

RESEARCH ARTICLE

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Inter-individual differentially methylated region-targeted EWAS reveals epigenetic signatures of early childhood adversity

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ABSTRACT

Aims: Adverse childhood experiences (ACEs), especially in early life, can affect psychosocial development and increase lifelong risk for mental disorders. ACEs are also known to induce persistent epigenetic changes. This study aimed to explore ACE-associated DNA methylation signatures using an epigenome-wide association study (EWAS) targeting inter-individual differentially methylated regions (DMRs).

Methods: We developed a targeted capture probe system covering ~1.3 million CpG sites within inter-individual DMRs. This system was applied to salivary DNA from drug-naïve children aged 6–12 years with exposure to multiple early-life ACEs ($n = 23$) or who had no ACEs ($n = 21$).

Results: We identified 15 novel DMRs significantly associated with ACEs. A cluster of six CpG sites within an exon of the *EIF4G2* gene showed consistently increased methylation in children with ACEs, with strong inter-site correlations. Enrichment analysis indicated that genes near these DMRs are involved in neurodevelopmental disorders, suggesting that early adversity may influence brain development through epigenetic mechanisms.

Conclusion: Our findings suggest that early adversity may contribute to lasting epigenetic modifications in children. The identified DMRs may serve as noninvasive biomarkers for retrospective ACE assessment and provide insights into the biological embedding of early-life stress.

PLAIN LANGUAGE SUMMARY

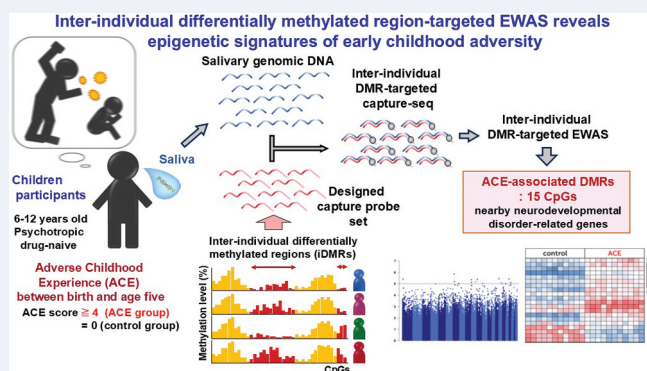
Children who have hard experiences early in life – like abuse, neglect, or stress – may be more likely to have problems with their mental health later. These experiences can leave lasting effects on the body. One way this happens is through DNA methylation, a process that can switch genes on or off without changing the DNA itself. In our study, we looked at saliva from children aged 6 to 12. We found 15 places in the DNA where children who had stressful experiences were different from those who did not. One important gene, *EIF4G2*, showed higher methylation in children with early stress. Other differences were near genes important for brain development. This shows that early stress can leave biological marks. In the future, these marks may help us understand how stress affects children and identify those who might need extra support.

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Article highlights

- In this study, we analyzed salivary DNA to explore methylation regions associated with ACEs, focusing on children who were psychotropic medication-naïve and had experienced multiple ACEs in early childhood.
- We conducted an inter-individual DMR-targeted EWAS using our custom-designed capture probes and identified 15 novel DMRs associated with ACEs, including a CpG cluster near the *EIF4G2* gene that showed increased methylation in children with ACEs.
- The identified ACE-associated DMRs were located near genes implicated in neurodevelopmental disorders, suggesting that ACEs may influence developmental trajectories through epigenetic mechanisms.
- These findings deepen our understanding of the role of early adversity in epigenetic embedding and support the potential of saliva-based epigenetic biomarkers in child psychiatry.

1. Introduction

Early childhood, particularly the first few years of life, is a critical period for the maturation of the nervous system and cognitive development [1–4]. During this period, children need proper care and support for healthy development. Emotion regulation and forming close relationships are also important for building social skills. Exposure to physical or psychological abuse, neglect, or inadequate caregiving during this period, regardless of whether the individual consciously perceives it as “adversity,” can negatively impact cognitive development, social behavior, and stress reactivity [1–4]. These developmental changes and stress responses have lifelong consequences, including an increased risk of mental disorders in adulthood [5].

Understanding adverse childhood experiences (ACEs) is essential to accurately evaluate a child’s developmental trajectory. In pediatric psychiatry, recognizing ACEs can provide essential insights relevant for early intervention and appropriate care [3]. However, ACEs have traditionally been assessed through interviews with children or their caregivers, making objective evaluation highly challenging. Retrospective recall of early childhood ACEs, particularly those occurring between birth and five years of age, is inherently difficult owing to the limitations of early memory. Moreover, obtaining reliable information from caregivers is often hindered by memory biases and subjective interpretations of parenting practices. In cases of abuse, direct caregiver reporting may not be possible. Thus, developing a method to objectively estimate the history of ACEs in children would be of significant value in the field of child and adolescent psychiatry.

In recent years, genomic research in psychiatry has advanced remarkably, and genome-wide association studies (GWAS) meta-analyses examining early-life adversity based on large datasets from multiple cohorts have been reported. These studies have discussed the role of irreversible genetic factors, such as children’s inherent developmental traits and genetic susceptibility underlying experiences of maltreatment [6].

Meanwhile, epigenetic modifications of the genome, including DNA methylation and histone modifications, have attracted considerable attention as mechanisms through which environmental exposures during specific developmental periods can exert long-lasting effects on gene expression. Analysis of epigenetic modifications, particularly DNA methylation, provides a promising approach for assessing ACEs. Methylation levels in

the transcriptional regulatory regions of genes related to brain development including corticotropin-releasing hormone (*CRH*), arginine vasopressin (*AVP*), proopiomelanocortin (*POMC*, which encodes ACTH), *FKBP5*, and *NR3C1* (which encodes the glucocorticoid receptor, GR), are altered by ACEs [7,8]. These changes can lead to altered gene expression, affecting cortisol levels via the hypothalamic-pituitary-adrenal (HPA) axis. Chronic dysregulation of the HPA axis, which plays a central role in maintaining systemic homeostasis, and abnormalities in the stress response can broadly affect brain functions, including cognition [2,3]. In addition to the HPA axis, genes such as brain-derived neurotrophic factor (*BDNF*), *SLC6A4* (encoding the serotonin transporter), glutamate decarboxylase 1 (*GAD1*), Reelin (*RELN*), and oxytocin receptor (*OXT*) are also subject to epigenetic transcriptional regulation via DNA methylation in response to ACEs. Altered expression of these genes is known to influence brain development and the regulation of neural function [9,10]. This phenomenon has been demonstrated in rodent models as well as in human studies [7]. Furthermore, early-life stress alters CpG methylation patterns, and these epigenetic changes persist into adulthood [8,11,12]. Notably, while early-life stress induces these methylation changes, similar stressors encountered later in life do not elicit significant alterations [7]. This is part of the early-life epigenetic programming of the stress-response systems, often associated with the sensitive period of development [13]. Therefore, the DNA methylation status in specific genomic regions has been proposed as a potential biomarker that records and retains experiences of early life stress.

