Effect of prenatal exposure to phthalates on epigenome-wide DNA methylations in cord blood and implications for fetal growth: The Hokkaido Study on Environment and Children's Health

Ryu Miura[a], Atsuko Araki[a], Toru Ishihara[a,b], Kunio Miyake[c], Chihiro Miyashita[a], Tamie Nakajima[d], Sumitaka Kobayashi[a], Mayumi Ishizuka[e], Takeo Kubota[f], Reiko Kishi[a,*]

[a]Hokkaido University Center for Environmental and Health Sciences, Sapporo, Japan

[b]Graduate School of Human Development and Environment, Kobe University, Kobe Japan

[c]Departments of Health Sciences, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

[d]College of Life and Health Sciences, Chubu University, Aichi, Japan

[e]Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[f]Faculty of Child Studies, Seitoku University, Chiba, Japan

*Correspondence: e-mail: rkishi@med.hokudai.ac.jp

ABSTRACT

Prenatal exposure to phthalates negatively affects the offspring's health. In particular, epigenetic alterations, such as DNA methylation, may connect phthalate exposure with health outcomes. Here, we evaluated the association of di-2-ethylhexyl phthalate (DEHP) exposure *in utero* with cord blood epigenome-wide DNA methylation in 203 mother-child pairs enrolled in the Hokkaido Study on Environment and Children's Health, using the Illumina HumanMethylation450 BeadChip. Epigenome-wide association analysis demonstrated the predominant positive associations between the levels of the primary metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), in maternal blood and DNA methylation levels in cord blood. The genes annotated to the CpGs positively associated with MEHP levels were enriched for pathways related to metabolism, the endocrine system, and signal transduction. Among them, methylation levels of CpGs involved in metabolism were inversely associated with the offspring's ponderal index (PI). Further, clustering and mediation analyses suggested that multiple increased methylation changes may jointly mediate the association of DEHP exposure *in utero* with the offspring's PI at birth. Although further studies are required to assess the impact of these changes, this study suggests that differential DNA methylation may link phthalate exposure *in utero* to fetal growth and further imply that DNA methylation has predictive value for the offspring's obesity.

Keywords: EWAS, DEHP, MEHP, increased methylation, ponderal index

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Abbreviations: EDC, Endocrine-disrupting chemicals; EWAS, Epigenome-wide association studies; DMR, Differentially methylated regions; DEHP, di-2-ethylhexyl phthalate; CpG, cytosine-guanine dinucleotide; PI, Ponderal index; MEHP, mono(2-ethylhexyl) phthalate; BMI, Body mass index; FDR, False discovery rate; DMCpG, differentially methylated CpG, DRHM-CpGs, DEHP-related higher methylated CpGs; KEGG, Kyoto Encyclopedia Genes and Genomes; SD, Standard deviation; TSS200, 200 bases from the transcription start site; IGR, Intergenic region; GO, Gene Ontology; MAPK, Mitogen-activated protein kinase.

1. Introduction

Phthalates are widely used plasticizers (Koch et al. 2013) included in the composition of consumer products, such as food packages, toys, and personal care products, which can lead to chemical exposure through ingestion, inhalation, and skin adsorption (Ait Bamai et al. 2015; Jensen et al. 2015). They are potential endocrine-disrupting chemicals (EDCs) and have been found to exert various adverse effects that negatively impact an individual's health. In particular, phthalate exposure *in utero* has been linked to adverse birth outcomes, such as decreased birth size (Minatoya et al. 2017; Song et al. 2018; Whyatt et al. 2009) preterm birth (Ferguson et al. 2017; Huang et al. 2014), pregnancy loss (Gao et al. 2017), and reduced anogenital distance in infants (Swan et al. 2015). Prenatal exposure to phthalates can also affect childhood health outcomes, such as behavioral problems (Engel et al. 2010; Engel et al. 2009; Minatoya et al. 2018b; Tellez-Rojo et al. 2013), obesity (Buckley et al. 2016; Kim and Park 2014), and allergic diseases (Ait Bamai et al. 2018; Jaakkola and Knight 2008; Whyatt et al. 2014). Based on these, although phthalates are rapidly metabolized and excreted**,** early life exposure to phthalates may contribute to long-term health outcomes (Koch et al. 2013). However, the potential mechanisms underlying their long-lasting effects have not been fully elucidated. Epigenetic modifications, e.g., DNA methylation, may represent potential mechanisms by which phthalate exposure *in utero* exerts long-term effects. Several studies have indicated that epigenetic changes may connect EDC exposure in the developmental stage with long-term adverse health outcomes (Barouki et al. 2018; Ho et al. 2017; McLachlan 2016; Tapia-Orozco et al. 2017). In addition, animal studies have demonstrated that developmental phthalate exposure was associated with DNA methylation changes in the offspring (Abdel-Maksoud et al. 2015; Manikkam et al. 2013; Martinez-Arguelles and Papadopoulos 2015; Rajesh and Balasubramanian 2015; Sekaran and Jagadeesan 2015; Wu et al. 2010). Several human cohort studies have also shown that

