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Interaction of *DRD4* Methylation and Phthalate Metabolites Affects Continuous Performance Test Performance in ADHD

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Abstract

Objective: We investigated the interaction effect between the methylation of dopamine receptor D4 (DRD4) and phthalate exposure in ADHD on continuous performance test (CPT) variables. **Method:** Urine concentrations of mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), and mono-n-butyl phthalate (MBP) were tested. The methylation status was analyzed for CpG sites of *DRD4*. Multivariable linear regression models were applied to investigate the interaction effects of methylation and phthalate levels. **Results:** There was a significant interaction effect of the methylation of CpG26 and CpG28 with the MEHHP level on omission errors in ADHD patients, but not in controls. The post hoc analysis revealed a significant correlation between the MEHHP concentration and omission errors in the methylated group, but not in the unmethylated group. **Conclusion:** The interaction between the methylation status of CpG sites of *DRD4*, particularly CpG26 and CpG28, and phthalate metabolite levels affects the attention level in ADHD patients. (*J. of Att. Dis. XXXX; XX(X) XX-XX*)

Keywords

ADHD, methylation, *DRD4*, phthalate exposure, continuous performance test

Introduction

ADHD is a complex polygenetic disorder, in which genetic factors contribute to 75% of its heritability, while environmental factors are responsible for the remaining 25% (Faraone et al., 2005). The dopamine receptor D4 (DRD4) exon III 48-base pair variable number tandem repeat (VNTR) is one of the leading candidate genes in ADHD (Faraone & Mick, 2010). In Caucasian studies, the 7-repeat (7R) allele has been found to be a risk factor in ADHD (Li, Sham, Owen, & He, 2006), but these study results are non-applicable in Asians, as the allele frequency is very rare (Lichter et al., 1993). Korean studies have mainly focused on the 4-repeat (4R) allele (Hong et al., 2012; Ji, Paik, Park, & Lim, 2013; H. Kim, Kim, Kim, Kim, & Kim, 2017).

The variables of the continuous performance test (CPT) are among the most widely studied endophenotypes of ADHD (Alemany et al., 2015; Andreou et al., 2007; Kebir, Tabbane, Sengupta, & Joober, 2009). CPT refers to neuropsychological tests that measure an individual's sustained and selective attention, both of which are found to be impaired in ADHD patients (DuPaul, Anastopoulos, Shelton, Guevremont, & Metevia, 1992). A previous study found a significant protective effect of the 4R allele on the performance of a CPT (B. Kim et al., 2009), Studies on the relationship between the 7R allele and CPT variables have reported inconsistent results, in which some studies have found better performance in 7R carriers, whereas others have reported worse performance associated with the 7R allele (Kebir & Joober, 2011). The discrepancy among genetic study results may be partially explained by gene–environment interaction, in which a certain genetic variant can increase vulnerability (Buitelaar, 2005).

Phthalate metabolites have been found to contribute to ADHD symptoms and neuropsychological deficits found in ADHD patients (S. Park et al., 2014). Phthalates are a group

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of endocrine-disrupting synthetic plasticizers and solvents that are used in a wide variety of commercial products, including food packaging, medical and cleaning equipment, cosmetics, and toys (Kobrosly et al., 2014). Among phthalates, di-n-butyl-phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP) have been widely studied in neurodevelopment studies (Ejaredar, Nyanza, Ten Eycke, & Dewey, 2015). Secondary oxidized DEHP metabolites include mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), which are valuable markers of DEHP exposure. Mono-n-butyl phthalate (MnBP) is a metabolite of DBP and is used as a biomarker of DBP exposure. Previous studies have reported significant associations between phthalate metabolite levels and CPT performance (B. N. Kim et al., 2009; S. Park et al., 2014).

The postulated mechanism underlying the association between phthalates and ADHD is an alteration in the expression of various genes, including *DRD4*, by phthalate metabolites (Masuo, Morita, Oka, & Ishido, 2004). On the basis of this assumption, S. Park et al (2014) conducted a gene–environment interaction study and found evidence of interaction between the *DRD4* 4R genotype and phthalate metabolite levels on CPT variables in ADHD patients (S. Park et al., 2014). However, this study was limited by the lack of consideration of epigenetic processes. Epigenetic factors have an important role in gene and environment interactions, as environmental factors influence the genomic expression through epigenetic mechanisms (Bagot & Meaney, 2010). DNA methylation is the most frequently studied epigenetic mark (Reik, 2007; Suzuki & Bird, 2008). In this process, a methyl group is attached to 5′-cytosine residues at cytosine–guanine sequences (CpG) in the DNA (Bird, 1986), usually resulting in inhibition of RNA transcription and gene expression (Akbarian, 2010). Therefore, the methylation level, rather than the genotype per se may be more important when determining genetic effects.