In addition to studies focusing on specific candidate genes, epigenome-wide association studies (EWAS) have been used to identify DMRs associated with ACEs across the genome. Among these, the Avon Longitudinal Study of Parents and Children (ALSPAC), a large-scale prospective cohort study, has provided valuable insights. Dunn et al. reported that the timing of adversity, specifically, exposure before the age of three, has a greater impact on DNA methylation patterns than either the cumulative burden or the recentness of adversity [14]. Despite numerous previous studies, conclusive ACE-associated DMRs remain largely unidentified, except for a few candidate biomarkers, such as *NR3C1* and *FKBP5* [8].

In conducting genome-wide analysis, it was known that DNA methylation profiles vary between individuals and that these differences are worth investigating; however, the specific genomic regions exhibiting such inter-individual variability had not been thoroughly explored. Through whole-genome bisulfite sequencing of over 100 individuals, we identified significant inter-individual DMRs, which we termed common DNA methylation variations (CDMVs) [15]. Here, we performed an EWAS using a CDMV-targeted capture probe to enrich next-generation sequencing (NGS) targets, a technique that we developed previously [16]. While conventional EWAS typically employs bead chip-based methods, our inter-individual DMR-targeted EWAS approach offers the potential to identify distinct candidate sites. We classified participants into two groups – namely, the ACE and non-ACE (control) groups – based on strict criteria. The ACE group comprised children who had experienced at least four of the ten ACEs during early childhood, whereas the control group included only

those with no history of ACEs. Although the sample size in this study was relatively small, our rigorous selection criteria enabled the identification of several DMRs with significant methylation differences. Most of these were isolated DMRs; however, we also identified a CpG cluster in which methylation levels were correlated with ACEs.

2. Materials and methods

2.1. Study participants and measurements

This study was approved by the Ethics Committee of Iwate Medical University (Approval ID: MH2021-178). All procedures were conducted in accordance with the approved guidelines. The study was conducted in accordance with the guidelines of the Declaration of Helsinki. The participants were Japanese children aged 6–12 years at the time of the survey. A total of 51 children and their parents or caregivers agreed to participate in the study. Participants were recruited between March 2022 and March 2024 from the child and adolescent psychiatry and pediatrics departments at Iwate Medical University Hospital and its affiliated hospitals on a completely voluntary basis. The study was explained both orally and in writing by the trained research coordinators. Written informed assent was obtained from the children and written informed consent was obtained from their parents or caregivers.

The following assessments were conducted: ACEs were assessed using a structured 10-item yes/no questionnaire completed by parents or caregivers, in which they were asked whether the child had experienced any of the 10 types of adversity between ages 0 and 5 years. To reduce the risk of underreporting or missed adversity experiences, the assessment was administered in a face-to-face format by a trained pediatrician, child and adolescent psychiatrist, or clinical psychologist, using empathic and supportive inquiry to obtain accurate background information. Because the participants consisted of children who visited pediatric or child and adolescent psychiatry outpatient clinics, as well as their siblings and caregivers, information regarding family environment and caregiving circumstances was confirmed through multiple clinical encounters, including during recruitment and study-related interviews. The 10 ACE items included the following: (1) Physical abuse; (2) Psychological abuse; (3) Sexual abuse; (4) Alcohol or drug abuse by household members; (5) Witnessing domestic violence (DV); (6) History of mental illness among household members; (7) Parental separation or divorce; (8) History of imprisonment or detention among household members; (9) Physical neglect; and (10) Emotional neglect [17] (Supplementary Materials, ACE Assessment questionnaire).

Cognitive functioning was assessed using the Full-Scale IQ (FS-IQ) derived from the Wechsler Intelligence Scale for Children-Fourth Edition (WISC-IV) [18]. The WISC-IV was administered through interviews with the children. Two in the ACE group could not complete the test. Additional information on the upbringing, health status, family environment, and economic situation was collected via caregiver questionnaires. All data used for analysis were systematically anonymized to prevent personal identification.

2.2. Sample collection

Saliva samples were collected using an Oragene DISCOVER kit (OGR-675; DNA Genotek, Ottawa, Canada). After anonymization, genomic DNA was extracted according to the manufacturer's protocol. No Proteinase K digestion was performed, as sufficient DNA yield and quality are obtained without it. DNA purity was assessed by measuring the absorbance at 260/280 nm (A260/280) and 260/230 nm (A260/230) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA yield was determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA fragment size and integrity, including the DNA Integrity Number (DIN) were evaluated using the TapeStation system with Genomic DNA ScreenTape and reagents (Agilent Technologies, Santa Clara, CA, USA).

2.3. Preparation of sequencing libraries

Participants were selected based on the criteria of having an ACE score ≥ 4 and no history of psychotropic medication use. Because the use of psychotropic medications, such as selective serotonin reuptake inhibitors (SSRIs), anxiolytics, and mood stabilizers, is known to potentially influence the epigenetic state of genomic DNA, samples from participants with a history of such medication use were excluded from the analysis [19,20]. From saliva samples collected from 51 participants, by matching age and sex, a total of 44 genomic DNA samples, derived from participants in the ACE ($n=23$) and control ($n=21$) groups were analyzed. Aliquots of genomic DNA (1.1 μ g), eluted in 50 μ L of TE buffer, were sheared into 150–200 bp fragments using a Covaris LE220 focused ultrasonicator (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries for inter-individual DMR-targeted sequencing were prepared using a customized Agilent SureSelect Human Methyl-Seq Custom Capture Kit, which contained probes targeting CDMVs [15]. The Agilent Bravo automated library preparation system (Agilent Technologies, Santa Clara, CA, USA) was used according to the manufacturer's instructions, and bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Libraries (17 pM) were pooled, spiked with 20% PhiX Control v3 (Illumina, San Diego, CA, USA), and subjected to paired-end sequencing (2 $\times$ 125 bp) using a HiSeq 2500 system (Illumina, San Diego, CA, USA).

