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SLC6A3 gene methylation may be associated with alcohol use disorder, but personality and life stress may not influence methylation

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Original Research

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Abstract

Background. DNA methylation plays a crucial role in gene regulation and has been implicated in various neuropsychiatric disorders, including alcohol use disorder (AUD). The rs27072 polymorphism within the SLC6A3 gene has been studied in addictive disorders; however, its role in epigenetic modifications remains unclear. This study investigates the methylation levels of CpG sites near rs27072 and their potential associations with AUD, personality traits, and environmental stressors.

Materials and methods. One hundred twenty-four male participants (66 patients with AUD and 58 controls) were analyzed for DNA methylation at CpG islands proximal to the rs27072 locus. The personality traits and life stress events were assessed in all participants.

Results. AUD patients had a lower methylation level than healthy controls ($p = 0.003$ for total average). However, the results changed to borderline significance after adjusting for clinical covariates in the analysis ($p = 0.042$), and the genotype at rs27072 did not modulate the methylation levels. There is high novelty seeking ($p < 0.001$), and more bad life events in patients with AUD than healthy controls ($p < 0.001$). Additionally, no significant correlations were found between methylation levels and personality traits or life stress scores ($p > 0.05$).

Conclusions. The methylation of the SLC6A3 gene may be marginally associated with AUD; however, the rs27072 genotype, personality, and life stress may not be directly linked to epigenetic modifications. Cross-sectional epigenetic studies may not establish causality; future studies with larger, more diverse cohorts and longitudinal designs are warranted to elucidate the complex interplay in AUD pathophysiology.

Introduction

Alcohol use disorder (AUD) represents one of the most prevalent mental health conditions globally, with profound public health implications. The harmful use of alcohol resulted in approximately 3 million deaths globally in 2016, accounting for 5.3% of all deaths and an economic burden worldwide.[1] Twin and adoption studies have consistently demonstrated the substantial genetic contribution to AUD, with heritability estimates ranging from 50% to 60%.[2] While genetic predisposition forms a critical foundation in understanding AUD, recent evidence underscores the importance of epigenetic modifications as mediators of gene–environment interactions, offering additional layers of explanation in the development of AUD.[3] Epigenetic mechanisms, such as DNA methylation, dynamically regulate gene expression without altering the underlying DNA sequence. DNA methylation occurs at cytosine residues within CpG dinucleotides and represses transcription by altering chromatin structure or blocking the binding of transcription factors.[4] Aberrant methylation patterns have been implicated in several neuropsychiatric disorders, including AUD.[5] These modifications are particularly significant in gene–environment interactions, where environmental stressors such as trauma or adverse life events may influence methylation patterns and alter gene expression.[6]

The dopaminergic system plays a central role in addiction through its regulation of reward circuits and reinforcement of behaviors, creating robust feedback loops that encourage continued substance use.[7] The dopamine transporter (*DAT1*, *SLC6A3*) gene, located on chromosome 5p15.3, is associated with dopamine transporter regulation, linking it directly to reward processing and addiction-related behaviors. The *SLC6A3* has emerged as a key target in epigenetic studies, with its role in addiction-related pathways highlighting the interplay between methylation and gene regulation in the development of AUD.[8] Previous research has primarily focused

on single-nucleotide polymorphisms (SNPs) within this gene, exploring their potential association with substance use disorders and other neuropsychiatric conditions.[9,10] Studies investigating DNA methylation patterns in the promoter region of *SLC6A3* have reported some evidence suggesting altered methylation in addiction but inconsistent findings across cohorts.[11,12] Additionally, studies have not investigated the methylation of the *SLC6A3* exon to AUD. The rs27072 SNP, located within exon 15 of the *SLC6A3* gene, has drawn our interest. Although it has been associated with conditions such as drug addiction and alcohol withdrawal symptoms, there is no established clear relationship between rs27072 methylation and AUD.[9,13]

In addition to genetic and epigenetic factors, personality traits further shape the risk and progression of AUD, interacting dynamically with environmental stressors and gene–environment mechanisms.[14,15] Furthermore, adverse life events, which exacerbate stress and trauma, may amplify the impact of these traits through epigenetic mechanisms, creating a complex web of interactions that contribute to the development and maintenance of AUD.[14,16] Given the multifaceted burden of AUD and the intricate mechanisms underlying its pathogenesis, exploring the interplay of genetic, epigenetic, and psychological factors becomes essential. Such investigations hold the potential to fill critical gaps in understanding how these elements converge in shaping AUD susceptibility. Addressing this gap could illuminate new pathways for understanding AUD's pathophysiology and identify biomarkers for diagnosis and intervention.