prenatal phthalate exposure correlates with DNA methylation changes in selected candidate genes, using placenta (LaRocca et al. 2014; Zhao et al. 2016; Zhao et al. 2015) or cord blood samples (Huang et al. 2018; Huen et al. 2016; Montrose et al. 2018; Tindula et al. 2018). Recently, a few epigenome-wide association studies (EWASs) were published, allowing a unbiased assessment of epigenetic modifications associated with environmental factors (Christensen and Marsit 2011). Among them, one study reported that phthalate exposure altered the placental methylome and DNA methylation modification on the epidermal growth factor receptor significantly mediated the associated effects from phthalates exposure on early placental function (Grindler et al. 2018). Moreover, several differentially methylated regions (DMRs) in cord blood associated with prenatal phthalate exposure have been identified (Solomon et al. 2017). Genes with these regions are implicated in the inflammation reaction, cancer, endocrine function, and male fertility. Another study also investigated genome-wide DNA methylation changes in cord blood associated with prenatal exposure to the most common phthalate, di-2-ethylhexyl phthalate (DEHP), and suggested that DNA methylation in genes involved in the androgen response, spermatogenesis, and cancer-related pathways may be affected by prenatal exposure to this chemical (Chen et al. 2018). Although existing evidence supports the role of prenatal phthalate exposure in modifying DNA methylation, few studies have focused on the potential effects of phthalate exposure-associated methylation changes on the developing fetus and later in life.

Here, using an epigenome-wide approach, we aimed to elucidate the relation between prenatal DEHP exposure and cord blood DNA methylation from participants of the Hokkaido Study. Furthermore, we explored whether DNA methylation at the identified loci mediated the effect of prenatal DEHP exposure on the ponderal index (PI) at birth as an indicator of fetal growth.

2. Materials and Methods

2.1 Study population

Details of participants enrolled in the Sapporo cohort of the Hokkaido Study on Environment and Children's Health were previously described (Kishi et al. 2017; Kishi et al. 2013; Kishi et al. 2011).

2.2 Measurement of the primary metabolite of DEHP; mono(2-ethylhexyl) phthalate (MEHP)

Maternal blood samples were obtained during the hospital examination of participants and stored at −80 °C. Concentrations of MEHP in maternal blood, as an indicator of DEHP exposure, were measured via gas chromatography mass spectrometry at Nagoya University, as described (Araki et al. 2017; Araki et al. 2014; Jia et al. 2015). The detection limit was 0.28 ng/mL.

2.3 450K DNA methylation analysis

Umbilical cord bloods were collected immediately after birth and then stored at −80 °C. Cord blood DNA methylation levels at 485,577 CpGs was measured using the Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA, USA) by G&G Science Co., Ltd. (Fukushima, Japan). Details of the 450K methylation analysis have been described previously (Miura et al. 2019; Miura et al. 2018). After quality control (Aryee et al. 2014), functional normalization (Fortin et al. 2014) and reducing the batch effects (Leek et al. 2012), β-values, ranging from 0-1 for 0% to 100% methylated, at 426,413 CpG probes were obtained.

2.4 Data analysis

Among the 514 participants, 203 mother-infant pairs had detectable MEHP levels in maternal blood and cord blood DNA methylation data. Data analyses methods were previously described (Miura et al. 2019; Miura et al. 2018). Briefly, the associations of the *β*-values with MEHP natural log (ln)-transformed concentrations were determined using robust linear regression analysis (Fox and Weisberg 2011) with the *limma* package in the R/Bioconductor, which was adjusted for maternal age, level of education, pre-pregnancy body mass index (BMI), smoking status during pregnancy, blood sampling periods, gestational age, infant sex, and estimates of cord blood cell counts for $CD4^+$ T cells, $CD8^+$ T cells, monocytes, granulocytes, B cells, and nucleated red blood cells. The proportion of cord blood cells was estimated using the *minfi* package in the R (ver.3.3.2)/Bioconductor (ver. 3.3). We selected covariates previously reported to be associated with exposure or cord blood DNA methylation. For multiple comparisons, *p*-values were adjusted using a false discovery rate (FDR) to obtain *q*-values. Since we obtained a reduced number of FDR-significant findings, we evaluated the differentially methylated CpGs (DMCpGs) with an uncorrected *p*-value < 2.5E-04. We also assessed DEHP-related higher methylated CpGs (DRHM-CpGs) for functional enrichment with Kyoto Encyclopedia Genes and Genomes (KEGG) pathways (Kanehisa et al. 2002) via the gometh function of the *missMethyl* package in R/Bioconductor (Phipson et al. 2016).

To ascertain whether MEHP levels were associated with the characteristics of participants, we utilized the Spearman's correlation test, Mann–Whitney *U* test, and Kruskal–Wallis test.

Moreover, we examined associations between methylation levels (*β*-values) at DRHM-CpGs and the PI at birth using a multivariate regression model adjusted for maternal age, level of education, parity, pre-pregnancy BMI, smoking status during pregnancy, gestational

age, and infant sex, with JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). The PI was calculated as follows: PI (kg/m^3) = birth weight (kg) / (birth length (m))^3.

After identification of CpGs related to the PI, we tested the methylation patterns of these CpGs for mediation in the association between maternal MEHP levels and the PI, using a structural equation model from *lavaan* in R ver. 3.6.3. CpGs inversely associated with the PI and with *p*-value < 0.1 were selected, and z-scores for methylation levels were calculated. To determine inter-individual patterns in DNA methylation, we performed hierarchical clustering with Euclidean distance and the Ward D2 agglomeration method (Clifford et al. 2011) in R and stratified participants by methylation profile. In the mediation analysis, methylation levels (β) or the methylation cluster was used as a mediator, and models were adjusted for ln(MEHP), maternal age, gestational age, and infant sex in the association between the methylation ~~cluster~~ and the PI, and for maternal age, smoking during pregnancy, and blood sampling periods in the association between ln(MEHP) and the methylation. These factors were associated with the PI and methylation, respectively, with $p < 0.1$ in the regression analysis. The clustering approach enables us to clarify whether the methylation in each identified CpGs had occurred simultaneously or independently. In addition, they allow to adequately incorporate the mediators into the model considering the inter-individual patterns in DNA methylation.