2.4. DNA methylation profiling in targeted DMRs

Raw sequencing data were converted to the FASTQ format using the Illumina bcl2fastq2 Conversion Software v2.20. Data quality was assessed using FastQC v0.11.5. Adapter sequences were trimmed using Trim Galore v0.4.2, and reads shorter than 20 bp were discarded. The remaining reads were aligned to the Genome Reference Consortium Human Reference 37 (GRCh37/hg19) obtained from the UCSC Genome Browser [21], using Novoalign v3.6.5.

Post-alignment processing was performed according to previously reported methods [16]. Methylated CpGs were detected using NovoMethyl v1.4, and the methylation levels

at the targeted CpGs were calculated as beta values using R v4.0.5. CpGs harboring genetic variants of either dinucleotide were excluded. The DNA methylation levels were calculated by dividing the number of unconverted cytosines in the mapped reads by the total number of converted and unconverted cytosines. CpGs with extreme depth ($<6\times$ or $>300\times$) and low call rate ($<50\%$) were excluded. To evaluate the potential batch effects, principal component analyses were conducted using the *prcomp* function in the R Stats package.

2.5. Epigenome-wide association study (EWAS)

The EWAS was conducted using a linear regression model to identify DMRs associated with ACEs. The results were adjusted for age and sex using functions from the *minfi* Bioconductor package [22], with minor modifications. All analyses were conducted under identical conditions for both phases. The genome-wide suggestive significance threshold was set at $p < 1.00 \times 10^{-5}$, and the Bonferroni-corrected threshold was set at $p < 3.89 \times 10^{-8}$ ($0.05/1,282,919$). Statistical analysis scripts are provided as Supplementary Materials (Supplementary Materials, EWAS script file). Genes adjacent to the candidate CpG sites and surrounding sequences were obtained using the UCSC Genome Browser [21]. GENCODE and RefSeq tracks were used to identify adjacent genes. dbSNP and CpG Island tracks were used to confirm the presence of single nucleotide polymorphisms (SNPs) and CpG islands/shores, respectively. Enrichment analysis was performed using Metascape [23], and significant results with a p -value $< 10^{-4}$ are shown.

2.6. Pyrosequencing

Genomic DNA (500 ng) was treated with bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA), in accordance with the manufacturer's instructions. PCR amplification was performed using the PyroMark PCR Kit (Qiagen, Venlo, Netherlands) under the following conditions: initial denaturation at 95°C for 15 min; 45 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR products were processed for pyrosequencing according to the PyroMark Q96 ID User Manual v5 (Qiagen, Venlo, Netherlands). Pyrosequencing was performed using the PyroMark Gold Q96 Reagent Kit (Qiagen, Venlo, Netherlands) on a PyroMark Q96 ID system. DNA methylation levels between the ACE and control groups were compared using a linear regression model in R, adjusted for sex and age. The primer sequences are listed in Supplementary Materials (Supplementary Table S3).

2.7. Statistical analyses

Statistical analyses using R for the EWAS and pyrosequencing were described in their respective sections. The Mann – Whitney U test and Pearson correlation coefficients were calculated using the Bell Curve for Excel software (Social Survey Research Information Co., Ltd, Tokyo, Japan). A two-sided test was used for all analyses. Box-and-whisker plots, including dot plots, were generated using the Bell Curve for Excel software.

3. Results

3.1. Demographics of the participants

In this study, we compared two groups of children: those who had an ACE score of 4 or more (ACE group, $n = 23$), and those with no history of ACEs (control group, $n = 21$) (Table 1). The target ACE period was defined as birth to five years of age, a critical developmental window identified in prior research [14]. Participants were eligible if they were between 6 and 12 years of age at the time of enrollment (Table 1). The most frequent ACEs in the ACE group included having a family member with a psychiatric disorder, parental separation or divorce, and physical or emotional abuse whereas the prevalence of sexual abuse was low (Supplementary Table S1).

3.2. Candidate ACE-associated DMRs

Using genomic DNA extracted from saliva, we conducted an EWAS targeting 1,282,919 CpG sites using the CDMVv3 capture probe, focusing on regions with high inter-individual DNA methylation variability. Although salivary DNA can be contaminated with bacterial DNA, the mapping rate of the sequencing reads to the human reference genome was 93.4%, indicating that the CDMVv3 capture probe effectively minimized contamination.

Although no CpG sites reached the Bonferroni-corrected genome-wide significance threshold ($p < 3.89 \times 10^{-8}$), 15 candidate CpG sites exceeded the suggestive threshold for statistical significance ($p < 1.0 \times 10^{-5}$) after adjusting for age and sex (Figure 1, Table 2). Half of the 15 ACE-associated CpG sites were located within gene bodies (Table 2). Notably, CpG sites at Chr19:804040 and Chr11:10827494 were located in the coding exons of *PTBP1* and *EIF4G2*, respectively. Several candidate CpG sites were adjacent to non-coding RNAs, such as *MIR4745*, *SNAI3-AS1*, *PPP1R26-AS1*, *LINC02907*, and *LINC01535*. Three CpG sites (Chr19:804040, Chr11:10827494, and Chr12:3397286) were located within the transcriptional regulatory region, approximately 5 kb upstream of the transcription start site of the respective neighboring genes. Three other CpG sites were located in intergenic regions approximately 50 kb away from the nearest annotated genes (Table 2).

Both the mean and variance of DNA methylation levels across samples varied substantially across the 15 candidate CpG sites (Figure 2(a)). Most of the CpG sites affected by ACEs showed increased methylation levels in the ACE group (13/15 CpGs, 86.7%). However, CpG sites on Chr19:804040 and Chr10:102758335 exhibited decreased methylation in the ACE group (Figure 2(a), Table 2). While methylation levels among the ACE-associated CpG sites did not show uniformly high correlations, some pairs were strongly correlated, with Pearson's r exceeding 0.6 (Figure 2(b)).