The primary purpose of this study was to investigate the interaction between *DRD4* methylation and phthalate metabolite levels on CPT variables. The secondary research goal was to investigate the association between methylation of *DRD4* CpG sites and CPT variables.

Materials and Methods

Participants

Online Supplement Figure S1 depicts an explanatory diagram of recruitment and assessment of participants. This study included 249 children and adolescents with ADHD and 98 healthy controls (HCs) between 6 and 17 years of age, who were recruited between August 2010 and February 2015. For this study, participants from two studies that were conducted using the same protocol were combined into a single subject pool; detailed explanations of both study protocols and the combined protocol have been provided elsewhere (J. I. Kim et al., 2016). The first study initially recruited 90 ADHD patients and 33 HCs; after excluding five ADHD patients with missing CPT data and one HC with missing genetic data, 85 ADHD patients and 32 HCs were assessed (Hong et al., 2015). The second study initially recruited 191 ADHD patients and 78 HCs; after excluding four patients with missing CPT data, six patients missing phthalate data and 17 patients with missing genetic data from the ADHD group and one subject with missing CPT data and 11 subjects with missing genetic data from the HC group, 164 ADHD patients and 66 HCs were assessed (S. Park et al., 2015).

All the ADHD patients were medication-naïve, of Korean ethnicity, and had visited the child and adolescent psychiatry outpatient clinic at the Seoul National University Hospital. ADHD and other psychiatric comorbidities were confirmed according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.; *DSM-IV*; American Psychiatric Association, 1994) by board-certified child and adolescent psychiatrists using the Kiddie Schedule for Affective Disorders and Schizophrenia—Present and Lifetime version (K-SADS-PL; Kaufman et al., 1997; Y. S. Kim et al., 2004). The exclusion criteria for ADHD were as follows: IQ < 70; a hereditary genetic disorder; current or past history of brain trauma, organic brain disorder, seizure, or any neurological disorder; autism spectrum disorder, communication disorder, or learning disorder; schizophrenia or any other childhood-onset psychotic disorder; major depressive disorder or bipolar disorder; Tourette's syndrome or a chronic motor/vocal tic disorder; obsessive compulsive disorder; and/or a history of methylphenidate treatment lasting for more than 1 year or having taken the drug within the past 4 weeks. The HC group included typical-development children and adolescents who were free of any psychiatric diagnoses according to the K-SADS-PL. The same exclusion criteria for the ADHD group were applied to the HC group with the additional criterion of an ADHD diagnosis.

IQ was measured using the Korean Educational Developmental Institute's Wechsler Intelligence Scale for Children (K. S. Park, Yoon, Park, & Kwon, 1996), and the severity of ADHD symptoms was measured using the parent-completed ADHD Rating Scale-IV (ADHD-RS; So, Kim, Ko, & Koh, 2002). Written informed consent was obtained from all parents/guardians and adolescents, and the children provided verbal assent to participate after sufficient explanation of the study prior to enrollment. All study protocols were approved by the institutional review board of Seoul National University Hospital.

Determination of Methylation Status

Genomic DNA was extracted from whole blood using an Intron_G-DEX™ IIb Genomic DNA Extraction kit (Intron, South Korea). A bisulfite sequencing procedure was performed

to determine the CpG methylation profiles located upstream of the *DRD4* coding regions; the experiment was carried out as previously described (Xu et al., 2015) with some modifications. The bisulfite conversion was performed with the EpiMark Biosulfite Conversion Kit (New England Biolabs Inc., Ipswich, MA, the United States) according to the manufacturer's instructions. The bisulfite conversion reaction was performed in a reaction mixture volume of 140 μL that contained 1 μg of genomic DNA isolated from blood and 130 μL of bisulfite mix, which was prepared by adding 650 μL of nuclease-free water and 250 μL of solubilization buffer. The bisulfite conversion reaction steps consisted of denaturation at 95°C for 5 min, incubation at 65°C for 30 min, denaturation at 95°C for 5 min, incubation at 65°C for 60 min, denaturation at 95°C for 5 min, incubation at 65°C for 30 min, and a final incubation at 18°C to 20°C for up to 12 hr.

Following the completion of the conversion reaction, the desulfonation reaction and sample clean-up procedures were carried out using the EpiMark spin column provided with the conversion kit. First, the entire sample was loaded onto the EpiMark spin column and washed with 500 μL of wash buffer. Next, the desulfonation reaction was performed by adding 500 μL of desulfonation reaction buffer to the column and incubating it at room temperature for 15 min. After the incubation, the column was washed with 500 μL of wash buffer, and the samples were eluted into 20 μL of elution buffer.