The flow for the analyses is represented in Supplementary Figure S1.

2.5 Ethics

This study was conducted with written informed consent from all subjects. The study protocol was approved by the institutional Ethical Board for Human Gene and Genome Studies at the Hokkaido University Graduate School of Medicine and the Hokkaido

University Center for Environmental and Health Science. All experiments were performed in accordance with the relevant guidelines and regulations.

3. Results

3.1 Study population

The characteristics of the subjects are shown in Table 1. The median MEHP concentration in maternal blood was 10.3 ng/mL (interquartile range: 5.8–15.3 ng/mL), with a 100% detection rate. The average ± standard deviation (SD) of the mothers' age was 29.8 ± 4.9 years. Maternal blood sampling periods were significantly associated with MEHP levels (p-value < 0.01). Of the 203 newborns, 94 (46.3%) were male. The mean gestational age, birth weight, and birth length were 39.9 weeks, 3137.5 g, and 48.5 cm, respectively. The MEHP level was negatively correlated with the PI ($\rho = -0.133$, $p = 0.059$).

3.2 EWAS of DEHP exposure in utero

In adjusted robust linear regression models, there were two CpGs with significant epigenome-wide methylation alteration (FDR q-value < 0.05): one located at 200 bases from the transcription start site (TSS200) of *ZC3H10* (cg26409978) and another mapped to *SDK1* (cg00564857), as shown in Figure 1A. Maternal MEHP levels showed more positive association with methylation levels than negative association, as seen in the volcano plot. For instance, of 271 DMCpGs with uncorrected p-values < 2.5E-04, 253 (93.4%) were positively associated with MEHP levels (Figure 1B). The list of the DMCpGs with p-values < 2.5E-04 is available in the Supplemental Table S1.

We had very few findings with a significant false discovery rate (FDR) to confirm the effect of prenatal DEHP exposure on DNA methylation changes. We examined the location of the DRHM-CpGs with p-value < 2.5E-04 in gene features and CpG islands; notably, we

found statistically significant differences in the association with MEHP levels considering the expected proportions (for gene features, X^2 p-value = 0.004; for CpG islands, X^2 p-value = 0.01; Figure 2). A decrease of methylation level in island and an increase in the intergenic region (IGR) were particularly observed.

Next, we compared our results to those of a published study on the association between prenatal phthalate exposures and DNA methylation in cord blood that used Illumina HumanMethlation450 BeadChips (Solomon et al. 2017). In this study, the authors identified seven DMRs associated with MEHP levels in maternal urine at 26 gestational weeks using two different approaches (see Supplementary Table S2). We extracted the results of our EWAS at CpGs in the DMRs identified by (Solomon et al. 2017) (Table 2). Since the CpGs included in each region showed methylation alteration in the same direction, the average the partial regression coefficients were shown in Table 2. Although no CpG was associated with maternal MEHP levels with genome-wide statistical significance in our cohort, six of the seven DMRs showed increased methylation changes. Among them, five DMRs that mapped to *MUC4*, *C5orf63*, *CNPY1*, *SVIL-AS1*, and *FIBIN*, showed the same positive direction as those identified by (Solomon et al. 2017).

3.3 Gene Ontology (GO) analysis

To investigate the biological processes influenced by DEHP-associated increased methylation, we tested for KEGG pathway (Kanehisa et al. 2002) enrichment among the 253 DRHM-CpGs with $p < 2.5E-04$. We observed 12 enriched pathways with FDR < 0.05. GO analyses of the data obtained from EWAS are inclined for cancer-related genes (Harper et al. 2013) and relatively healthier children were included in the analysis; therefore, the enriched pathways excluding cancer and human disease pathways are listed in Table 3. The most significant pathway was “metabolic pathway,” with FDR = 2.4E-08. We also observed three

pathways involved in the endocrine system—GnRH signaling pathway, renin secretion, and cortisol synthesis and secretion—and two pathways involved in signal transduction: the mitogen-activated protein kinase (MAPK) and Notch signaling pathways.

3.4 Methylation for mediation in the association between prenatal DEHP exposure and the offspring's PI at birth

Initially, we conducted multiple regression analyses to examine the association between the PI and methylation levels at 16 DRHM-CpGs on genes involved in metabolic pathways (Table 3). Of those, methylation levels at 12 DRHM-CpGs were inversely related to the PI (Figure 3). In particular, the methylation levels at cg27433759:*PIK3CG*, cg10548708:*ACAA1*, and cg07002201:*FUT9* were associated with PI with p-value < 0.1. Although the methylation levels at the three CpGs were positively correlated (Supplementary Table S3), we could not determine whether the methylation in each identified CpGs had occurred simultaneously or independently. To clarify this, we stratified samples based on the methylation levels (z-scores) at those three CpGs using hierarchical clustering. This approach revealed two distinct methylation clusters: the increased methylation cluster (cluster 1, n = 59) and the decreased methylation cluster (cluster 2, n = 144) (Supplementary Figure S1). Cluster 1 exhibited significantly higher methylation levels at all three CpGs than cluster 2. We then examined the differences in MEHP levels and PI between both clusters. Cluster 1 showed higher MEHP levels and lower PI than cluster 2 (Supplementary Figure S2). These results demonstrated that the increased methylation in cg27433759:*PIK3CG*, cg10548708:*ACAA1*, and cg07002201:F*UT9* associated with higher MEHP levels and lower PI simultaneously occurred in the current participants. Finally, we tested the methylation cluster for mediation in the association between MEHP levels and the PI (Figure 4). The mediation path through the methylation cluster explained 28.8% (indirect/total) of the effect