This study aims to explore the methylation patterns of CpG islands surrounding the rs27072 polymorphism and their associations with AUD. Furthermore, it integrates epigenetics, specific personality, and life stress events to advance our understanding of AUD's multifactorial etiology.

Materials and methods

Study design and participants

This cross-sectional study was conducted at Tri-Service General Hospital (TSGH) in Taiwan between June 2021 and June 2024. The study protocol was approved by the TSGH Institutional Review Board (IRB number: 1-107-05-011), and written informed consent was obtained from all participants before study enrollment. All participants were unrelated Han Chinese individuals residing in Taiwan. A total of 66 male patients of Han Chinese descent were recruited from the alcohol treatment program at TSGH. Eligible participants were adults aged 20 years or older who met the DSM-5 criteria to confirm AUD and assess for any co-occurring mental illness. To ensure the independence of our samples, we conducted family history interviews with each participant. Only individuals whose families had resided in Taiwan for at least 3 generations were included to minimize genetic heterogeneity. AUD patients with concurrent organic brain disease, severe medical conditions, or other major psychotic disorders and other substance use disorders (except nicotine) were excluded from the study. In addition, age-matched healthy male controls (n = 58) were recruited from the community through advertisements and underwent identical screening procedures as the patients.

Personality and life events assessment

Personality traits were assessed using the Chinese version of the Tridimensional Personality Questionnaire (TPQ) during euthymic phases. TPQ is a 68-item self-report questionnaire that measures novelty seeking (NS) and harm avoidance (HA). Internal consistency analysis revealed Cronbach's alpha values of 0.70 and 0.87 for NS and HA subscales, respectively. Recent life events were evaluated using the Chinese Life Event Questionnaire (LEQ), which demonstrated robust test–retest reliability with kappa values of 0.949 for the positive events score (Pes) and 0.889 for the negative events score (Nes). The LEQ assessed 82 items across 11 life domains, with impact ratings ranging from "no effect" to "significant effect" on a 4-point scale.[17]

DNA methylation and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using DNAzol kit (Invitrogen, USA). All DNA samples had to meet quality criteria, with absorbance ratios (A260/280: 1.65–1.85 and A260/230: 1.8–2.1) and a minimum concentration of 50 ng/μl. Sodium bisulfite conversion was performed on 2 μg of genomic DNA using the Epitect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. 2 μl of each sample were used as templates in PCR reactions with the following primer, Left primer: GGATTTTGTATGAATTTGTGGTTTTT, Right primer: ATATAAAACTCCCTCCCTCCCTACT. Amplifications and cloning have been previously described in the literature.[18] The sequence contains 231 bp of exon15 of the SLC6A3 gene, which includes SNP rs27072. It covers 5 CpG methylation islands. However, we exclude the CpG-5 island site from the analysis because the site is SNP rs27072 itself, which cannot be methylated in the TT allele. The genotypes of rs27072 were genotyped using TaqMan assays incorporating FAM and VIC® dyes. Thermocycling and data collection were conducted using the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The procedure was replicated for 10 randomly selected samples to ensure genotyping accuracy and validated against results from restriction fragment length polymorphism analysis and Sanger sequencing.

Statistical analysis

All statistical analyses were conducted using SPSS version 22. Descriptive statistics were used to summarize the data, with means and standard deviations reported for continuous variables and percentages for categorical variables. All tests were 2-tailed, with a significance level of $p < 0.05$. Independent samples t-tests were conducted to compare methylation levels between patients with AUD and healthy controls. We performed a 2-step linear-regression framework. Step 1 was fitted to the full sample, comprising individuals with AUD and healthy controls; this model adjusted for age, family history of AUD, and current nicotine-use disorder (NUD). Family history was coded as positive when participants reported at least one third-degree relative with problem drinking, and NUD was defined by matching two or more DSM-5 criteria during the clinical interview. Step 2 focused exclusively on the AUD cohort and re-estimated the model after introducing 2 clinical variables that are meaningful only within this group (DSM-5 AUD severity and age of AUD onset), while retaining the covariates from Step 1. AUD severity followed standard DSM-5 cut-offs (At least 2 points or above), and age of onset reflected the self-reported age at which those criteria were first satisfied. For both steps, model assumptions regarding linearity and multicollinearity were examined, with statistical significance set at $p < 0.05$.