The bisulfite-treated DNA was then subjected to a polymerase chain reaction (PCR) procedure in which the nested PCR method was utilized to amplify the CpG island regions of *DAT1* and *DRD4*. The following primers were used: for the first PCR of the *DRD4* gene, primers DRD4_F1 (TAGGTTATTTTTTTTTGGTGAAGA) and DRD4_R1 (TCACCCTAATCCACCTAATATCT); and for the second PCR of the *DRD4* gene, primers DRD4_F2 (TTGTTTAGGGTTAGAGGGG) and DRD4_R2 (ATCCACCTAATATCTAACAAAACC).

The first-step PCR reaction was performed in a 25-μL volume containing 20 to 100 ng of bisulfite-treated DNA, 1 × PCR buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 μM of each primer for the first PCR, and 0.625 units of EpiMark Taq polymerase (New England Biolabs). The reaction steps consisted of denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 1 min, with a final extension at 68°C for 5 min. The second-step PCR reaction was carried out using 1 μL of a 1:10 dilution of the first-step PCR product in a total volume of 25 μL under the same conditions as the first-step procedure but using the second PCR primers and an annealing temperature of 50°C. Following the PCR procedure, any unincorporated primers and dNTPs were removed by adding a one tenth volume of Exo-Sap (ExoProStar 1; GE Healthcare, Little Chalfont, the United Kingdom), incubating it for 15 min at 37°C, and then incubating it for 15 min at 85°C for enzyme inactivation.

The resulting PCR products were directly subjected to DNA sequencing and the methylation status of each CpG island was manually determined by visual inspection. The percentage of methylation was calculated as the peak height of C versus the peak height of C plus the peak height of T for each CpG site as shown in the sequencing chromatogram. Sites between 0% and 10% were classified as unmethylated (0%, U/U), and sites between 90% and 100% were classified as fully methylated (100%, M/M). Sites between 10% and 90% were classified as hemimethylated (50%, U/M).

Phthalate Metabolite Level Measurement

The spot urine samples were collected in 50-mL sterile specimen containers at the outpatient clinic in the morning and were refrigerated at −20℃ immediately. Refrigerated specimens were transported to the laboratory within 2 hr. We measured the metabolites of DEHP and DBP among several phthalate compounds because these are the most widely used phthalates in personal care products (e.g., DEHP in various kinds of plastic products, including vinyl flooring, paints, toys, and plastic bags and DBP in cosmetic products, such as perfume, aftershaves, and shampoo) and have been most widely linked to adverse health effects. DEHP is metabolized to the primary metabolite MEHP and subsequently to secondary metabolites, MEHHP and MEOP, and DBP is metabolized to the primary metabolite MBP. The metabolites measured in this study included MEHP, MEOP, and MBP because these metabolites have been reported to be associated with cognitive function and behaviors of children in previous studies. The urine phthalate metabolite concentrations were measured with high-performance liquid chromatography tandem mass spectrometry (Agilent 6410 triple Quad LCMS; Agilent Technologies, Santa Clara, CA, the United States). Urine of 500 μL were buffered with 30 μL of 2.0 mol/L sodium acetate (pH 5.0) and then spiked with a mixture of isotope phthalate monoester standards (100 ng/mL) and 10 μL of β-glucuronidase. The sample was incubated at 37℃ for 3 hr to deconjugate the glucuronidated phthalate metabolites. After incubation, 100 μL of 2 nmol/L hydrogen chloride was added to collect phthalate monoesters. The extract was dried with nitrogen gas and reconstituted with 1 ml of high-performance liquid chromatography-grade water in a 2-ml glass vial. One blank and one quality control (QC) sample were included in each batch of samples. The QC sample was spiked with pooled urine and a mixture of phthalate monoester standard (100 ng/mL). The supernatants were purified by solid phase extraction with disposable Agilent C18 1.8 μm (2.1 × 50 mm). The mobile phase was 0.1% acetic acid water: 0.01% acetic acid acetonitrile (90:10, v/v) at a flow rate of 0.25 mL/min, and the phthalates were monitored at target masses of 221, 293, and 291 and internal standard masses of 225, 297, and 295. We used the value

(micrograms per liter)/creatinine (grams per liter) for dilution correction in the analyses. Because the concentrations were not normally distributed, we used their log10-transformed values.

