厚生労働科学研究補助金（化学物質リスク研究事業）
分担研究報告書

フタル酸エステル類（DEHP）胎児期曝露による臍帯血 DNA メチル化の網羅的解析と出生時体格との関連

Association between cord blood DNA methylations by fetal exposure of phthalates and ponderal index at birth in epigenome-wide study

研究代表者	岸　玲子	北海道大学環境健康科学研究教育センター	特別招へい教授
研究分担者	三浦　りゅう	北海道大学環境健康科学研究教育センター	特任助教
研究分担者	荒木　敦子	北海道大学環境健康科学研究教育センター	特任准教授
研究分担者	松浦　英幸	北海道大学大学院・農学研究院	教授
研究分担者	篠原　信雄	北海道大学大学院・医学研究院	教授

研究要旨

Exposure to phthalate in utero is associated with adverse health outcome of the offspring. Differential DNA methylation at specific CpG sites may link phthalate exposure to health impacts. We examined the association of prenatal Di-2-ethylhexyl phthalate (DEHP) exposure with genome-wide DNA methylation changes in cord blood in 203 mother-child pairs in the Hokkaido Study on Environment and Children's Health, using the Illumina HumanMethylation 450 BeadChip. We found that the primary metabolite of DEHP: mono (2-ethylhexyl) phthalate (MEHP) levels in maternal blood were predominantly associated with hypermethylation in cord blood DNA. The genes annotated to hypermethylated CpGs associated with maternal MEHP levels were enriched for pathways related to metabolism, endocrine system, and signal transduction. Among them, hypermethylated CpGs involved in metabolism were inversely associated with offspring's ponderal index (PI). Further, mediation analysis suggested that multiple hypermethylation changes may jointly mediate the association between prenatal DEHP exposure and offspring's PI. Although additional studies are needed to determine the functional consequences of these changes, our findings imply differential DNA methylation may link

A. 研究目的

研究協力者
増田　秀幸 (北海道大学環境健康科学研究教育センター，特任助教)

Phthalates are widely used plasticizers (Koch et al. 2013) for consumer products including toys, food packages, personal care products, and other household items, leading to widespread exposure to these chemicals through diet, inhalation, and dermal adsorption (Ait Bamai et al. 2015; Jensen et al. 2015). Phthalates have potential for endocrine-disrupting chemicals (EDCs) and have been found to be associated with multiple adverse effects on human health. In particular, 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 of infants (Swan et al. 2015). Prenatal exposure can also affect childhood health

outcomes such as behavioral problems (Engel et al. 2009; Engel et al. 2010; Minatoya et al. 2018b; Tellez-Rojo et al. 2013), obesity (Buckley et al. 2016; Kim and Park 2014), and allergic disease (Ait Bamai et al. 2018; Jaakkola and Knight 2008; Whyatt et al. 2014). Early-life exposure to phthalates may contribute to fetal origins of disease; however, actual mechanisms accounting for long-term effects remain unclear.

As phthalates are rapidly metabolized and excreted, epigenetic modification, such as DNA methylation, may be a potential mechanism by which phthalate exposure in utero exerts the long-term effects. Accumulating evidence suggests that epigenetic alternations may link developmental EDC exposure with susceptibility to diseases later in life (Barouki et al. 2018; Ho et al. 2017; McLachlan 2016; Tapia-Orozco et al. 2017). Animal studies have demonstrated the association between developmental phthalate exposure and 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). Serval human cohort studies showed that prenatal phthalate exposure was associated with DNA methylation in selected candidate genes using placenta (LaRocca et al. 2014; Zhao et al. 2015; Zhao et al. 2016) 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) that can allow a hypothesis-free assessment of epigenetic alterations in relation to the environmental factors (Christensen and Marsit 2011) were published. One study reported phthalate exposure altered placental methylome and identified epidermal growth factor receptor (EGFR) as a critical candidate gene mediating the effects of phthalates on early placental function (Grindler et al. 2018). Several differential methylation regions in cord blood associated with prenatal phthalate exposure were identified (Solomon et al. 2017). Genes with those regions were involved in inflammatory response, cancer, endocrine function, and male fertility. Another study also examined the association of genome-wide DNA methylation in cord blood with prenatal exposure to the most common phthalates, Di-2-ethylhexyl phthalate (DEHP) and suggested that DNA methylation in genes involved in the androgen response, spermatogenesis, and cancer-related pathway may be affected by prenatal phthalate exposure (Chen et al. 2018). Although existing evidence supports the role of prenatal phthalate exposure in modifying DNA methylation, little is known about potential effects of the exposure-associated methylation on fetal development and later in life. Using epigenome-wide approach, we aimed to explore association between prenatal DEHP exposure and DNA methylation changes in cord blood collected from the participants of the Hokkaido study. Furthermore, we studied whether the DNA methylation at identified loci mediated the effect of DEHP exposure in utero on ponderal index at birth as an indicator of fetal growth.