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Result

The study included a total of 124 male participants, comprising 66 patients diagnosed with AUD and 58 control participants. Demographic analysis revealed that the mean age of the AUD group was 37.18 ± 10.65 years, while the mean age of the control group was 35.52 ± 5.61 years. The mean age difference was not statistically significant between the AUD and control groups (p = 0.115). Among the 66 patients diagnosed with AUD, 30 (54.5%) reported a first-degree family history of AUD. The mean age of onset for AUD in the AUD group was 26.7 ± 6.3 years, and 62 patients (93.9%) reported concurrent nicotine use. Based on DSM-5 criteria, the mean severity score was 9.3 ± 0.9; all patients met the criteria of severe (i.e., at least 6 criteria). In contrast, none of the 58 control participants reported a first-degree family history of AUD or current substance use, nor did they have any mental illness.

The DNA methylation CpG islands near rs27072 exhibited statistical differences between AUD patients and controls during initial analysis. At CpG-1island, the mean methylation level was significantly lower in AUD patients (mean = 0.39 ± 0.11) compared to controls (mean = 0.45 ± 0.12), and p = 0.019. CpG-2 island also exhibited lower methylation levels in the AUD group (mean = 0.66 ± 0.09) relative to controls (mean = 0.70 ± 0.08), although this difference did not reach statistical significance (p = 0.072). A more pronounced reduction in methylation was observed at CpG-3 island (p = 0.009), where the AUD group displayed significantly lower levels (mean = 0.62 ± 0.16) compared to the control group (mean = 0.73 ± 0.09). CpG-4 island methylation levels, however, did not differ significantly between the 2 groups (mean = 0.61 ± 0.09 for AUD versus mean = 0.63 ± 0.09 for controls), and p = 0.345. The average methylation level of these 4 CpG islands (CpG-1,2,3,4) also shows significantly lower levels (mean = 0.57 ± 0.08) compared to the control group (mean = 0.63 ± 0.08) and p = 0.003 (Figure 1A).

To further investigate whether the observed methylation differences remained significant after adjusting for clinical covariates, 2-step multiple linear regression models were performed. Step 1: We examined the association between diagnostic group (AUD versus control) and average methylation level across the 4 CpG sites, controlling for age, AUD family history, and NUD. The result is shown in Table 1. The model showed borderline statistical significance overall (p = 0.048, data not shown), with diagnosis group being the only significant predictor (B = 0.092 ± 0.045, p = 0.042). When each CpG site was examined separately, the borderline associations observed in the unadjusted analysis for CpG-1 (p = 0.066) and CpG-3 (p = 0.075) were attenuated and no longer reached conventional significance, indicating that demographic or smoking-related factors at least partly confounded these site-specific effects.

Step 2 was conducted within the AUD group only, allowing for the inclusion of AUD-specific clinical factors. Covariates in this model included age, AUD family history, nicotine use, DSM-5 AUD severity, and age of AUD onset. The overall model was not significant (p = 0.670, data not shown). Within the AUD group, none of these clinical variables, including family history of AUD, AUD severity, and age of onset, were related to average methylation (see Table 2).

Psychological assessments further revealed significant differences between AUD patients and controls in different personality