CPT

A Korean version of the computerized CPT was administered to assess the impulsivity and inattention of the participants (Shin, Cho, Chun, & Hong, 2000). The participants were instructed to respond to the target stimulus (square containing a triangle) but not to the nontarget stimuli (square containing either a square or a circle); the visual stimuli were presented on a screen for 100 ms every 2 s. Performance was assessed based on three variables: (a) omission errors (failure to respond) as a measure of inattention, (b) commission errors (false response) as a measure of impulsivity, and (c) response time variability (the *SD* of the response times of correct responses) as a measure of sustained attention. All data were automatically transformed into T-scores adjusted for age relative to a normal population of 847 children between 5 years and 15 years of age (Shin, Cho, Chun, & Hong, 2000); lower T-scores indicated better performance.

Statistical Analysis

The demographic and clinical characteristics of the ADHD and HC groups were compared using independent *t* tests for continuous variables and chi-square tests or Fisher's exact tests for categorical variables. Due to the rarity of the M/M group at some sites, the U/M and M/M groups were combined into a single methylated group for the methylation analyses. Thus, the methylation status of each CpG site was categorized into two groups: unmethylated (U/U) and methylated (U/M + M/M). The distribution of methylation groups of each CpG site was compared between the ADHD and HC groups using chi-square tests or Fisher's exact tests.

The interaction between the methylation status of each CpG site and each phthalate metabolite level on CPT variables was calculated using hierarchical multivariable linear regression analyses: the methylation group of each CpG site and phthalate metabolite level was placed in the first block, and the interaction of these two was put into the second block. We performed the linear regression analyses in the ADHD and HC groups separately to determine whether the significant interactions were specific to the ADHD group. We conducted further post hoc analyses to evaluate the association between the phthalate metabolite concentration and CPT variables in the methylated and unmethylated groups, separately.

All statistical analyses were performed with SPSS ver. 22.0 software (SPSS Inc., Chicago, IL, the United States), and a two-tailed p-value < 0.002 (0.05 / 28 CpG sites) was considered to indicate statistical significance.

Results

The demographic and clinical characteristics of the ADHD and HC group are presented in Table 1. The mean age and IQ of the ADHD group was significantly lower than those of the HC group, and the proportion of male subjects was significantly higher. The MEHHP, MEOHP, and MBP levels were significantly higher in the ADHD group compared with the MBP group ($p < 0.001$). No CpG sites showed significant differences in methylation statuses between the ADHD and HC groups, but there was a nominally significant association between methylation group distribution and diagnostic groups in CpG6 and CpG22 ($p = .034$ and $p = .004$, respectively, Table S1). There was a higher percentage of subjects in the methylated group in the ADHD group compared with the HC group (ADHD vs. HC: CpG6 92% vs. 90.8%, CpG22 94% vs. 82.6%).

The methylation groups of CpG26 and CpG28 showed significant interaction with MEHHP levels on omission errors (25.95, 95% confidence interval [CI] = [10.37, 41.53] for CpG26; 23.01, 95% CI = [9.18, 36.84] for CpG28; $p = 0.001$, Table 2), and the methylation group of CpG26 had a significant interaction effect with MEHHP levels on response time variability (23.77, 95% CI = [9.93, 37.60]; $p = .001$, Table 3) in the ADHD group. Post hoc analyses revealed that in the methylated group of CpG26, there was a significant positive correlation between omission errors and MEHHP levels (14.60, 95% CI = [28.14, 54.15]; $p < .001$), but this correlation was not found in the unmethylated group (Table 4, Figure 1). There also was a significant positive correlation between omission errors and MEHHP levels in the methylated group of CpG28 (17.03, 95% CI = [8.37, 25.70]; $p < .001$), but not in the unmethylated group (Table 4, Figure 2).

There were no significant interactions between the methylation status and phthalate metabolite levels on any CPT variable in the HC group. Moreover, there were no CpG sites that showed significant main effects on CPT variables in the ADHD and HC groups.

Discussion

This is the first study to investigate the interaction between *DRD4* methylation and phthalate metabolite levels on CPT scores. Although there were no CpG sites that showed a main effect on CPT performance, the methylation of CpG26 and CpG28 interacted with phthalate metabolites and affected the inattention and response time variability found in ADHD patients, but not in healthy children and adolescents. These results highlight the importance of considering both methylation and environmental factors when designing a genetic study (Han et al., 2015). Simino et al. similarly proposed that the interactions between epigenetic phenomena and environmental factors may be synergistic (Simino, Rao, & Freedman, 2012).

Table 1. Demographic and Clinical Characteristics of the ADHD and HC Groups.