of MEHP levels on the PI, although methylation levels at each of the three CpGs did not mediate statistically significant effects (Supplementary Table S4). Since the methylation levels at the three CpGs were positively correlated (Supplementary Table S3), we considered total methylation levels at the three CpGs and observed a mediation effect with *p*-value < 0.05 considering the methylation cluster as the mediator, which explained 32.7 % of the effect of MEHP levels on PI (Supplementary Table S4).

4. Discussion

Here, we assessed the effect of prenatal DEHP exposure on DNA methylation in cord blood and found that maternal MEHP levels were predominantly associated with increased methylation changes. The genes annotated to DRHM-CpGs were enriched for pathways related to metabolism, the endocrine system, and signal transduction. Further, clustering and mediation analyses suggested that the increased methylation changes related to metabolic pathways may link prenatal DEHP exposure to fetal growth (as indicated by the offspring's PI at birth).

As we described previously (Araki et al. 2014), maternal MEHP levels from subjects in-between the second and third trimester (median = 10.3 ng/mL) were higher than those at 18 weeks of gestation (median = 1.18 ng/mL). Additionally, in most cases, phthalate metabolite levels in blood samples are noticeably higher than in urine samples (Frederiksen et al. 2010).

Noteworthy, we found two DMCpGs with FDR < 0.05: cg26409978 located in TSS200 of zinc finger CCCH-type domain-containing 10 (*ZC3H10*)) and cg00564857 mapped to *SDK1* (sidekick cell adhesion molecule 1), both showing increased methylation changes. We also observed a preference for methylation positively associated with MEHP levels with *p*-values < 2.5E-04. In a previous study using the 450K platform, (Solomon et al. 2017)

reported seven DMRs associated with MEHP levels in maternal urine at 26 gestational weeks (n = 332, median: 3.63 μg/g creatinine). Our study differs in sample size, matrices, sampling time, and analysis methods; nonetheless, when we evaluated the direction of methylation changes in these DMRs, increased methylation in five of them was replicated in our data set (Table 2). The observed phthalate-induced increased methylation was also consistent with a previous study that demonstrated a positive association between prenatal levels of high molecular weight phthalate and cord blood methylation region of *MEG3* (Tindula et al. 2018). These results suggested that maternal MEHP would predominantly induce higher methylation in the offspring. However, other studies on cord blood methylation alterations have also reported prenatal phthalate-induced decreased methylation. A previous study demonstrated a negative association between maternal levels of monoethyl phthalate, a metabolite of diethyl phthalate, with *Alu* methylation and a similar but weaker association with the methylation of *LINE-1* (Huen et al. 2016). In addition, mono-n-butyl phthalate and monobenzyl phthalate in maternal urine samples were inversely associated with *Alu* methylation (Huang et al. 2018). Another study showed that a negative association of maternal phthalate concentrations with the methylation of the metabolism-related genes *IGF2* and *PPARA* (Montrose et al. 2018), as well as *LINE-1* methylation. The differences in metabolite type, measuring time, and level of phthalates may account for these disparities.

We also observed an enrichment of DRHM-CpGs in the IGR, with a decrease within CpG islands (Figure 2). Previous studies showed that disease-associated and environmentally induced DMCpGs, such as those resulting from obesity or exercise intervention, have accumulated in the IGR or open seas (Grundberg et al. 2013; Huang et al. 2015; Ronn et al. 2013; Zhu et al. 2018), suggesting that DNA methylation may also be dynamically regulated outside CpG islands. The enrichment of DMCpGs within the IGR may affect the function of gene expression regulators located within the region. A recent study showed that the

methylation levels at CpGs in the IGR were anticorrelated to the nearest gene expression (Zhu et al. 2018).

Since prenatal DEHP exposure was predominantly associated with increased methylation changes, we conducted GO analysis for 253 DRHM-CpGs with $p < 2.5E-04$ to examine the effects of DEHP-associated increased methylation on the biological processes. The analysis showed the accumulation of CpGs with DEHP-induced higher methylation in metabolic pathways. The effects on these pathways are accordant with those reported in previous epidemiological studies, which have shown that phthalate exposure *in utero* is associated with fetal metabolic outcomes, such as decreased birth size (Minatoya et al. 2017; Watkins et al. 2016; Whyatt et al. 2009) and adipokine levels, i.e., markers of metabolic function in cord blood (Ashley-Martin et al. 2014; Minatoya et al. 2018a; Minatoya et al. 2017). It is possible that increased methylation associated with exposure to DEHP in utero may affect metabolic outcomes due to down-regulation of the expression of certain genes involved in metabolic pathways.

Given the above, we hypothesized that these methylation changes would disrupt fetal growth. Therefore, we examined the association between methylation levels at 16 DRHM-CpGs in metabolic pathways and the PI at birth, an indicator of fetal growth, and found that methylation levels at 12 CpGs were negatively associated with the PI (Figure 3). We also analyzed the association of two CpGs that survived FDR correction (*ZC3H10*: cg26409978 and *SDK1*: cg00564857) with PI and found that both the CpGs were inversely related to PI; however, it was not statistically significant (β = -6.6, 95% CI: -59.5 to 46.2 for cg26409978, β = -6.9, 95% CI: -16.9 to 3.2 for cg00564857).