Table 1. Demographic characteristics of the participants in this study.

	control group	ACE group	p -value
n	21	23	
sex (male [%])	61.90	65.20	
Age $\pm$ SD [years]	9.23 ± 1.61	10.13 ± 1.35	0.0762
ACE score $\pm$ SD	0	4.78 ± 1.00	7.2×10^{-17}
FS-IQ score $\pm$ SD	100.76 ± 11.28	88.40 ± 10.88	0.0015

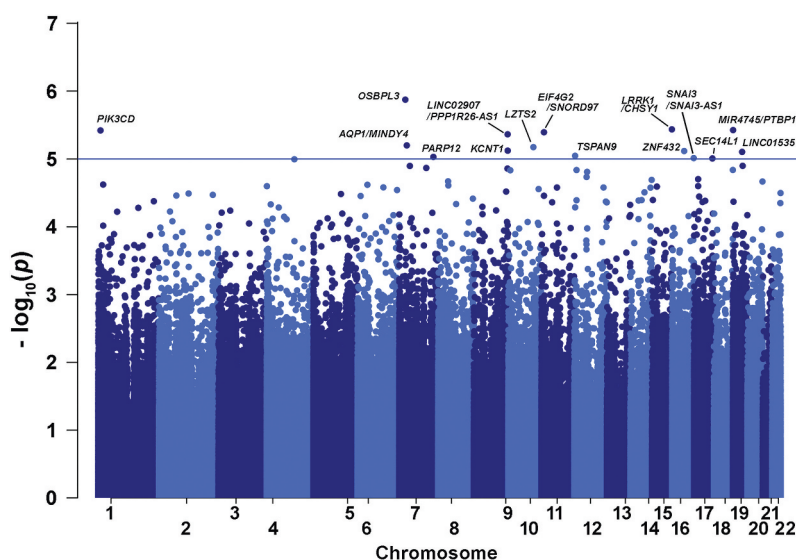


Figure 1. Manhattan plots for the association between DNA methylation and adverse childhood experiences. The Manhattan plot displays the negative logarithm of the p -values $[-\log_{10}(p\text{-value})]$ against chromosomal positions. The genome-wide suggestive threshold is indicated with a blue line at $p < 1.00 \times 10^{-5}$. The Bonferroni-corrected significance threshold is set at $p < 3.89 \times 10^{-8}$. Genes adjacent to candidate CpG sites are labeled.

Table 2. The associated CpG sites ($p\text{-value} < 10^{-5}$) from inter-individual DMR-targeted EWAS analyses of ACEs adjusted for sex and age.

Chromosome	Position	$p\text{-value}$	Methylation level $\pm$ SD (%)			Gene symbol	Genomic context	Distance(bp)	CpG island
			Control group	ACE group	Δ				
7	24905869	1.33×10^{-6}	86.16 ± 4.91	91.90 ± 4.24	5.74	<i>OSBPL3</i>	intron4		
15	101667085	3.65×10^{-6}	38.93 ± 12.29	55.11 ± 13.38	16.18	<i>LRRK1</i> <i>CHSY1</i>	intergenic	48623 48847	
19	804040	3.74×10^{-6}	77.63 ± 6.05	70.60 ± 6.96	-7.03	<i>PTBP1</i> <i>MIR4745</i>	exon3 promoter	901	CpG shore
1	9751353	3.79×10^{-6}	84.94 ± 4.08	91.23 ± 4.36	6.29	<i>PIK3CD</i>	intron1		CpG shore
11	10827494	4.03×10^{-6}	33.27 ± 8.22	44.24 ± 7.48	10.96	<i>EIF4G2</i> <i>SNORD97</i>	exon4 upstream	4339	CpG shore
9	138296858	4.32×10^{-6}	71.92 ± 8.49	82.54 ± 7.17	10.63	<i>LINC02907</i> <i>PPP1R26-AS1</i>	intergenic	58450 57715	
7	30940024	6.29×10^{-6}	60.19 ± 9.76	74.02 ± 7.27	13.83	<i>MINDY4</i> <i>AQP1</i>	downstream upstream	8022 11445	
10	102758335	6.68×10^{-6}	3.78 ± 4.89	0.00	-3.78	<i>LZTS2</i>	intron1		CpG island
9	138645525	7.53×10^{-6}	58.61 ± 13.34	79.49 ± 14.32	20.88	<i>KCNT1</i>	intron3		
16	49517917	7.63×10^{-6}	67.59 ± 11.33	84.60 ± 12.37	17.01	<i>ZNF423</i>	downstream	3519	
19	37760616	7.85×10^{-6}	86.99 ± 8.95	96.94 ± 5.53	9.95	<i>LINC01535</i>	downstream	4179	CpG shore
12	3397286	8.97×10^{-6}	85.24 ± 8.32	95.60 ± 4.28	10.36	<i>TSPAN9</i>	upstream	1556	
7	139753725	9.32×10^{-6}	89.68 ± 3.56	93.83 ± 1.91	4.15	<i>PARP12</i>	intron4		
16	88748644	9.69×10^{-6}	52.24 ± 7.21	63.50 ± 8.01	11.26	<i>SNAI3</i> <i>SNAI3-AS1</i>	intron1 intron2		CpG shore
17	75039443	9.81×10^{-6}	95.70 ± 2.26	98.78 ± 1.59	3.08	<i>SEC14L1</i>	intergenic	45794	CpG shore

Position: Location of the CpG site in the human genome assembly GRCh37/hg19. Methylation level: Mean $\pm$ SD of the methylation level, as determined by NGS results. Δ (Delta): Difference in methylation levels between the ACE group and the control group. Distance (bp): Number of base pairs from the CpG site to the nearest gene body. Genomic context: the location of DMRs relative to gene elements, including introns, exons, and intergenic regions. CpG island: Region of DNA with high GC content and a high frequency of CpG dinucleotides, based on the CpG Islands track in the UCSC Genome Browser. CpG shore: Region located within 2 kb of a CpG island.

3.3. Correlation of *EIF4G2* CpG cluster methylation with ACE

We also examined the methylation status of CpG sites neighboring the candidate loci. Most candidate CpG sites showed changes independent of the neighboring CpGs. However, Chr11:10827494 showed a strong correlation of the methylation levels with nearby CpGs (Figure 3). These sites were located in exon 4 of the *EIF4G2* (eukaryotic initiation factor 4 gamma 2) gene (Figure 3(a)). *EIF4G2* is a translation initiation factor that regulates non-canonical eIF4E-independent translation and contributes to neuronal differentiation and synaptic plasticity

in neurons [24]. Given its role in neuronal development, the observed methylation changes may reflect a link between early adversity and altered neurodevelopmental trajectories. Among the eight CpG sites within this exon, six adjacent sites within a 58-bp region (Chr11:10827484–10827541) were strongly correlated with ACEs (Figure 3(a–c)). Methylation levels among the six CpG sites were also highly correlated with each other (Figure 3(d,e), Supplementary Figure S1). This DMR is located approximately 2 kb downstream of the *EIF4G2* transcription start site, and its involvement in the transcriptional regulation of this gene remains unclear. However, it lies approximately 4