B. 研究方法

Study population.

Participants were enrolled in the Sapporo cohort of the Hokkaido Study on Environment and Children's Health (Kishi et al. 2011; Kishi et al. 2013; Kishi et al. 2017). Briefly, we recruited pregnant women at 23–35 weeks of gestation between 2002 and 2005 from the Toho Hospital

(Sapporo, Japan). After the second trimester during their pregnancy, the participants completed the self-administered questionnaire containing baseline information including family income, educational level, parity history, and pregnancy health information including smoking status, alcohol consumption, and caffeine intake. Information on pregnancy complications, gestational age, infant sex, and birth size was obtained from medical records.

Measurement of the primary metabolite of DEHP:
Maternal blood samples were obtained at the time of their hospital examination and stored at −80℃ prior to analysis. Levels of mono (2-ethylhexyl) phthalate (MEHP) were measured in maternal blood by gas chromatography mass spectrometry (GC-MS) at Nagoya university as described previously (Araki et al. 2014; Araki et al. 2017; Jia et al. 2015). The detection of limit (LOD) was 0.28 ng/mL.

450K DNA methylation analysis.
Umbilical cord blood samples were taken immediately after birth, and then stored at −80℃. After DNA extraction using a Maxwell® 16 DNA Purification Kit (Promega, Madison, WI, USA), cord blood DNA methylation at 485,577 CpGs was quantified using the Infinium HumanMethylation 450 BeadChip (Illumina Inc., San Diego, CA, USA) by G&G Science Co., Ltd. (Fukushima, Japan). Details for the 450K methylation analysis are described elsewhere (Miura et al. 2018). Samples were run across five plate batches and were assigned randomized location across plates. After quality control (Aryee et al. 2014), functional normalization (Fortin et al. 2014) was applied to the raw data, and normalized beta (β) values, ranging from 0-1 for 0% to 100% methylated, were obtained for the 292 cord blood samples. Probes with a detection p-value >0.05 in more than 25% of samples, single nucleotide polymorphism (SNP)-affected probes, cross-reactive probes identified by Chen et al. (Chen et al. 2013), and probes on sex chromosomes were removed. As a result, 426,413 CpG probes were included in the working set. We applied the ComBat method on M-values (logit-transformed β-values) to adjust methylation data for the sample plate to reduce a potential bias due to batch effects (Leek et al. 2012). The M-values were back-transformed to β-values that were used for subsequent data analyses.

Data analysis.
Among the 514 participants of the Sapporo Cohort Study, 203 mother-infant pairs had both exposure and DNA methylation data. Cord blood cell proportion was estimated by the method implemented in the R/Bioconductor package minfi (Bakulski et al. 2016). Using limma package in R, robust linear regression analysis (Fox and Weisberg 2011) and empirical Bayesian method (Smyth 2004) were applied to determine the associations of β-value at each CpG site with MEHP natural log (ln)-transformed concentrations, adjusted for maternal age, educational levels, pre-pregnancy body mass index (BMI), smoking during pregnancy, blood sampling periods, gestational age, infant sex, and cord blood cell estimates for $CD4^+$ T cells, $CD8^+$ T cells, granulocytes, monocytes, B cell and nucleated red blood cells. Adjustment covariates were selected from factors previously reported to be associated with exposure or cord blood DNA methylation. For multiple comparisons, p-values

were adjusted by a false discovery rate (FDR) to obtain q-values. Because of too few FDR-significant findings, we evaluated the differentially methylated CpGs (DMCpGs) with uncorrected p-value <2.5E-04. We also assessed hypermethylated DMCpGs (hyper-DMCpGs) for functional enrichment with KEGG pathways (Kanehisa et al. 2002) via the gometh function in the missMethyl package in R/Bioconductor (Phipson et al. 2016). Statistical analyses were performed using minfi, sva, and limma packages in R ver. 3.3.2 and Bioconductor ver. 3.3.

The Spearman's correlation test, Mann-Whitney U-test, and Kruskal-Wallis test were applied to determine whether maternal and offspring characteristics were associated with MEHP levels.