Characteristic	ADHD (*n* = 249)	HC (*n* = 98)	*p* value
Age (years), *M* (*SD*)	8.9 (2.4)	10.4 (3.0)	<.001
Sex (male), *N* (%)	191 (76.7)	54 (55.1)	<.001
Intelligence quotient, *M* (*SD*)	105.6 (14.4)	113.7 (12.5)	<.001
Yearly family income > US$25,000, *N* (%)	154 (69.7)	57 (62.6)	.227
ADHD subtype, *N* (%)			
Predominantly inattentive	94 (37.9)		
Predominantly hyperactive-impulsive	16 (6.5)		
Combined	112 (45.2)		
NOS	26 (10.5)		
Maternal education, years, *M* (*SD*)	14.6 (2.3)	14.1 (3.5)	.325
Paternal education, years, *M* (*SD*)	14.8 (1.9)	14.6 (2.0)	.279
ADHD rating scale score, *M* (*SD*)			
Total score	24.9 (10.9)	6.1 (7.2)	<.001
Inattention subscore	14.8 (5.6)	3.8 (4.0)	<.001
Hyperactivity-impulsivity subscore	10.1 (6.6)	1.8 (2.1)	<.001
CPT variables, *M* (*SD*)			
Omission errors	64.8 (20.2)	54.1 (15.7)	<.001
Commission errors	65.0 (19.3)	54.9 (14.7)	<.001
Response time variability	55.4 (11.9)	55.2 (11.8)	<.001
Phthalate metabolite levels			
MEHHP	1.6 (0.4)	1.5 (0.4)	<.001
MEOHP	1.6 (0.3)	1.4 (0.4)	<.001
MBP	1.8 (0.3)	1.6 (4.0)	<.001

Note. ADHD = attention-deficit hyperactivity disorder; HC = healthy control; NOS = not otherwise specified; CPT = continuous performance test; MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP = mono-(2-ethyl-5-oxohexyl) phthalate; MBP = mono-n-butyl phthalate.

This was also the first study to examine the association between *DRD4* methylation and CPT scores in ADHD patients. However, there were no significant associations between methylation of any CpG site and CPT variables. The results of this study are contradictory to the results of other studies that have investigated the association between *DRD4* methylation and cognition. Dadds, Schollar-Root, Lenroot, Moul, and Hawes (2016) reported that the mean methylation of *DRD4* showed significant correlations with cognitive problems reported by mothers in ADHD children (Dadds et al., 2016). Another study by Cheng et al. (2014) found a significant negative correlation between the mean methylation of 6 CpG sites in the *DRD4* CpG island and p300 in male schizophrenia patients (Cheng et al., 2014). The p300 wave is often used as a cognitive function metric and has been proven relative to schizophrenia cognitive impairment (Gao et al., 2012). The studies by Dadds and Cheng differ from the present study because they used the mean methylation level of *DRD4*. Cognitive problems were not objectively measured using neuropsychological tests, but rather by maternal report in the study by Dadds et al. The strength of our study is that it used an objective neuropsychological test and examined the methylation of each CpG site. However, the small sample size may have limited the statistical power, and future studies with larger samples sizes are warranted.

Genetic studies on the *DRD4* variant have been rather challenging in Asian populations, including Koreans, as the 7R allele is very rare (Lichter et al., 1993). The few studies focusing on the 4R allele in the Korean population have obtained mixed results, which may be due to the lack of consideration of environmental factors (Y. S. Kim et al., 2005). Gene × environment studies on *DRD4* in Caucasians have been focused on the 7R allele, and they have investigated the effects of prenatal maternal stress, parental support, stressful life events, and prenatal smoking (Grizenko et al., 2012; Pluess, Belsky, & Neuman, 2009; Sanchez-Mora et al., 2015). None of these studies have investigated the interaction between *DRD4* methylation and environmental factors. Further studies on the interactions of *DRD4* methylation and various environmental factors other than phthalate exposure are warranted in the future.

Although the mechanisms leading to the interaction between *DRD4* methylation and phthalate exposure are largely unknown, the most plausible explanation can be derived from previous study results on the toxicity of phthalates on the dopaminergic system. Animal studies have shown hyperkinetics in rats administered with

Table 2. Main and Interaction Effects of CpG Methylation and MEHHP Concentration on Omission Errors of the CPT.