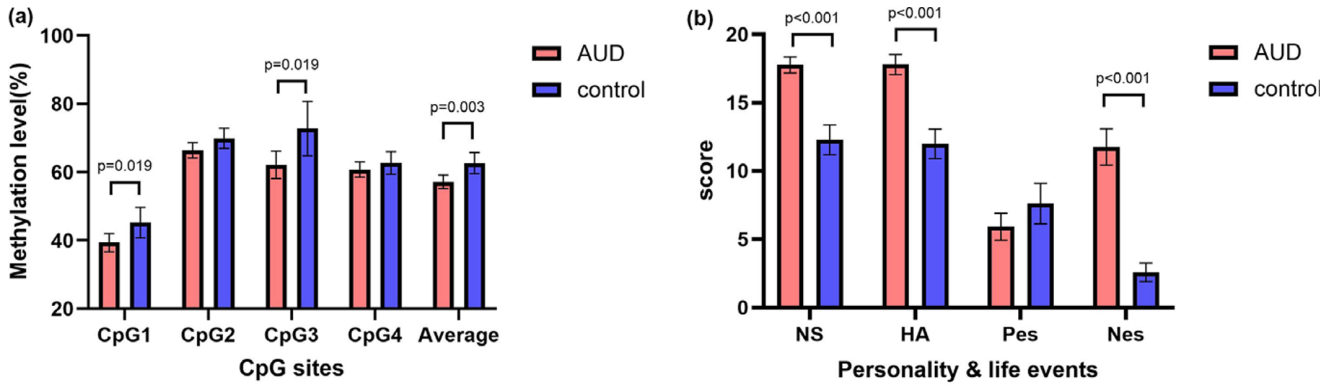

Figure 1. (a) Comparison of Methylation Levels at CpG Sites Between AUD and Control Groups Methylation levels (%) at CpG1, CpG2, CpG3, CpG4 sites, and their average in AUD (red) and control (blue) groups. Error bars represent the 95% confidence interval (CI). (b) Comparison of Personality and Life Events Score Between AUD and Control Groups Comparison of scores for Novel Seeking (NS), Harm Avoidance (HA), Positive life events (Pes), and Negative life events (Nes) between AUD (red) and control (blue) groups. Error bars represent the 95% confidence interval (CI).

Table 1. Association of DAT Methylation Level Around rs27072 Between the AUD Group and Healthy Controls Using Linear Regression, Adjusting for Age, AUD Family History, Gender, and NUD

	Groups		Age		AUD family history		NUD	
Variants	B(SE)	p	B(SE)	P	B(SE)	p	B(SE)	p
CpG–1 island	−0.113(0.074)	0.066	0.001(0.002)	0.685	−0.007(0.028)	0.816	0.059(0.058)	0.307
CpG–2 island	−0.061(0.049)	0.214	0.000(0.002)	0.901	0.013(0.022)	0.559	0.020(0.046)	0.663
CpG–3 island	−0.178(0.99)	0.075	0.000(0.003)	0.920	0.026(0.046)	0.568	0.063(0.094)	0.502
CpG–4 island	−0.016(0.050)	0.757	−0.001(0.002)	0.695	0.006(0.023)	0.792	−0.005(0.047)	0.915
Average	0.092(0.045)	0.042	0.000(0.001)	0.906	0.010(0.02)	0.639	0.034(0.04)	0.419

Average: average methylation level of CpG1 to CpG4; Abbreviations: Nicotine use disorder; AUD, Alcohol use disorder.

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Table 2. Linear Regression Data for DAT Methylation Level Around rs27072 in the AUD Group Only, Adjusting for Age, AUD Family History, Gender, NUD, AUD Onset Age, and DSM-5 Severity

	Age		AUD family history		NUD		AUD onset age		DSM-5 severity	
Variants	B(SE)	p	B(SE)	p	B(SE)	p	B(SE)	p	B(SE)	p
CpG–1 island	−0.001(0.003)	0.786	−0.011(0.028)	0.700	0.062(0.059)	0.297	0.003(0.002)	0.274	−0.001(0.015)	0.934
CpG–2 island	0.000(0.002)	0.969	0.012(0.024)	0.625	0.034(0.051)	0.506	−0.001(0.002)	0.611	−0.013(0.013)	0.341
CpG–3 island	−0.003(0.004)	0.452	0.016(0.043)	0.709	0.084(0.090)	0.357	0.003(0.004)	0.377	−0.015(0.024)	0.526
CpG–4 island	−0.002(0.002)	0.380	0.002(0.023)	0.940	0.029(0.049)	0.557	0.000(0.002)	0.859	−0.033(0.013)	0.011
Average	0.001(0.002)	0.466	0.005(0.021)	0.823	0.052(0.044)	0.241	0.001(0.002)	0.471	−0.016(0.012)	0.182

Average: average methylation level of CpG1 to CpG4; Abbreviations: NUD, Nicotine use disorder; AUD, Alcohol use disorder.