We examined associations between methylation levels at hyper-DMCpGs and ponderal index (PI) at birth by a multivariate regression model adjusted by maternal age, educational levels, parity, pre-pregnancy BMI, smoking during pregnancy, gestational age, and infant sex, using JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). PI was calculated as follows; PI (kg/m^3) = birth weight (kg) / (birth length (m))^3. Then, we tested the CpGs for mediation in the association between MEHP levels in maternal blood and PI using PROSESS (Hayes 2013), a macro implemented in SPSS (IBM, Armonk, NY, USA). In addition to the same possible cofounders as named above, blood sampling periods were included as covariate in the mediator and outcome regression models.

Ethics.

Written informed consents were obtained from all participants. 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 approved the study protocol. All experiments were performed in accordance with relevant guidelines and regulations.

C. 研究結果

Study characteristics

The characteristics of the participants with the corresponding median MEHP concentrations in cord blood are described in Table 1. The median of MEHP concentration was 10.3 ng/mL (Interquartile range (IQR): 5.8 – 15.3 ng/mL) with 100% of detection rate. The average ± standard deviation (s.d.) age of the mothers was 29.8 ± 4.9 years. Maternal blood sampling periods were significantly associated with MEHP levels. Of the 203 newborns, 94 (46.3%) were male. The mean gestational age was 39.9 weeks, birth weight was 3137.5 g, and birth length was 48.5 cm. The MEHP level was negatively correlated to PI (ρ = -0.133, p=0.059).

Epigenome-wide association study of DEHP exposure in utero

In adjusted robust linear regression models, there were 2 CpGs with epigenome-wide significant methylation changes (FDR q-value < 0.05): one located in 200 bases from transcription start site (TSS200) of *PARP12* (cg26409978), and another mapped to *SDK1* (cg00564857) as shown in Figure 1A. DEHP exposure was more frequently associated with hypermethylation than with hypomethylation as seen in volcano plots (Figure 1B). For instance, of 271 DMCpGs with uncorrected p-value <2.5E-04, 253 CpGs (93.4%) were hypermethylated (Figure 1B). We examined the location of the hyper-DMCpGs with p-value <2.5E-04 in gene features and CpG islands. As

shown in Figure 2, there were statistically significant differences associated with DEHP exposure compared with the expected proportions (for gene features, X^2 P-value = 0.004; for CpG islands, X^2 P-value = 0.01). Decrease in island and increase in the intergenic region (IGR) were particularly observed.

Next, we considered our results in relation to a published study of association between prenatal phthalate exposure and cord blood DNA methylation using Illumina HumanMethlation450 Beads chips (Solomon et al. 2017). In the study, the authors identified seven differentially methylated regions (DMRs) associated with MEHP levels in maternal urine at 26 weeks gestation by using two differential approaches. We examined the direction of methylation changes in the DMRs identified by Solomon et al. in our data set (Table 2), in which we averaged methylation levels of each CpG site because the CpGs included in each region showed the same direction of methylation changes. Although no CpG reached statistical significance in our cohort, six of the seven DMRs showed the same direction as those identified by Solomon et al. (Table 2), of which five DMRs mapped to *MUC4*, *C5orf63*, *CNPY1*, *SVIL-AS1*, and *FIBIN* were hypermethylated, suggesting that prenatal DEHP exposure would predominantly induce hypermethylation.

Gene Ontology (GO) analysis

To investigate the underlying biology that may be affected by DEHP-associated hypermethylation changes, we tested for Kyoto Encyclopedia Genes and Genomes (KEGG) pathways (Kanehisa et al. 2002) enrichment among the 253 hyper-DMCpGs with p <2.5E-04. We observed twelve enriched pathways with FDR <0.05. GO analyses of the data obtained using 450K chip are known to be biased for cancer-related genes (Harper et al. 2013); therefore, the enriched pathways excluded 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 endocrine system: GnRH signaling pathway, Renin secretion, and Cortisol synthesis and secretion, and two pathways involved in signal transduction: mitogen-activated protein kinase (MAPK) signaling pathway and Notch signaling pathway.

The methylation for mediation in the association between prenatal DEHP exposure and offspring’s PI at birth

First, we performed multiple regression analyses to examine the association between PI and methylation levels at sixteen hyper-DMCpGs on the genes involved in metabolic pathways (as shown in Table 3). Of those, methylation levels at twelve hyper-DMCpGs were inversely related to PI (Figure 3). In particular, methylation levels at cg27433759: *PIK3CG*, cg10548708: *ACAA1*, and cg07002201: *FUT9* were associated with PI with p-value <0.1. Then, we considered averaged methylation levels at the three CpGs and observed mediate effect with Sobel test p-value <0.05 (Table 4), which explained 30.4 % of the effect of MEHP levels on PI.