	CpG methylation		Log MEHHP		Methylation × log MEHHP	
Variables	β [95% CI]	*p* value	β [95% CI]	*p* value	β [95% CI]	*p* value
CpG3	−36.80 [–83.15, 9.54]	.119	−12.13 [–38.87, 14.61]	.372	21.79 [–5.88, 49.45]	.122
CpG4	19.79 [–60.02, 99.60]	.626	25.17 [–23.44, 73.77]	.309	−17.24 [–66.34, 31.87]	.490
CpG5	−28.31 [–75.24, 18.63]	.236	−6.87 [–34.71, 20.97]	.627	16.13 [–12.60, 44.86]	.270
CpG6	−10.68 [–49.59, 28.22]	.589	−1.98 [–24.24, 20.27]	.861	11.16 [–12.23, 34.55]	.348
CpG7	−30.84 [–100.12, 38.44]	.381	−2.61 [–44.53, 39.31]	.903	11.26 [–31.23, 53.75]	.602
CpG8	−35.93 [–68.41, –3.45]	.030	−11.22 [–30.48, 8.05]	.252	22.49 [1.86, 43.11]	.033
CpG9	−41.66 [–77.96, –5.35]	.025	−16.87 [–37.54, 3.80]	.109	28.06 [6.18, 49.95]	.012**
CpG10	−4.16 [–58.15, 49.83]	.879	3.20 [–28.62, 35.02]	.843	5.23 [–27.36, 36.82]	.752
CpG11	2.78 [–24.88, 30.43]	.843	8.62 [0.81, 16.43]	.031	−0.87 [–18.06, 16.31]	.920
CpG12	−25.98 [–49.62, –2.34]	.031	−1.89 [–13.07, 9.29]	.739	16.10 [1.98, 30.22]	.026
CpG13	−10.07 [–40.25, 20.12]	.512	0.78 [–15.20, 16.76]	.923	9.53 [–8.15, 27.22]	.289
CpG14	−17.41 [–43.85, 9.02]	.196	0.90 [–12.43, 14.24]	.894	9.95 [–5.61, 25.51]	.209
CpG15	−30.05 [–53.94, –6.17]	.014	−2.66 [–13.46, 8.15]	.628	18.30 [4.33, 32.27]	.010
CpG16	−29.30 [–70.04, 11.44]	.158	−6.36 [–29.88, 17.16]	.594	16.01 [–8.58, 40.60]	.201
CpG17	−32.43 [–56.02, –8.85]	.007	−2.71 [–13.56, 8.14]	.624	17.87 [3.97, 31.78]	.012
CpG18	−100.67 [–197.70, –3.64]	.042	−43.13 [–95.20, 8.93]	.104	52.12 [–0.40, 104.64]	.052
CpG19	−52.02 [–109.14, 5.10]	.074	−27.75 [–63.33, 7.83]	.126	37.21 [0.96, 73.47]	.04
CpG20	14.49 [–38.99, 67.97]	.594	17.14 [–14.21, 48.48]	.282	−9.37 [–41.50, 22.76]	.566
CpG21	−26.49 [–79.37, 26.39]	.325	−1.37 [–31.33, 28.58]	.928	9.99 [–20.79, 40.76]	.523
CpG22	−32.52 [–76.04, 11.01]	.142	−15.20 [–42.33, 11.93]	.271	24.70 [–3.34, 52.75]	.084
CpG23	49.21 [–103.72, 5.29]	.077	−23.23 [–55.55, 9.09]	.158	32.90 [–0.17, 65.96]	.051
CpG24	13,45 [–13.27, 40.17]	.322	10.10 [–2.21, 18.00]	.012**	−7.24 [–23.54, 9.07]	.383
CpG25	−12.78 [–47.95, 22.39]	.475	7.27 [–0.11, 14.65]	.053	7.52 [–13.13, 28.17]	.474
CpG26	−39.05 [–65.37, –12.73]	.004	−11.35 [–24.87, 2.17]	.100	25.95 [10.37, 41.53]	.001**
CpG27	−7.83 [–39.21, 23.55]	.624	4.08 [–13.64, 21.80]	.651	4.89 [–14.35, 24.13]	.617
CpG28	−35.20 [–58.44, –11.96]	.003	−5.98 [–16.84, 4.88]	.279	23.01 [9.18, 36.84]	.001**

Note. CPT = continuous performance test; MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate; HC = healthy control; CI = confidence interval.
**$p < .002$.

6-hydroxydopamine and also reduced immunoreactivity for tyrosine hydroxylase, which is a rate-limiting enzyme with regard to the production of dopamine, induced by phthalate metabolites (Ishido et al., 2004; Shaywitz, Yager, & Klopper, 1976). Other studies have reported alterations in the expression patterns of various genes, including the D4 receptor and dopamine transporter in the midbrain caused by phthalate metabolites (Masuo et al., 2004). As methylation of a CpG site usually results in inhibited expression of a gene (Goll & Bestor, 2005), we can hypothesize that in the methylated group, the expression of *DRD4* was suppressed, resulting in decreased density of the D4 receptor and a compensatory increase in the sensitivity to dopamine. This could increase the susceptibility to the neurotoxic effects of phthalate, leading to increased inattention and deficits in sustained attention.