Table 3. Whether the Genotype Variants of rs27072 Modulate Methylation Levels, Personality Traits, and Life Event Severity

Group	AUD (n = 66) Mean ± SD			Health control (n = 58) Mean ± SD		
Genotype	CC (n = 36)	CT + TT (n = 30)	p	CC (n = 34)	CC + CT (n = 24)	p
Methylation levels (%)						
CpG1	37.64 ± 10.85	41.43 ± 10.35	0.154	44.18 ± 13.38	46.67 ± 9.15	0.582
CpG2	67.42 ± 7.25	65.17 ± 11.07	0.325	70.24 ± 8.47	69.50 ± 6.56	0.803
CpG3	62.44 ± 16.54	61.83 ± 16.26	0.881	69.53 ± 22.4	77.33 ± 18.44	0.330
CpG4	61.22 ± 9.85	60.27 ± 8.34	0.676	62.47 ± 9.49	63.0 ± 7.77	0.875
Average	57.18 ± 8.0	57.17 ± 8.22	0.998	61.6 ± 9.33	64.12 ± 5.98	0.418
Psychosocial factors (score)						
NS	18.53 ± 4.66	16.87 ± 4.72	0.156	11.88 ± 3.79	12.83 ± 3.07	0.479
HA	17.28 ± 6.19	18.43 ± 5.56	0.432	11.24 ± 6.45	13.08 ± 4.96	0.412
Pes	9.17 ± 9.36	4.23 ± 4.82	0.008	7.76 ± 8.47	9.83 ± 7.48	0.503
Nes	13.78 ± 12.25	9.37 ± 8.48	0.101	1.94 ± 2.36	3.50 ± 4.83	0.258

Average: average methylation level of CpG1 to CpG4;
Abbreviations: AUD, alcohol use disorder; NS, novel seeking; HA, harm avoidance; Pes, positive event scores; Nes, negative event scores.

traits and life event severity. Novel Seeking (NS) scores were markedly higher in the AUD group, with a mean score of 17.73 ± 4.72 compared to a mean score of 12.98 ± 4.00 in the control group ($p < 0.001$). Harm Avoidance (HA) scores were also higher in the AUD group (mean = 12.80 ± 5.59) relative to controls (mean = 12.00 ± 5.09), although this difference was not statistically significant ($p = 0.776$). Regarding life event severity, the positive events score (Pes) did not differ significantly between the 2 groups (AUD versus Controls $p = 0.343$). However, the negative event score (Nes) was significantly higher in AUD patients, with a mean score of 13.77 ± 10.85 compared to 2.59 ± 3.60 in controls ($p < 0.001$) (Figure 1B).

The genotype frequency for rs27072 in AUD patients was 36 (54.5%) for the *C/C* genotype, 27 (40.9%) for the *C/T* genotype, and 3 (4.5%) for the *T/T* genotype. The genotype frequency in healthy controls was 34 (58.6%) for the *C/C* genotype, 20 (34.5%) for the *C/T* genotype, and 4 (6.9%) for the *T/T* genotype. There is no significant difference in genotype (rs27072) between patients with AUD and controls ($p = 0.697$). We merged with the minor allele T as a genotype group (*C/T*+ *T/T*) because the sample of the *T/T* genotype is rare (<5 subjects). Thereafter, we conducted an independent t-test to assess whether the genotype differences influence methylation, life stress, and personality (Table 3). The results showed that genotype differences did not significantly affect methylation levels in AUD patients and controls (Figure 2A,B), personality scores (NS, HA) and negative event scores (Nes) (Figure 2C,D), respectively. Although the scores of the positive event (Pes) differ significantly between genotypes in the AUD group ($p = 0.008$), the significance becomes borderline after Bonferroni correction for multiple comparisons (the significance should be less than 0.05/18 = 0.003, as shown in Table 3).

No significant Pearson correlations were identified between the individual or average methylation levels of CpG island sites 1–4 and the assessed personality traits of HA or NS. Additionally, no significant correlations were observed with positive or negative life event scores (Pes or Nes) (data not shown). These findings indicate that changes in individual and average methylation levels at CpG island sites 1–4, proximal to rs27072, are not linearly correlated with psychological traits or environmental factors.