Among them, three CpGs, cg27433759:*PIK3CG*, cg10548708:*ACAA1*, and cg07002201:*FUT9*, approached statistical significance (p-value < 0.1). *PIK3CG* (phosphatidylinositol-4,5-bisphosphate 3-kinase) encodes a class I catalytic subunit of

phosphoinositide 3-kinase (PI3K), which phosphorylates inositol lipids and is related to the pathway affecting insulin-like growth factor 1 (IGF1)-Akt (Matheny et al. 2017) and erythropoietin-induced JAK-STAT (Cokic et al. 2012) signaling pathways. *ACAA1* (acetyl-CoA acetyltransferase 1) encodes an enzyme operative in the β-oxidation system of the peroxisomes and is involved in fatty acid metabolism (Islam et al. 2019). FUT9 (fucosyltransferase 9) belongs to the glycosyltransferase family and is involved in glycosphingolipid biosynthesis (Ogasawara et al. 2011). Hierarchical cluster analysis confirmed that the separation of samples at the DNA methylation level positively correlated with MEHP levels (Supplementary Figures S1 and S2), indicating that the inter-individual increased methylation changes could be induced by prenatal DEHP exposure. Furthermore, although each CpG did not show significant mediation in the association between prenatal DEHP exposure and offspring's PI, both the methylation clusters and the total methylation at the three CpGs represented significant mediation effects (p-value < 0.05) and explained 28.8% and 32.7 % of the effect of MEHP levels on the PI (Figure 4 and Supplementary Table S4), respectively. In addition, the direct effects are non-significant after adding the both mediators in the models. Since the direct effects are not closer to the zero than the indirect effects, the mediators not completely but quite robustly mediate the association between maternal MEHP levels and offspring's PI. These results suggest that multiple DEHP-induced higher methylation may jointly contribute to the effects of DEHP exposure *in utero* on fetal development.

GO analysis also showed that DEHP-induced increased methylation was associated with the MAPK signaling pathway, including nine genes (Table 3). Of those, four genes, namely *MAP2K6*, *CACNA1D*, *CACNA1C*, and *MAP3K3*, were also involved in the endocrine system, as shown in Table 3. Recently, an experimental study showed that MEHP has an impact on MAPK pathways as well as on peroxisome proliferator-activated receptor γ

(PPARγ) transcriptional activity, leading to the disturbance in lipid metabolism and human villous cytotrophoblast differentiation (Shoaito et al. 2019). MAPK signaling modulates a diverse range of cellular functions, cellular functions cell proliferation, differentiation, and migration. In addition to the metabolic pathway, possibly, increased methylation on the genes related to the MAPK signaling pathway may link prenatal phthalate exposure to adverse health outcomes. The effect of methylation changes identified herein, specifically in the MAPK signaling pathway, on long-term health outcomes warrant further longitudinal studies.

Nonetheless, there were some limitations in this study. First, MEHP levels were measured only once between the second and third trimesters. Consequently, we need to consider that a single MEHP measurement could represent a long-term prenatal exposure due to the short half-life of MEHP. In addition, among several metabolites of DEHP, only MEHP levels were measured. MEHP is the primary metabolite of DEHP, but other secondary metabolites, such as mono(2-ethyl-5-hydroxyhexypentyl) phthalate and mono(2-ethyl-5-carboxyl) phthalate, have been detected in maternal serum (Hart et al. 2014). Further, although DEHP is the most common phthalate, there are several phthalates coexisted in the environment, such as *di*-butyl phthalate, dimethyl phthalate, and diethyl phthalate. These chemicals, including other secondary metabolites of DEHP, should be considered and fully examined in the future. Second, since urine samples were unavailable in this study, only blood samples were used to measure maternal MEHP levels. Recently, most studies have measured urinary phthalate levels, which keeps the risk of a potential contamination to a minimum. In this study, we cautiously handled all samples to prevent *ex vivo* hydrolysis of DEHP and contamination. In addition, we calculated the background levels of MEHP and confirmed that external contamination was of no consequence. Third, DEHP is known to affect multiple tissues. Notably, whether the association of prenatal DEHP exposure with cord blood DNA methylation that we observed potentially represents methylation changes in

other tissues is unknown. Moreover, replication analysis using a different population or gene expression analysis is important to validate the result from epigenome-wide analysis. Without validation analysis is also a limitation of this study. Fourth, this study limited participants to mothers who delivered vaginally, meaning that relatively healthier children were included in the analysis. Therefore, the effects of DEHP exposure on DNA methylation might be underestimated. Fifth, cord blood DNA methylation and the PI at birth were cross-sectional. Subsequently, the cause and effect relation between them was undetermined. Lastly, we analyzed CpGs showing a *p*-value < 2.5E-04 (not epigenome-wide significance), to confirm the effect that prenatal DEHP exposure had on DNA methylation. We cannot exclude the possibility that some results might be false positives.

5. Conclusion

Collectively, this EWAS identified increased methylation changes associated with prenatal DEHP exposure. The DEHP-associated increased methylation changes may jointly contribute to the effects of prenatal exposure to this chemical on fetal development.