D. 議論

We examined the effect of prenatal DEHP exposure on DNA methylation in cord blood and found that maternal MEHP levels were predominantly associated with hypermethylation. The genes annotated to hyper-DMCpGs were

enriched for pathways related to metabolism, endocrine system, and signal transduction. Further, mediation analysis suggested that a part of hypermethylation may mediate the association between prenatal DEHP exposure and offspring's ponderal index.

As we described previously (Araki et al. 2014), the levels of MEHP in this cohort (median = 10.3 ng/mL) were higher than those in pregnant women at 18 weeks (median = 1.18 ng/ml). Additionally, in most cases, the levels of phthalate metabolites were considerably higher in urine samples (Frederriksen et al. 2010). We found two DMCpGs with FDR < 0.05: cg26409978 located in TSS200 of *PARP12* (poly(ADP-Ribose) polymerase family member 12; previous name: zinc finger CCCH-type domain containing 1 (*ZC3H1*)) and cg00564857 mapped to *SDK1* (Sidekick Cell Adhesion Molecule 1). Both CpGs showed hypermethylation. We also observed the preference of hypermethylation associated with MEHP levels with p-value <2.5E-04. In the previous study using the 450K platform, Salomon et al. (Solomon et al. 2017) reported the seven DMRs associated with MEHP levels in maternal urine at 26 weeks gestation (n=332, median: 3.63 μg/g-creatinine). Our study differs in sample size, matrices, collecting timing, and analysis methods; nonetheless, when we evaluated the direction of methylation changes in those DMRs, hypermethylation in the five DMRs were replicated in our data set (Table 2). Phthalate-induced hypermethylation 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). It is plausible that maternal MEHP would predominantly induce offspring's hypermethylation. However, others on cord blood methylation alterations reported prenatal phthalate-induced hypomethylation. One study demonstrated an inverse association between prenatal concentrations of monoethyl phthalate, a metabolite of diethyl phthalate (DEP), with cord blood methylation of Alu repeats, and a similar but weaker association with *LINE-1* methylation (Huen et al. 2016). Maternal urinary mono-n-butyl phthalate (MBP) and monobenzyl phthalate (MBzP) were negatively associated with Alu methylation (Huang et al. 2018). Another study showed that maternal phthalate concentrations were negatively associated with methylation on *LINE-1* and metabolism-related genes; *IGF2* and *PPARA* (Montrose et al. 2018). The differences in metabolite type, the measuring time, and level of phthalates may account for the disparities.

We also observed an enrichment of hyper-DMCpGs in the IGR, with decrease within CpG island (Figure 2). Disease associated- and environmentally induced-DMCpGs, such as obesity or exercise intervention, have been shown to be enriched within 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 with in the IGR may affect functional process of regulatory elements, such as enhancers or insulators, located within the IGR. Recent study showed that the methylation levels at CpGs in the IGR were anticorrelated with nearest gene expression (Zhu et al. 2018).

GO analysis showed that DEHP-associated hypermethylation was associated with metabolic

pathway, endocrine system, and MAPK signaling pathway. This is consistent with previous work. For instance, epidemiological studies showed that phthalate exposure in utero has been associated with fetal metabolic outcomes, such as birth size (Minatoya et al. 2017; Watkins et al. 2016; Whyatt et al. 2009) and adipokine levels, markers of metabolic function, in cord blood (Ashley-Martin et al. 2014; Minatoya et al. 2017; Minatoya et al. 2018a). Prenatal exposure has also been linked to steroid hormone levels in infants (Araki et al. 2014; Araki et al. 2017; Lin et al. 2011). Recently, an experimental study showed that MEHP has an impact on MAPK pathways as well as an effect on peroxisome proliferator–activated receptor γ (PPARγ) transcriptional activity, which together promote disturbances in lipid metabolism and in human villous cytotrophoblast differentiation (Shoaito et al. 2019).