Discussion

We investigated methylation levels of CpG island near SNP rs37072 in a Han Chinese male population with AUD. Our study identified methylation on 2 CpG islands (CpG1 and CpG3), and the total methylation average (CpG1 + CpG2 + CpG3 + Cp4) is borderline associated with the development of AUD. Our findings imply that methylation levels near SNP rs37072 of the *SLC6A3* gene may be borderline associated with the AUD status. Methylation

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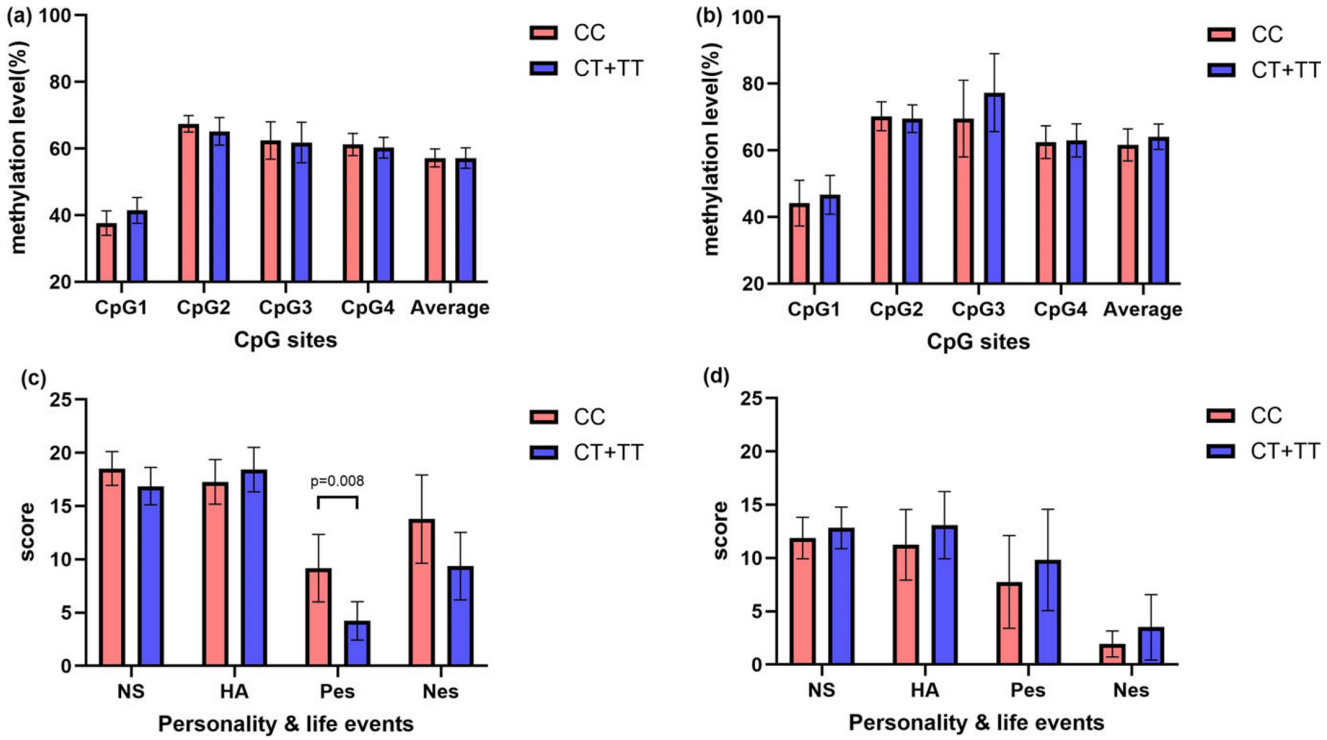

Figure 2. Methylation levels and personality/life events across genotypes in AUD and healthy individuals. (a and b) Methylation levels (%) at CpG1, CpG2, CpG3, CpG4 sites, and their average in individuals with CC genotype (red) and CT+TT genotype (blue). Figure 2A shows data from AUD participants, and Figure 2B shows data from healthy individuals. Error bars represent the 95% confidence interval (CI). (c and d) Scores for Novel Seeking (NS), Harm Avoidance (HA), Positive life events (Pes), and Negative life events (Nes) in individuals with CC genotype (red) and CT+TT genotype (blue). Figure 2C shows data from AUD participants, and Figure 2D shows data from healthy individuals. Error bars represent the 95% confidence interval (CI).