DNA methylation alterations in cord blood may be involved in modulating the postnatal growth trajectory. In addition, recent studies showed the sex-specific effects of phthalate exposure on DNA methylation (Chang et al. 2020; Svobada et al. 2020). Additional studies with larger sample sizes are needed to fully elucidate the influence of prenatal DEHP exposure on cord blood DNA methylation changes and the subsequent effects on infant long-term outcomes, including sex-specific health outcomes.

CRediT author contribution statement

T.K. and R.K conceived and supervised the study. R.M., A.A., T.I., K.M., C.M., T.N., K.S., and M.I. contributed to data curation, formal analysis, investigation, and methodology. R.M., A.A., K.M., C.M., K.S., and R.K. contributed to funding acquisition. R.M., A.A., T.I., C.M, and R.K contributed to writing – original draft. All authors contributed to writing – review editing. All authors reviewed the final version of the manuscript.

Declaration of competing Interest

The authors declare no competing interests.

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Figures

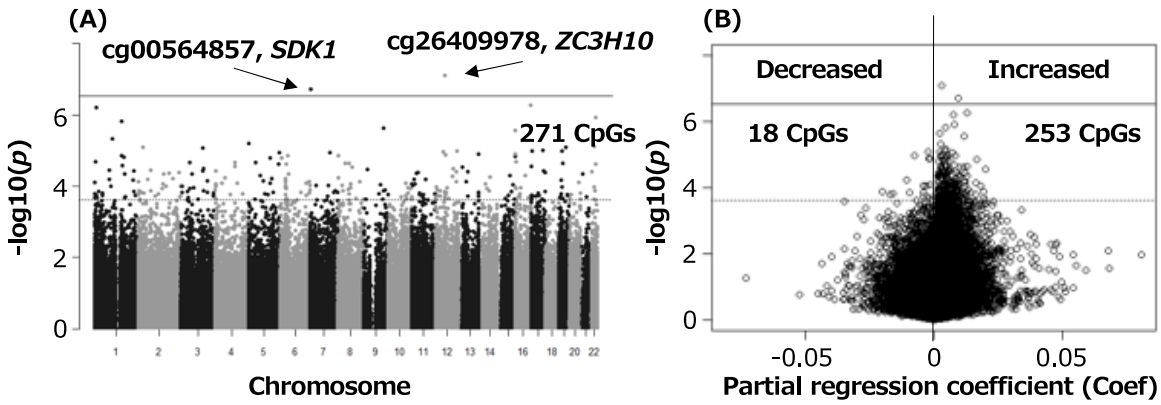

Figure 1. Manhattan (A) and volcano (B) plots of the epigenome-wide DNA methylation associations with prenatal exposure to DEHP.

Adjusted for maternal age, level of educational, pre-pregnancy BMI, smoking status during pregnancy, blood sampling periods, gestational age, infant sex, and estimates of cord blood cell counts. Horizontal solid lines represent the significance threshold of an FDR < 0.05. Horizontal dotted lines represent the threshold of a *p*-value < 2.5E-04.

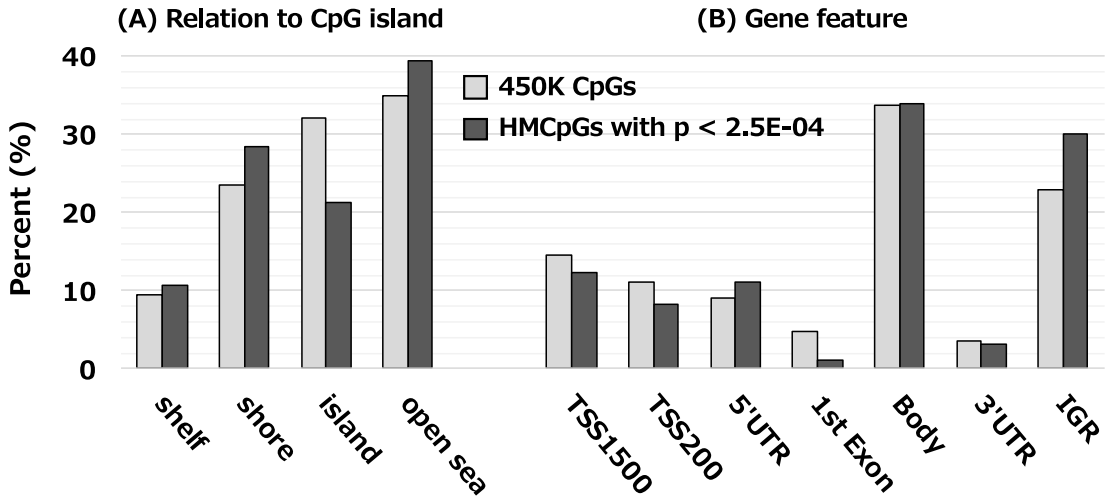

Figure 2. Location of DRHM-CpGs with $p < 2.5E-04$ (253 CpGs) compared to that of all CpGs in the methylation array.

X^2 test: (A) $p = 0.004$, (B) $p = 0.01$.