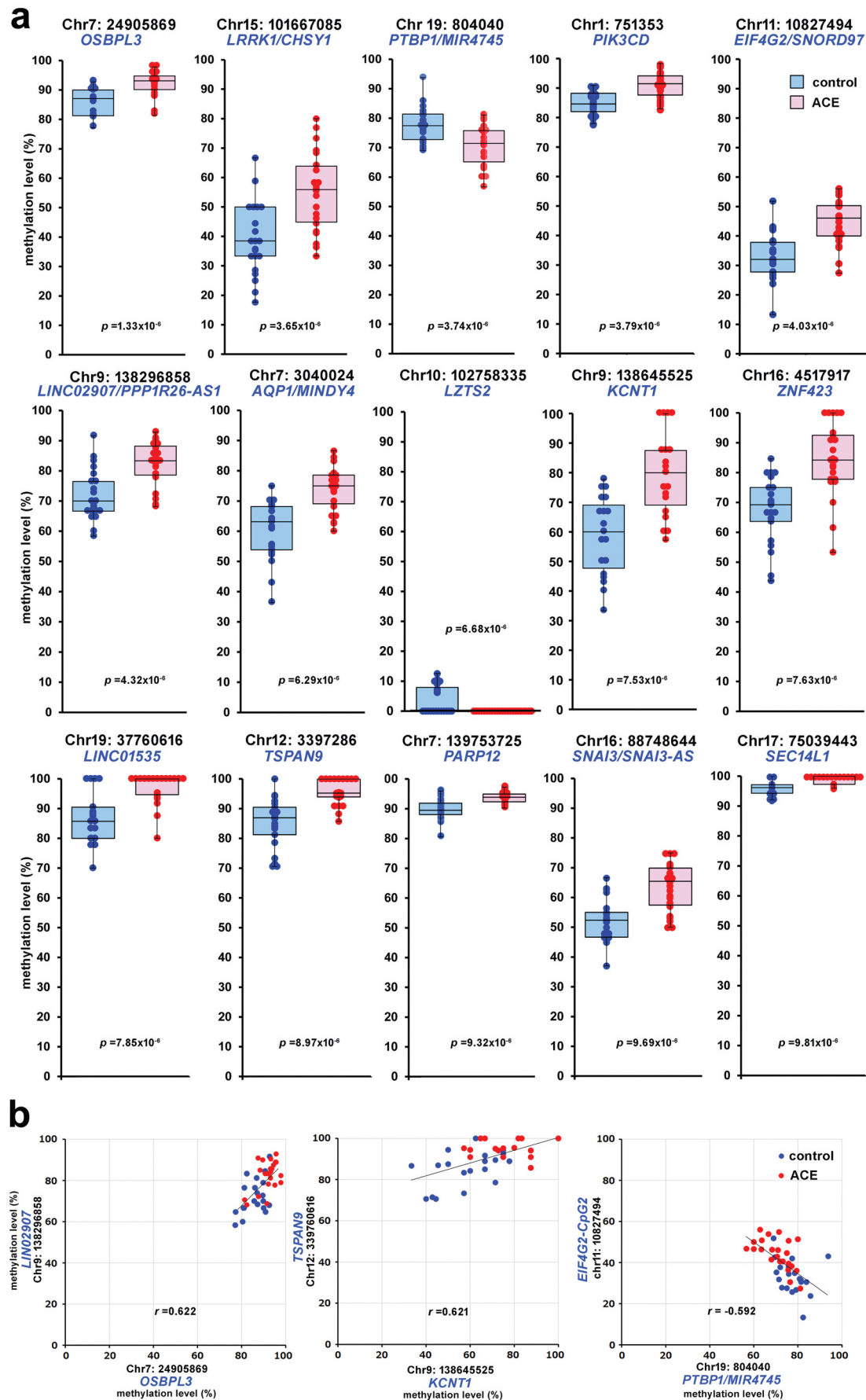


Figure 2. DNA methylation levels of top-ranked candidate CpG sites and their correlations. (a) Box-and-whisker plots with overlaid dot plots show the methylation levels (%) of each of the 15 top-ranked candidate CpG sites in the control and adverse childhood experience (ACE) groups, as determined via next-generation sequencing analysis. Blue: control group; red: ACE group. The box shows the median and quartiles, and the error bars show the minimum and maximum values. (b) Scatter plots display correlations

kb upstream of the *SNORD97* (small nucleolar RNA, C/D Box 97) gene, suggesting a potential role in regulating the transcription of *SNORD97* (Table 2, Figure 3(a)). Although *SNORD97* is a non-coding RNA, it has been implicated in the pathogenesis of amyotrophic lateral sclerosis [25].

To validate the methylation levels estimated via NGS, we conducted locus-specific methylation analysis of the top candidate DMRs using pyrosequencing of bisulfite-treated genomic DNA (Supplementary Figure S2). Although the absolute methylation values varied slightly between the methods, because of the sequence context sensitivity of pyrosequencing, the direction and significance of group differences were consistently replicated. This supports the reliability of the sequencing-based data.

3.4. Enrichment analysis using Metascape

We performed gene set enrichment analysis using Metascape on genes adjacent to 122 CpG sites meeting a threshold of $p < 1.0 \times 10^{-4}$ (Figure 4, Supplementary Table S2) [23]. In the biological pathways and processes category, significant enrichment was observed for the negative regulation of peptide hormone secretion, signaling by GPCR, aquaporin transport, and potassium channels (Figure 4(a)). These pathways are believed to be involved in stress responses, homeostasis regulation, and neurotransmission. Notably, neurodevelopmental disorder exhibited the strongest enrichment among disease-associated terms ($p < 1.0 \times 10^{-5}$), with Fragile X syndrome being the second most-enriched (Figure 4(b)). Although the p -value is below 1.0×10^{-4} , it is also interesting that “delayed speech and language development” is included, as it is closely linked to the features of autism spectrum disorder (ASD) and intellectual disability (ID) (Figure 4(b)). These findings suggest that the genes adjacent to the ACE-associated DMRs may mediate the impact of early adversity on the onset of neurodevelopmental disorders through epigenetic mechanisms.

3.5. Correlation between ACE-associated DMRs and FS-IQ scores

With regard to cognitive development, children in the ACE group exhibited significantly lower FS-IQ scores compared to the control group (Supplementary Figure S3). Across all participants, the ACE score was negatively correlated with FS-IQ ($r = -0.513$, $p = 0.0006$). We also explored the correlation between the FS-IQ scores and methylation levels at ACE-associated candidate CpG sites identified in this study. Methylation levels at approximately half of the candidate CpG sites showed Spearman correlation coefficients of approximately $r = -0.3$ with FS-IQ (Supplementary Figure S3). For example, Chr7:24905869 (*OSBPL3*) and Chr7:30940024 (*AQP1/MINDY4*) showed moderate negative correlations with FS-IQ ($r = -0.396$ and $r = -0.353$, respectively), whereas Chr16:88748644 (*SNAI3/SNAI3-AS1*) showed no meaningful correlation ($r = -0.048$, $p = 0.7656$) (Supplementary Figure S3).