patterns have been extensively studied as a key epigenetic mechanism influencing gene expression and its role in neuropsychiatric disorders. Zheng et al. 2023 have highlighted the dynamic nature of DNA methylation in response to environmental factors, such as chronic stress and substance use, particularly in genes associated with neurotransmission.[19] In AUD, studies have identified altered methylation in genes involved in reward pathways, suggesting that epigenetic modifications may mediate the interplay between environmental exposures and genetic susceptibility.[20] Previous studies suggested that the methylation status of alcohol-associated CpG islands may change with alcohol consumption in adults.[21,22] However, these studies often do not account for the influence of life stress, environmental factors, or personality traits. Our study observed reduced methylation at specific CpG islands. This finding is consistent with the previous study in men with AUD, which reported lower average global methylation levels compared to controls.[23] However, it contrasts with previous findings that reported increased methylation levels in AUD patients,[8] and other work found no difference in brain tissue between cases and controls.[8,24] This discrepancy may underscore the importance of considering cohort-specific factors, such as ethnicity, environmental exposures, and psychosocial variables in future investigations.

Previous studies have indicated that the rs27072 polymorphism is associated with specific risks, such as bipolar disorder, amphetamine use disorder, and alcohol withdrawal. [9,25,26] Despite these findings, evidence linking rs27072 to alcohol-related phenotypes remains inconclusive. To reconcile discrepancies between our results and prior AUD methylation studies, one critical factor is the focus of methylation analysis. The variability in methylation patterns across different cohorts indicates the need for more targeted investigations into specific genes, such as *SLC6A3*.[8] Our findings contribute to this field by highlighting the potential role of rs27072-associated methylation changes in AUD. Previous studies predominantly examined the SLC6A3 promoter region, which is traditionally associated with transcriptional regulation.[19,20] In contrast, our study targeted methylation in the exon region of SLC6A3, which may play a distinct role in modulating gene expression through mechanisms such as splicing regulation or translation efficiency. This methodological difference could explain the observed divergence in findings. Future research should explore the functional implications of exon-specific methylation in SLC6A3 and its potential interaction with promoter methylation to provide a more comprehensive understanding of its role in AUD.

Personality and life stress events in the patient with AUD

Personality traits, NS and HA, have been noticed as predictors of AUD risk.[27] In this study, patients with AUD exhibited significantly higher scores on NS and HA than healthy controls (Figure 1A). These findings are consistent with prior research indicating that individuals with substance use disorders generally display high NS and HA traits relative to non-affected individuals.[9,28,29] High NS, characterized by impulsive behavior and reward-seeking behavior, has been linked to the onset of substance use, the transition to compulsive use, increased relapse risk, and a higher probability of developing addiction. High NS is also related to alcohol use in both human and animal models.[26,27,30] Similarly, heightened HA, reflecting a predisposition to stress and anxiety, appears to contribute to AUD risk. This supports earlier findings connecting

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drinking behavior with temperamental traits described in Cloninger's framework.[26,31] While high HA may initially discourage drinking, frequent alcohol consumption could amplify the risk of dependency as individuals seek to alleviate stress and anxiety through alcohol use.[32]

Our findings also reveal that individuals with AUD encounter more stressful life events than healthy controls (Figure 1B). However, the positive life events showed no significant difference between these 2 groups. According to the self-medication theory of addiction, individuals may resort to substances like alcohol or other addictive behaviors to mitigate physical symptoms or emotional distress.[16,17,33] The selection of a specific substance is shaped by its psychopharmacological properties and/or capacity to address the predominant emotional states the individual seeks to alleviate. Alcohol, as a central nervous system depressant, is particularly suited to reducing tension, anxiety, and discomfort arising from stressful life events, making it a commonly chosen coping mechanism.[34]

Different genotypes and methylation levels

Although research has identified SNPs that affect DNA methylation levels, which in turn may influence the risk of psychiatric conditions.[5] Our analysis revealed no significant association between the rs27072 genotype and methylation levels at the CpG island proximal to this SNP (rs27072) in AUD patients and healthy controls, respectively (Figure 2A,B). This finding suggests that different genotypes of rs27072 may not directly regulate epigenetic modifications in this region, contrasting with prior hypotheses that genetic variation at rs27072 could influence methylation patterns. Further research is needed to determine whether different methylation sites, such as those in promoter or exon regions, yield varying outcomes.