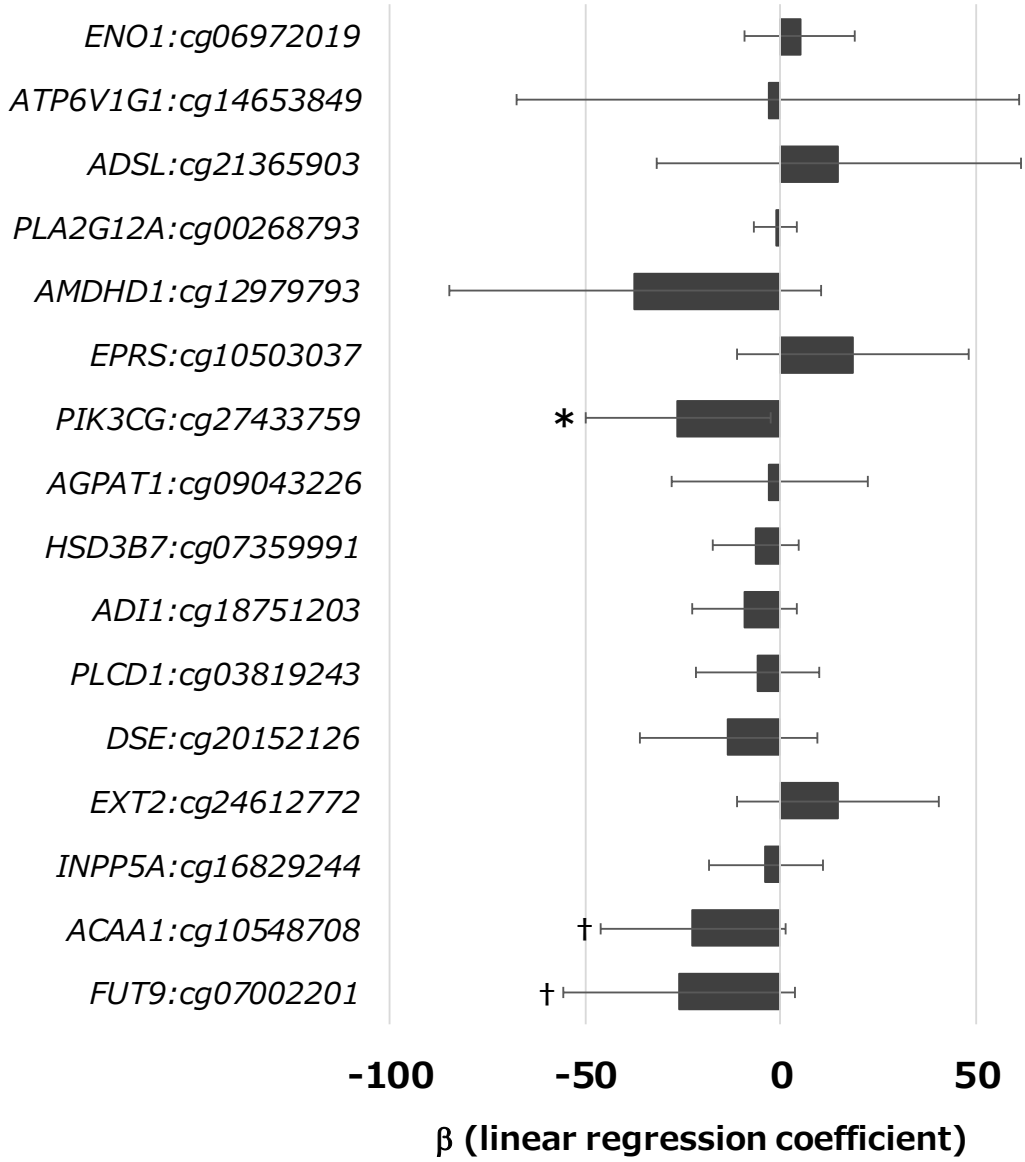

Figure 3. Linear regression coefficients (β) of the PI at birth in relation to the methylation levels, *ranging from 0–1 for 0% to 100% methylated,* at CpGs positively associated with MEHP with *p*-value < 2.5E-04, mapped to the genes involved in metabolic pathways (n = 203). *Linear regression coefficients (β) indicates PI changes with one unit increase in methylation levels.*

Error bars indicate a 95% confidential interval. Adjusted for maternal age, level of educational, parity, pre-pregnancy BMI, smoking status during pregnancy, gestational age, and infant sex.

$^{\dagger}p < 0.1$, $^{*}p < 0.05$.

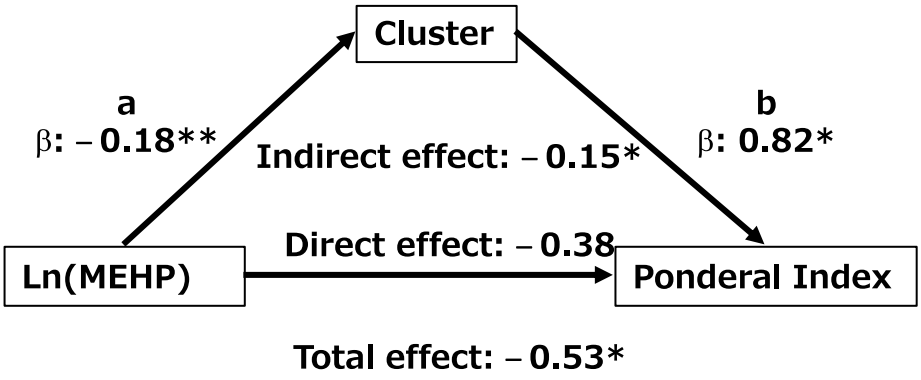

Figure 4. Mediator model for the association of prenatal MEHP exposure, methylation cluster for cg27433759, cg10548708, cg7002201, and PI at birth (n = 203).

Models were adjusted for maternal age and smoking status during pregnancy in path "a" and for ln(MEHP), maternal age, parity, gestational age, and infant sex in path "b."