4. Discussion

In this study, we identified novel DMRs associated with early childhood adversity in salivary DNA using inter-individual DMR-targeted EWAS. Although numerous studies have investigated the association between ACEs and epigenetic changes, most of them have included adolescent or adult participants. In such studies, ACEs were typically assessed through the participants’ recollection of their early childhood experiences. Relatively few studies have investigated the epigenetic effects of ACEs during childhood. Our study targeted children aged 6–12 years with multiple ACEs (ACE score ≥ 4). While the need for more objective approaches to evaluating ACE histories is widely recognized, studies such as ours must still rely on retrospective reports from the parents or caregivers who raised the child during early childhood. Importantly, in this study such information was not obtained through a self-report questionnaire; rather, it was elicited in face-to-face interviews conducted by experienced pediatricians, child and adolescent psychiatrists, or clinical psychologists, who employed empathic and supportive inquiry to maximize the accuracy of the assessment. Furthermore, this study focused exclusively on children who did not receive psychotropic medication. This is important, as some children with multiple ACEs receive pharmacological treatment for neurodevelopmental or affective symptoms, and certain psychotropic medications can influence epigenetic regulation [19,20].

Using data from the ALSPAC cohort study, Dunn et al. reported that the timing of adversity, specifically, exposure before the age of three years, had a greater impact on DNA methylation than either the accumulation or recency of adverse experiences [14]. Similarly, Schuurmans et al. also demonstrated strong associations between DMRs linked to perinatal and early childhood adversity and various psychiatric outcomes, including attention-deficit/hyperactivity disorder, depression, obsessive-compulsive disorder, and suicide attempts [26]. Based on these findings, we focused specifically on ACEs experienced during early childhood (up to age five years).

Inter-individual DMRs, identified in our previous study as CDMVs, were efficiently enriched in genomic regions associated with epigenetic disease risk [15]. Indeed, inter-individual DMR-targeted EWAS using a CDMV-capture probe has proven to be effective in identifying DMRs associated with kidney cancer and longevity [16,27]. Furthermore, by restricting our analysis to individuals with multiple ACEs who were psychotropic medication-naïve, a deliberately stringent criterion, we were able to identify 15 novel DMRs surpassing the suggestive significance threshold, despite the limited sample size. Notably, all 15 DMRs identified in this study are novel and have not been reported in previous EWAS.

Children in the ACE group exhibited significantly lower FS-IQ scores than the control group, and a negative correlation was observed between ACE scores and FS-IQ (Supplementary Figure S3). Several children in the ACE group fell within the borderline intellectual functioning range, defined as FS-IQ between 70 and

