect between 5-HTTLPR genotypes and paternal rejection (F = 0.491, *P* = 0.698) or paternal overprotection (F = 1.122, *P* = 0.372) on SLC6A4 methylation were found.

4. Discussion

Our study adopted a cross-sectional design to investigate the gene and environment interplay of the 5-HTTLPR variants and parenting styles on the SLC6A4 DNA methylation and abnormal eating symptoms in untreated ANs. First, the higher methylation level at CpG26.27.28, CpG31.32 and CpG37 of SLC6A4 gene may be implicated in AN pathogenesis, but not a biomarker to differentiate the two subtypes of AN. Besides, patients with CpG26.27.28 hypermethylation may have more severe AN symptoms and were more likely to experience paternal rejection and overprotection.

Studies have confirmed that peripheral blood SLC6A4 methylation levels correlate with levels in the brain [37], and most epigenetic studies adopted peripheral blood samples [39,40], which provides a rationale for our study. There have been few studies on SLC6A4 methylation in ED patients. An apparent correlation was found between SLC6A4 hypermethylation and AN in the present study, the results of which were in line with the previous observations in AN [41] and depression [40,42]. While some other studies of ANs did not find similar positive results [43,44], possibly due to the differences in the selected spliced fragments and modest samples. According to our results, increased CpG26.27.28 methylation level was associated with the severity of AN. Probably because SLC6A4 DNA hypermethylation causes the gene silencing, then the decrease of 5-HTT mRNA expression, which lead to the increase of 5-HT transporter and 5-HT reuptake and consequently decreased 5-HT concentration [29,45], The mechanistic effect may contribute to long-lasting sensitivities of the 5-HT system [46] and increase the susceptibility to AN. The chronicity of AN was noted to be associated with DNA methylation levels [47], possibly due to disruption of 5-HT homeostasis leading to changes in the structure and function of brain circuits in response to emotional stress regulation, which may contribute to psychopathological mechanisms [39,48].

Mechanisms of DNA methylation are thought to be involved in altered gene expression response to environmental exposure, which has been implicated in the etiology of ED [8]. Studies have pointed out the material role of 5-HTTLPR genetic variants in regulating

DNA methylation modifications [49] in patients with depression [40,45] and anxiety [50]. A prominent relationship was found between CpG DNA methylation reduction and 5-HTTLPR polymorphism in depression [51]. One study indicated 5-HTTLPR L/L genotype carriers displayed higher SLC6A4 DNA expression in response to early life stress [52]. While some studies showed that the 5-HTTLPR genotype S-allele carriers were increase the susceptibility to AN, as well as the concomitant instability and depressive symptoms, especially when confronted with problematic parenting styles [34,53]. However, the present study failed to show the impact of 5-HTTLPR genotypes on DNA methylation and the interaction effect between 5-HTTLPR genotypes and parenting styles on SLC6A4 methylation, probably due to the modest samples.

Negative parenting style was one of the most significant environmental stressors influencing their children's growth [46]. Some previous studies have investigated the relationship between parenting styles and eating disorders, one of which found that emotional warmth and understanding from parents can be helpful in reducing adolescents' susceptibility to eating disorders [54]. Adolescents who received the parenting styles of overprotection and authoritarian were more vulnerable to eating disorders, suggesting the essential role of negative parenting in EDs [22,23], which resembled what we found. Parenting styles could largely influence adolescents' abilities to cope with stressors and emotionally charged circumstances, and abilities of independence and self-control, which may be vulnerable to eating disorders and impulsive overeating [55,56]. The above findings provided family therapeutic directions for EDs, and achieving a balance between parents by making improvements of fathers' parenting role in the development of children is requisite.

The present study found ANs with CpG26.27.28 hypermethylation had more likely to experience parental rejection or overprotection. There are evidences that epigenetic alterations are involved in embedding the effect of early life experiences in the genome and mediating between environments and later behaviors [57,58]. While the present study did not find the apparent associations between other SLC6A4 DNA methylation levels and parenting styles, possibly because higher methylation levels may reflect effortful adaptation to the environments rather than risk markers of diseases [58,59], which provides a broader understanding for the adaptive value of methylation in response to environmental stressors.

Several limitations should be considered for the present study. Firstly, it is better to collect ANs who have nutritionally recovered to continue follow-up study, however, it is quite difficult to recruit these patients who have been recovered in the country for they usually will not come to the clinic any more. We have to admit that the weight of HCs was normal and the effects of malnutrition on SLC6A4 methylation should be considered, our group have already recruited HCs with low weight recently, some limitations will be improved in our subsequent study. Besides, the factors influencing DNA methylation were not assessed comprehensively enough, such as other environmental factors and patients' nutritional level. On the other hand, only a portion of participants completed the investigation on parenting styles which may affect statistical validity, moreover, the patients filled out this family parenting styles mainly through recall, which may have been somewhat biased. As the study was an exploratory study, we did not perform multiple testing for corrections to increase the likelihood of obtaining significant results.

In conclusion, our study suggested that hypermethylation of SLC6A4 promoter region may be implicated in untreated Chinese Han female AN patients. Participants with CpG26.27.28 hypermethylation had more likely to experience paternal rejection and overprotection which may heighten susceptibility to AN. Besides, increased CpG26.27.28 methylation level may account for more serious symptoms in untreated AN patients. While it failed to find the impact of 5-HTTLPR genotypes on DNA methylation. The present discovery provided a direction of the epigenetic and family treatment for AN patients and further study with larger samples is warranted.

Author contribution

Qianqian He analyzed and interpreted the data and wrote the paper. Cheng Lian, Sufang Peng, and Han Chen Contributed data. Qing Kang performed the experiments. Jue Chen conceived and designed the experiments.

Funding

This study was supported by Grants from Shanghai Jiao Tong University (YG2022ZD026); National Natural Science Foundation of China (81771461); Shanghai Municipal Health Commission (2019ZB0201); Shanghai Clinical Medical Research Center for Psychiatric and Psychological Disorders (19MC1911100); Shanghai clinical research center for mental health (SCRC-MH).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We would like to express our sincere gratitude to all participants who made contribution to the study and all of the editors and reviewers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2022.e12635.

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