There were some noteworthy limitations to this study. First, there was a significant difference in age, gender distribution, and IQ between the ADHD and HC groups. The sample size was relatively small for a gene–environment study, and the results must be interpreted carefully. The measurement of DNA methylation using peripheral blood DNA limited consideration of the differences in the epigenetic profiles according to tissue type. To confirm the mechanisms leading to the study results, further studies using postmortem brain tissues are required. The functional relevance of the methylation of each CpG site (e.g., mRNA expression or protein expression) was not investigated, and the neural mechanisms underlying the study results remain unclear. In addition, this study included only patients with a Korean genetic background, which may limit the generalizability of the findings regarding ADHD to other ethnicities. Methylation was not measured as a continuous variable, but rather in three groups according to the methylation level. Finally, the cross-sectional nature of this study limits a definite conclusion on causality.

Conclusion

This study demonstrated a significant interaction between the methylation status of CpG sites of a *DRD4* variant and

Table 3. Main and Interaction Effects of CpG Methylation and MEHHP Concentration on RTV of the CPT.

	CpG methylation		Log MEHHP		Methylation × log MEHHP	
Variables	β [95% CI]	*p* value	β [95% CI]	*p* value	β [95% CI]	*p* value
CpG3	−29.03 [−69.22, 13.16]	.181	−12.44 [−36.20, 11.33]	.304	17.27 [−7.31, 41.86]	.168
CpG4	−21.48 [−92.09, 49.14]	.550	−0.90 [−43.90, 42.11]	.967	4.80 [−38.64, 48.25]	.828
CpG5	−22.48 [−64.16, 19.21]	.289	−9.71 [−34.44, 15.01]	.440	14.28 [−11.24, 39.80]	.271
CpG6	0.77 [−33.97, 35.50]	.965	2.40 [−17.47, 22.26]	.812	1.40 [−19.49, 22.28]	.895
CpG7	−5.85 [−43.85, 32.16]	.762	9.44 [−28.06, 46.94]	.620	4.45 [−57.52, 66.42]	.888
CpG8	−19.37 [−48.39, 9.65]	.190	−5.96 [−23.17, 11.25]	.495	11.36 [−7.07, 29.78]	.226
CpG9	−20.59 [−53.08, 11.89]	.213	−9.94 [−28.44, 8.55]	.291	15.23 [−4.36, 34.81]	.127
CpG10	−1.43 [−49.34, 46.47]	.953	−0.06 [−28.29, 28.16]	.996	3.91 [−25.01, 32.82]	.790
CpG11	−2.59 [−27.13, 21.95]	.836	3.39 [−3.54, 10.33]	.336	2.48 [−12.77, 17.73]	.749
CpG12	−15.78 [−36.89, 5.34]	.142	−2.26 [−12.25, 7.73]	.656	9.83 [−3.08, 22.14]	.138
CpG13	−14.38 [−41.16, 12.40]	.291	−5.31 [−19.49, 8.87]	.462	11.36 [−4.33, 27.05]	155
CpG14	−18.07 [−41.50, 5.37]	.130	−4.18 [−16.00, 7.64]	.487	10.72 [−3.07, 24.51]	.127
CpG15	−32.39 [−52.43, −11.34]	.003	−8.08 [−17.60, 1.43]	.096	19.90 [7.69, 32.20]	.002
CpG16	−40.66 [−76.54, −4.78]	.027**	−15.81 [−36.53, 4.90]	.134	21.45 [−0.21, 43.10]	.052
CpG17	−29.47 [−50.40, −8.55]	.006	−6.61 [−16.23, 3.02]	.177	16.89 [4.56, 29.22]	.008
CpG18	−76.64 [−162.95, 9.67]	.082	−34.05 [−80.37, 12.26]	.149	38.26 [−8.46, 84.97]	.108
CpG19	−27.98 [−78.89, 22.93]	.280	−17.81 [−49.53, 13.90]	.270	22.17 [−10.14, 54.49]	.178
CpG20	10.71 [−36.78, 58.20]	.657	10.18 [−17.65, 38.01]	.472	−6.81 [−35.33, 21.72]	.539
CpG21	−49.53 [−96.28, −2.79]	.038	−20.87 [−47.35, 5.61]	.122	25.84 [−1.36, 53.04]	.062
CpG22	−36.73 [−75.35, 1.89]	.062	−19.86 [−43.93, 4.21]	.105	25.11 [0.22, 49.99]	.048
CpG23	−30.59 [−79.23, 18.05]	.217	−14.18 [−43.02, 14.66]	.334	18.73 [−10.78, 48.24]	.212
CpG24	−0.67 [−24.43, 23.10]	.956	3.68 [−3.34, 10.69]	.303	−0.50 [−15.00, 14.01]	.946
CpG25	−8.59 [−39.80, 22.62]	.588	2.87 [−3.68, 9.42]	.389	6.10 [−12.22, 24.43]	.512
CpG26	−39.86 [−63.22, −16.50]	.001**	−14.17 [−26.17, −2.17]	.021	23.77 [9.93, 37.60]	0.001**
CpG27	−6.59 [−34.44, 21.27]	.642	0.19 [−15.54, 15.92]	.981	4.14 [−12.94, 21.22]	.634
CpG28	−25.05 [−45.94, −4.16]	.019	−5.44 [−15.20, 4.32]	.273	14.84 [2.41, 27.28]	.020