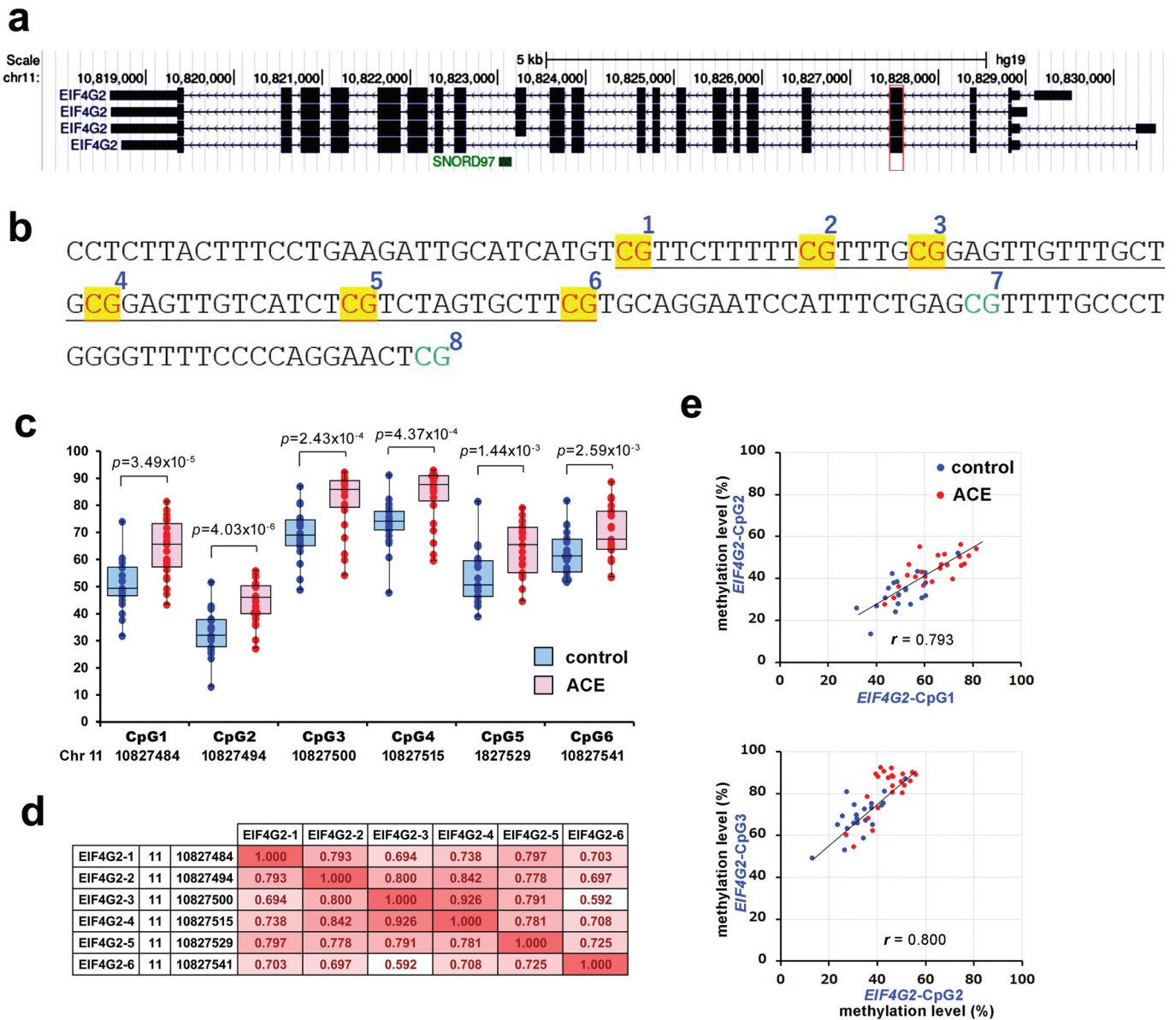


Figure 3. Characterization of a CpG cluster in exon 4 of the *EIF4G2* gene. (a) Schematic diagram showing the location of the candidate CpG cluster on chromosome 11 (positions 10827484–10827541), within exon 4 of the *EIF4G2* gene. (b) Sequence of the six CpG sites within the cluster. CpG sites showing correlated methylation patterns with adjacent sites are numbered and highlighted in red with yellow background. Flanking CpG sites without significant correlations are shown in green. (c) Box-and-whisker plots showing methylation levels of the CpG cluster in the control and adverse childhood experience (ACE) groups. Blue: control group; red: ACE group. The box shows the median and quartiles, and the error bars show the minimum and maximum values. (d) Matrix summarizing pairwise correlations of methylation levels among the six CpG sites in exon 4 of *EIF4G2*. (e) Scatter plots showing correlations between the methylation levels of *EIF4G2*-CpG1 and *EIF4G2*-CpG2 (upper), and between *EIF4G2*-CpG2 and *EIF4G2*-CpG3 (lower). Blue: control group; red: ACE group.

85. This result is consistent with prior evidence that ACEs negatively impact cognitive development [28]. Other studies have also reported that both the severity and cumulative burden of early adversity are negatively related to intellectual development [28,29]. Approximately half of the methylation levels of the candidate CpG sites identified in our study showed a moderate negative correlation with FS-IQ (Spearman's r ranging from -0.4 to -0.3) (Supplementary Figure S3). While many of the methylation levels of the CpG sites displayed positive correlations with ACE scores ($r = 0.4$ – 0.7), their correlations with FS-IQ were generally weaker. The presence of these moderate correlations, albeit not strong, remains worthy of consideration. Given that ACEs influence FS-IQ through a neurodevelopmental

processes, as widely discussed, this association should be carefully considered in epigenetic analyses. If FS-IQ is adjusted for in an EWAS, it may obscure ACE-related epigenetic alterations by selectively identifying DMRs that are either unrelated or resilient to such developmental effects.

Enrichment analysis suggested that genes adjacent to candidate DMRs were significantly associated with neurodevelopmental disorders, including X-linked Fragile X syndrome (Figure 4). Although we excluded sex chromosomes from our analysis due to the inclusion of both male and female participants, Fragile X syndrome – an X-linked disorder strongly associated with ID – emerged as a relevant enrichment signal. Among the genes located near the candidate CpG sites, *PTBP1*

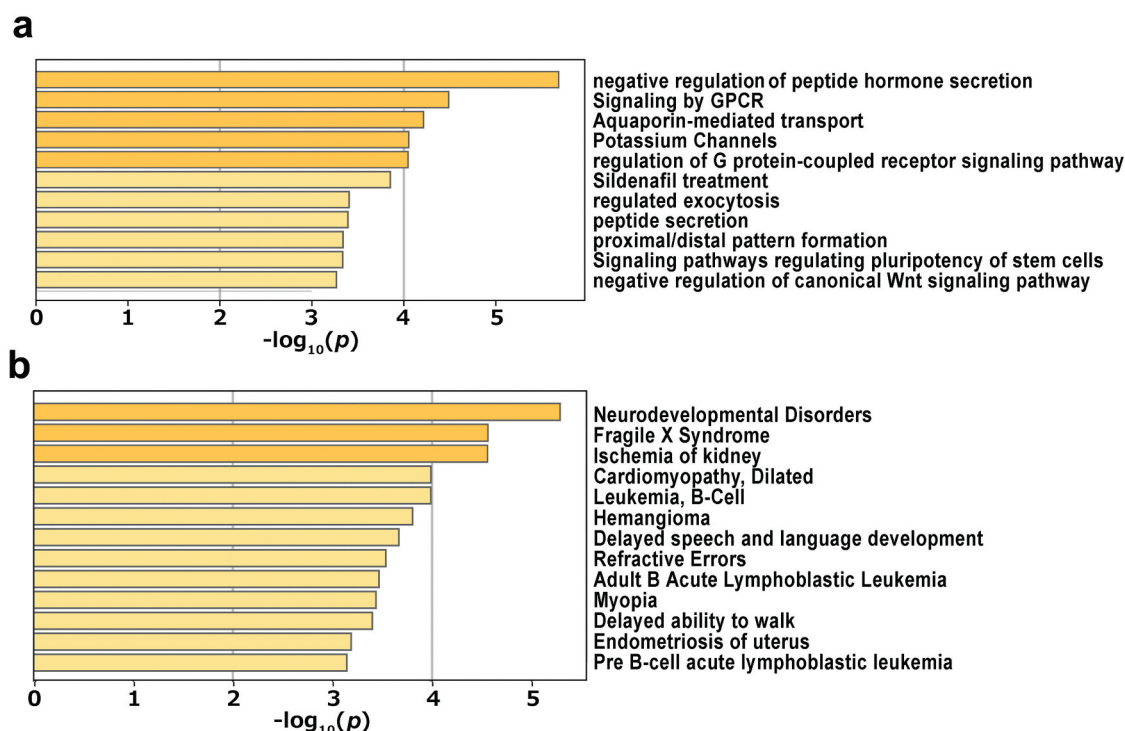


Figure 4. Metascape enrichment analysis of genes adjacent to differentially methylated CpG sites. (a) Pathway and process enrichment analyses were performed on genes adjacent to differentially methylated CpG sites using the following ontology sources: GO biological processes, reactome gene sets, and the KEGG pathway database. (b) Disease association analysis was conducted using the DisGeNET database. Bar charts display enrichment scores [$-\log_{10}(p\text{-value})$]. Gene sets with $p\text{-values} < 1.0 \times 10^{-3}$ are shown, with highlights for $p < 1.0 \times 10^{-4}$.