Effect sizes with $^{*}p < 0.05$ and $^{**}p < 0.01$ are shown.

Table 1. Characteristics of the study population and their relationships with maternal serum MEHP concentrations (n = 203).

		Mean ± SD/ N (%)	MEHP (ng/mL) ρ/ Median	25th	75th	*p*-value
Maternal characteristics						
Maternal age (year)[a]		29.8 ± 4.9	ρ = 0.038			0.594
Prenatal BMI (kg/m^2)[a]		21.2 ± 3.0	ρ = 0.049			0.485
Parity[b]	0	110 (54.2)	10.00	5.65	15.20	0.644
	≧1	93 (45.8)	10.37	6.00	15.65	
Educational level (year)[b]						
	≦12	93 (45.8)	10.37	5.92	14.66	0.831
	>12	112 (54.2)	9.92	5.65	15.42	
Annual household income (million yen)[c]						
	<3	39 (19.4)	11.53	6.03	16.60	0.379
	3–5	103 (51.2)	8.65	5.57	14.92	
	5–7	43 (21.4)	11.41	6.90	16.80	
	>7	16 (8.0)	9.83	5.42	13.48	
Smoking during pregnancy[b]						
	No	167 (82.3)	10.41	5.92	15.55	0.424
	Yes	36 (17.7)	7.80	5.23	14.11	
Alcohol consumption during pregnancy[b]						
	No	132 (65.5)	10.37	5.96	15.72	0.638
	Yes	70 (34.5)	10.22	5.40	15.09	
Caffeine intake during pregnancy (mg/day)[a]						
		143.0 ± 125.8	ρ = 0.064			0.374
Blood sampling period (week)[c]						
	<32	77 (37.9)	11.41	6.64	15.28	0.009
	32–35	48 (23.6)	12.40	6.64	17.32	
	≧35	78 (38.4)	7.08	5.00	13.80	
Infant characteristics						
Gestational age (week)[a]		39.9 ± 1.0	ρ = 0.000			0.998
Sex[b]	Male	94 (46.3)	9.86	6.32	14.42	0.673
	Female	109 (53.7)	10.41	5.63	16.31	
Birth weight (g)[a]		3137.5 ± 333.3	ρ = –0.066			0.352
Birth length (cm)[a]		48.5 ± 1.5	ρ = 0.057			0.416

PI (kg/m^3)[a]	27.4 ± 2.2	ρ = –0.133	0.059

[a]Spearman's correlation test (ρ)

[b]Mann–Whitney *U* test

[c]Kruskal–Wallis test

Table 2. Direction of cord blood DNA methylation changes associated with maternal MEHP levels at DMRs identified by Solomon et al. (2017) in the present study.

Gene	Chr	Start	End	Sapporo cohort				Salomon et al. 2017	
				Number of probes	Average Coef[a]	Min *p*-value[b]	Direction[c]	Max bFC[d]	Direction[c]
MUC4	3	195489306	195490169	8	0.018	0.223	+	0.297	+
C5orf63/FLJ44606	5	126408756	126409553	13	0.017	0.002	+	0.250	+
VTRNA2-1	5	135414858	135416613	16	−0.007	0.320	−	−0.895	−
RNF39	6	30038254	30039801	37	0.005	0.367	+	−0.833	−
CNPY1	7	155283233	155284759	10	0.004	0.082	+	0.171	+
SVIL-AS1	10	29698152	29698685	8	0.002	0.119	+	0.390	+
FIBIN	11	27015519	27016671	8	0.003	0.166	+	0.231	+

[a]Average partial regression coefficient at CpG sites in the region.

[b]Minimum *p*-value within the region.

[c]Direction of methylation change: +, increase; −, decrease.

[d]Fold change in the DNA methylation *M*-value per $\log_{10}$ unit increase in phthalate metabolite concentration.

Abbreviations: Chr, chromosome.

Table 3. Significantly enriched pathways (FDR < 0.05) for the gene targets of 253 DRHM-CpGs associated with MEHP levels (p < 2.5E-04).

KEGG orthology	KEGG pathway	Genes*	*p*-value
Metabolism	Metabolic pathways	*ENO1; ATP6V1G1; ADSL; PLA2G12A; AMDHD1; EPRS; PIK3CG; AGPAT1; HSD3B7; ADI1; PLCD1; DSE; EXT2; INPP5A; FUT9; ACAA1*	7.3E-11
Signal transduction	MAPK signaling pathway	*MAP2K6; EFNA3; CACNA1D; DAXX; FGF9; DUSP4; PPM1A; DUSP10; CACNA1C; MAP3K3*	3.0E-07
	Notch signaling pathway	*NUMBL; NCOR2; RFNG; CTBP1; NOTCH1*	6.4E-07
Endocrine system	GnRH signaling pathway	*MAP2K6; CACNA1D; ITPR2; CACNA1C; MAP3K3*	1.3E-04
	Renin secretion	*CACNA1D; ITPR2; CACNA1C*	6.9E-04
	Cortisol synthesis and secretion	*CACNA1D; ITPR2; CACNA1C*	1.2E-03
Circulatory system	Vascular smooth muscle contraction	*CACNA1D; PLA2G12A; CALD1; ITPR2; CACNA1C*	4.0E-04
Nervous system	Dopaminergic synapse	*CACNA1D; TH; ITPR2; CACNA1C*	7.4E-04

*Genes annotated to the DRHM-CpGs with p < 2.5E-04.