Note. RTV = response time variability, CPT = continuous performance test; MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate; HC = healthy control; CI = confidence interval;
**$p < .002$.

Table 4. Post Hoc Analyses of Significant Methylation Group × MEHHP Levels on CPT Variables.

	Methylated group		Unmethylated group	
Variables	β [95% CI]	*p* value	β [95% CI]	*p* value
Omission errors				
CpG26	14.60 [28.14, 54.15]	<.001**	−11.35 [−25.28, 2.58]	.107
CpG28	17.03 [8.37, 25.70]	<.001**	−5.98 [−16.76, 4.80]	.272
Response time variability				
CpG26	9.60 [2.82, 16.37]	.006*	−14.17 [−27.66, −0.68]	.040*

Note. CPT = continuous performance test; MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate; HC = healthy control; CI = confidence interval.
*$p < .05$. **$p < .002$.

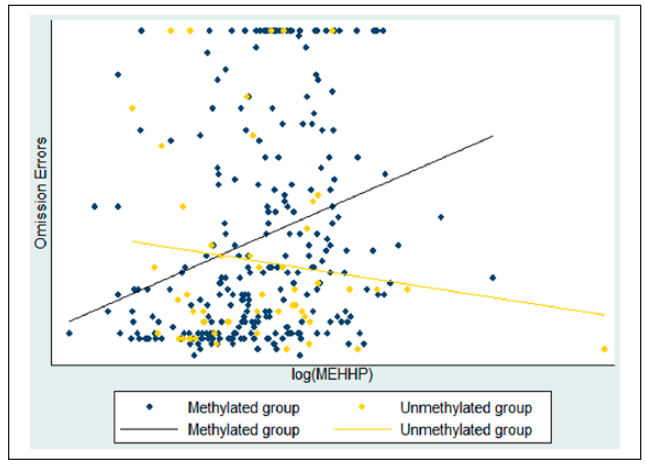

Figure 1. The post hoc analysis of the interaction between CpG26 methylation group and MEHHP levels on omission errors.
Note. MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate.

phthalate metabolite variants on CPT variables. Further prospective studies with large sample sizes that consider the effects of various epigenetic processes are required to broaden the results of this study.

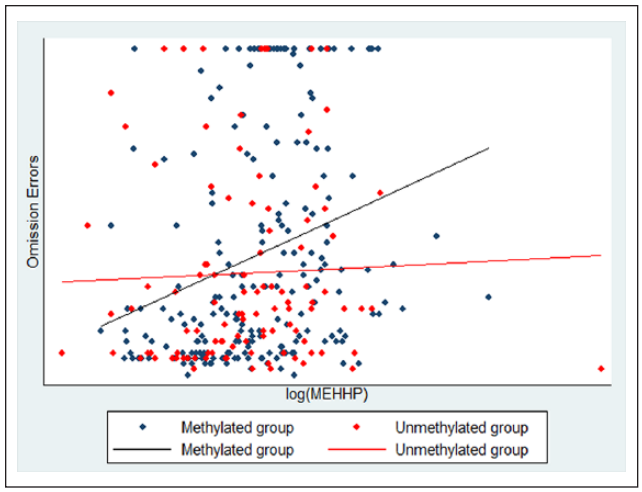

Figure 2. The post hoc analysis of the interaction between CpG28 methylation group and MEHHP levels on omission errors.
Note. MEHHP = mono-(2-ethyl-5-hydroxyhexyl) phthalate.

Declaration of Conflicting Interests

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Supplemental Material

Supplementary material for this article is available online.

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