(polypyrimidine tract binding protein 1), *PIK3CD* (PtdIns-3-kinase subunit P110-delta), *EIF4G2* (eukaryotic initiation factor 4 gamma 2), *KCNT1* (potassium channel, sodium activated subfamily T1), and *ZNF423* (zinc finger protein 423) are neurodevelopmental genes that have been associated with ASD and with developmental conditions including ID (Table 2) [24,30–35]. *LRRK1* (leucine rich repeat kinase 1), *CHSY1* (chondroitin sulfate synthase 1), and *SEC14L1* (SEC14-like lipid binding 1) are also involved in neurodevelopment and regulation of neural function (Table 2) [36–39]. These genes are implicated in various aspects of brain development and function, and their mutation or dysregulation is associated with the clinical features of ASD and ID. These results suggest that ACE-induced systemic stress responses – possibly mediated by stress hormones during sensitive developmental periods – may alter the activity of genes involved in brain development and intellectual functioning. In our EWAS, genes classically implicated in the HPA axis—*NR3C1*, *FKBP5*, and *CRH*—did not emerge as candidate DMRs associated with ACEs. Although the CDMV capture probe set covered genomic regions surrounding these loci, methylation levels did not differ significantly between the two groups. Notably, even meta-analytic evidence drawing on a large body of prior studies has reported null associations between ACEs and methylation in these HPA axis-related genes, suggesting that multiple factors – including sample size, tissue type, and statistical approaches such as covariate adjustments – may contribute to the inconsistency in findings [10]. In contrast, in our analysis, a CpG site located near *CRHBP* (corticotropin-releasing hormone binding protein) exceeded the threshold of $p < 1 \times$

10^{-4} ($p = 9.29 \times 10^{-5}$), indicating increased methylation levels in children with ACE exposure (Supplementary Table S2). Previous studies have reported trauma-related alterations in the methylation status of *CRHBP* [40], and this gene may play a role in regulating the HPA axis as a negative regulator of CRH. In addition, several candidate DMRs were found to be located near non-coding RNAs, such as *MIR4745*, *SNORD97*, *LINC02907* and *LINC01535* (Table 2). In recent years, lncRNAs have been increasingly recognized for their involvement in a wide range of biological processes, including cell proliferation, differentiation, brain development, and immune responses [39]. Emerging evidence suggests that lncRNAs participate in the regulation of DNA methylation, histone modifications, and chromatin remodeling, thereby contributing to region-specific epigenetic control of gene expression [41]. Although many aspects of their function remain to be elucidated, it is conceivable that certain non-coding RNAs may play a role in mediating the long-term effects of transient ACEs during critical developmental windows.

Although many of the DMRs identified in this study exhibited ACE-associated methylation changes that were independent of neighboring CpG sites, six CpGs within a cluster in exon 4 of the *EIF4G2* gene showed strong inter-CpG correlations in their methylation levels (Figure 3). This CpG cluster resides within a conserved coding region, which underscores the importance of elucidating how ACEs influence methylation in this region and its implications for gene regulation. *EIF4G2* regulates the survival, proliferation, and differentiation of neurons by promoting non-canonical cap-dependent translation and participating in the activation of *EIF4GI*-

variety of dependent canonical cap-dependent translation [42]. Moreover, EIF4G2-dependent control of translation plays a critical role in depolarization-induced translation of upstream open reading frames and downstream coding sequences, enabling localized protein synthesis, which is important for long-term potentiation [24]. Dysregulation of translation as mediated by FMRP, the product of the Fragile X syndrome gene, contributes to impaired cognitive development, underscoring the relevance of this pathway for proper neurodevelopment [43,44]. Consistently, EIF4G2 dysfunction produces Fragile X-like phenotypes in animal models [33,34]. These findings suggest that ACE-related dysregulation in EIF4G2-mediated translational control may contribute to the pathogenesis of neurodevelopmental and intellectual disabilities.

Although it is unclear as to how changes in the methylation levels at these CpG sites affect the expression or function of nearby genes, the proximity of several of the identified DMRs to genes implicated in neurodevelopmental disorders suggests that ACE-associated methylation changes may contribute to the pathogenesis of such conditions. In this study, we used saliva as the biological material for DNA methylation profiling. An association between ACEs and DMRs has been reported in previous studies analyzing peripheral blood, saliva, and postmortem brain tissue samples [11,12,45–47]. Although tissue-specific differences in methylation are well established, the methylation levels of a subset of genes relevant to brain function and psychiatric disorders are correlated with peripheral and brain tissues [48,49]. For example, allele-specific methylation of *FKBP5* correlates with glucocorticoid exposure in both blood and brain [50]. Smith et al. suggested that, under appropriate conditions, saliva samples may reflect methylation patterns observed in brain regions, supporting their utility for EWAS [51]. Given its noninvasive nature, saliva is particularly well suited for use as a biopsy material in pediatric populations. Our identification of ACE-associated DMRs using salivary DNA highlights the value of this approach. Furthermore, the identification of multiple ACE-associated candidate DMRs in this study opens up the possibility of developing a custom capture probe set for NGS that targets early ACE-associated DMRs. Methylation levels at individual candidate CpG sites can also be assessed using pyrosequencing-based quantification (Supplementary Figure S2). In particular, the ACE-sensitive CpG cluster within *EIF4G2* may serve as a valuable marker for targeted analyses.

A key limitation of the present study is the relatively small sample size, which resulted from the intentionally stringent inclusion criteria. While the use of custom-designed capture probes is a strength of this study, it also limits the validation of our findings against existing knowledge from analyses using commercial bead-chip methylation arrays, such as those included in the EPIDELTA and IMAGE-CpG databases [52,53]. Further replication and validation of our findings will require analyses using a larger sample size and broader inclusion criteria. In the future, expanding the analysis to include participants of different age groups, those with and without psychotropic medication, and those with ACE scores less than 4 may help establish the robustness and generalizability of our findings.

5. Conclusion

In conclusion, this exploratory study indicates that an inter-individual DMR-targeted EWAS approach may help identify novel DMRs associated with early childhood adversity using salivary DNA from children. Notably, several of these DMRs were located near genes implicated in neurodevelopmental disorders, suggesting that ACEs may leave lasting epigenetic marks on neurodevelopmental trajectories. Our findings provide a basis for the development of noninvasive retrospective biomarkers of early life adversity, with potential implications for early identification and intervention in vulnerable pediatric populations.

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Disclosure statement

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Ethical declaration

This study was approved by the Ethics Committee of Iwate Medical University (Approval ID: MH2021-178). All procedures were conducted in accordance with the approved guidelines. The study was conducted in accordance with the guidelines of the Declaration of Helsinki. All caregivers provided written informed consent and all children provided written informed assent.

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Data availability statement

The EWAS scripts used in this study are available in the Supplementary Materials (EWAS script file). The dataset analyzed in this study is provided in the Supplementary Materials (Supplementary Table S2), which lists CpG-specific methylation levels for both groups at sites with p -values $< 10^{-4}$. Because access to individual-level data requires approval from the Ethics Committee of Iwate Medical University and opt-out consent for the secondary use of data, please contact the corresponding author for further information.

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