Different genotypes and personality scores/life stress events

Previous genomic analysis revealed genetic associations with personality traits.[14] We examined whether the rs27072 genotype modulated personality traits and life stress events. The results demonstrated no significant genotype effect on these psychological and environmental measures, both in AUD patients and healthy controls (Figure 2C,D). This lack of association underscores the possibility that personality traits and life stressors influence the development of AUD through pathways independent of the rs27072 genotype. Further investigation is needed to capture the interaction between these psychosocial factors and genes/epigenetic modulation.

Methylation level and personality scores/life stress events

Finally, we investigated the relationship between methylation levels, personality scores (NS, HA), and life stress events. Our findings revealed no significant correlation, suggesting that the effects of these psychosocial factors may not mediate methylation changes in the SLC6A3 gene. This result contrasts with previous research suggesting a potential role of methylation in linking life stress and personality traits to neuropsychiatric outcomes.[6,11,35] Our study's absence of significant associations may reflect cohort-specific characteristics, such as limited sample size, different methylation areas, or the need for more comprehensive genomic analyses to detect subtle epigenetic effects.

Methylation level and other confounders

Previous studies have shown that various covariates, including clinical severity, age of onset, and the co-use of other substances, may influence gene epigenetic changes.[36] Demographic and historical factors also play a significant role; for instance, individuals who initiate alcohol use during adolescence exhibit a markedly increased risk of developing AUD later in life.[36] Twin and family studies have further estimated the heritability of AUD to be approximately 40–60%.[36] Thus, to further examine whether the observed methylation differences were independently associated with clinical or genetic variables, we performed 2 multiple linear regression analyses. The first-step model included all participants and adjusted for age, family history of AUD, and nicotine use. The second-step model was restricted to the AUD group and additionally incorporated DSM-5 AUD severity and age of drinking onset.

The first model showed borderline statistical significance, and the diagnosis group was the only significant predictor. These findings suggest that the observed group differences may be partially explained by alcohol use. However, the model accounted for only a limited proportion of the variance, and residuals did not meet the normality assumption, suggesting cautious interpretation is warranted.

The second model did not show a statistically significant result. This finding may suggest that, once AUD is established, individual differences in severity or onset parameters may contribute minimally to variations in methylation at the SLC6A3 CpG sites studied. Alternatively, the absence of significant associations may reflect insufficient statistical power, given the modest sample size and inter-individual variability in methylation.

Limitation

This study has several limitations that warrant consideration. First, the sample size was relatively small, which may have reduced the statistical power to detect subtle effects or associations, particularly in genotype analyses. Second, the study exclusively included male participants, limiting the generalizability of the findings to females, who may exhibit different genetic, epigenetic, or psychological responses related to AUD. Third, the distribution of genotypes was imbalanced, with a particularly small number of participants carrying the *T/T* genotype. This limited our ability to fully evaluate the potential effects of this genotype on methylation patterns and AUD-related traits. Fourth, this was a cross-sectional study, which precludes the assessment of temporal relationships or causality.[37] The Addiction Severity Index is a validated and informative measure; however, using DSM-5 criteria for evaluating clinical severity may not fully explain the quantitative alcohol use patterns. Finally, we lack genetic samples from first-degree relatives, which limits our ability to compare methylation levels within families directly. Further longitudinal and family-based studies are warranted to clarify whether methylation alterations are inherited traits or consequences of chronic alcohol use. One promising approach involves comparing AUD patients, healthy controls, and unaffected first-degree relatives, which may help differentiate heritable from exposure-driven effects. Integrating multi-omics and larger, more diverse samples is also recommended to improve generalizability and mechanistic understanding in future studies.

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Conclusions

Our findings suggest that lower methylation levels near rs27072 may be marginally associated with AUD diagnosis after adjusting for age, family history, and nicotine use. The rs27072 genotype was not significantly associated with methylation levels, personality traits, or life stress scores. These findings underscore the need for further studies with larger and more diverse samples, incorporating familial designs and longitudinal follow-up, to better elucidate the complex genetic, epigenetic, and psychosocial contributions to AUD pathophysiology.

Data availability statement. The current study data are available from the corresponding author upon reasonable request.

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Author contribution. SYH was responsible for the concept and design of the study. CYH, TEC, and SCK contributed to genotyping and data acquisition. SCK, TEC, and CYC performed data analysis. CYH provided statistical consultation. SCK, TEC, and YWY contributed to the interpretation of the findings. TEC, SCK, CLL, and SYH wrote the manuscript. All authors critically reviewed the content and approved the final version for publication.

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