Given the accumulation of DEHP-induced hypermethylations in metabolic pathway, we hypothesized that those methylation changes would disrupt fetal growth. We examined the association between methylation levels at sixteen hyper-DMCpGs in metabolic pathways and PI at birth, an indicator for fetal growth, and found that methylation levels at twelve CpGs were negatively associated with PI (Figure 3). Among them, three CpGs; cg27433759: *PIK3CG*, cg10548708: *ACAA1*, and cg07002201: *FUT9*, approached significance (p-value <0.1). *PIK3CG* (Phosphatidylinositol-4,5-bisphosphate 3-kinase) encodes a class I catalytic subunit of PI3K (Phosphoinositide 3-kinase) that phosphorylates inositol lipid and is related to the pathway affecting insulin-like growth factor (IGF1)-Akt signaling (Matheny et al. 2017) and development erythropoietin (EPO)-induced Jak-STAT pathway (Cokic et al. 2012). *ACAA1* (Acetyl-CoA Acyltransferase 1) encodes an enzyme operative in the beta-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 related to glycosphingolipid biosynthesis (Ogasawara et al. 2011). Although each CpG did not show significant mediation in the association between prenatal DEHP exposure and offspring's PI, the averaged methylation levels at the three CpGs represented significant mediate effect (Sobel test p-value <0.05) and explained 30.4 % of the effect of MEHP levels on PI (Table 4). This suggests that multiple hyper-DMCpGs may jointly contribute to effects of DEHP exposure in utero on fetal development. We assumed that there would be more DM-CpGs related to PI as not all genes hit KEGG pathways. Among thirty-eight hyper-DMCpGs with FDR < 0.25 (Supplementary Table S3), seven CpGs; cg05836256 (*LMF1*: Lipase Maturation Factor 1), cg21491711 (*DBN1*: Drebrin 1), cg01142096 (*ERICH1*: Glutamate Rich 1), cg12651645 (*PCSK6*: Proprotein Convertase Subtilisin/Kexin Type 6), cg04849589 (*CSNK1G3*: Casein Kinase 1 Gamma 3), cg01560642 (*TTC34*: Tetratricopeptide Repeat Domain 34), cg02735381 (*ALPK1*: Alpha Kinase 1) were related to PI with p-value <0.05, and two CpGs; cg26684601 (*JPH3*: Junctophilin 3), cg22493212 (*MIR4277*: MicroRNA4277), were with p-value <0.1 (Supplementary Figure S1). Notably, these genes are linked to metabolism, cell growth and development. LMF1 is related to lipoprotein metabolism (Hosseini et al. 2012). *DBN1* encodes a cytoplasmic actin-binding protein thought to play a role in the process of

neuronal growth (Shirao and Sekino 2017). DMR at *ERICH1* was identified in multiple sclerosis patients by 450K platform (Maltby et al. 2017). There is a link between PCSK6 and obesity (Du et al. 2016; Levenson et al. 2017). CSNK1G3 is involved the Hedgehog (Hh) signaling pathway that has numerous roles in the control of cell proliferation, tissue patterning, stem cell maintenance and development. TTC34 contains tetratricopeptide repeat domain which can regulate diverse biological processes, such as organelle targeting, protein import, and vesicle fusion (Zeytuni and Zarivach 2012). microRNAs are involved in post-transcriptional regulation in gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. JPH3 provides a structural foundation for functional cross-talk between cell surface and intracellular ion channels. Although not significant mediate effect, each CpG explained 12.5 % - 23.2 % of the effect of MEHP levels on PI.

The following limitations of this study should be considered. We measured MEHP levels only once from second to third trimester. There have been concerns about using a single MEHP measurement as a representation of the 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. Other secondary metabolites, such as mono(2-ethyl-5-hydroxyhexyl) phthalate and mono(2-ethyl-5-carboxyl) phthalate which have been detected in maternal serum (Hart et al. 2014), also be considered in future studies. Second, we used blood samples for exposure assessment as urine samples were not available in this study. The majority of the recent studies assessed phthalate levels in urine samples as urine samples can avoid the influence of external contamination. In this assay, all samples were handled carefully to avoid ex vivo hydrolysis of DEHP and external contamination. We measured background levels of MEHP and confirm that the influences of external contamination were null. Third, DNA methylation was measured using unfractionated cord blood. DEHP is known to affect multiple tissues. Whether the associations observed in this study may reflect associations between prenatal DEHP exposure and the methylation at target tissues is unknown. Lastly, we included participants for whom cord blood samples were available, thus limiting the scope only to mothers who delivered vaginally. It is thus possible that relatively healthier children were included in our analysis, and we may have underestimated the effects of DEHP exposure.

Despite these potential limitation, this epigenome-wide study identified hypermethylation changes associated to prenatal DEHP exposure. The DEHP-associated hypermethylations may jointly contribute to effects of prenatal exposure on fetal development. Further studies are needed to confirm our findings and to investigate their relevance to infant long-term outcomes.

E. 研究発表

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

			MEHP (ng/ml)			
		Mean ± SD N (%)	ρ Median	25th	75th	p
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 (weeks)[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

Ponderal Index (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

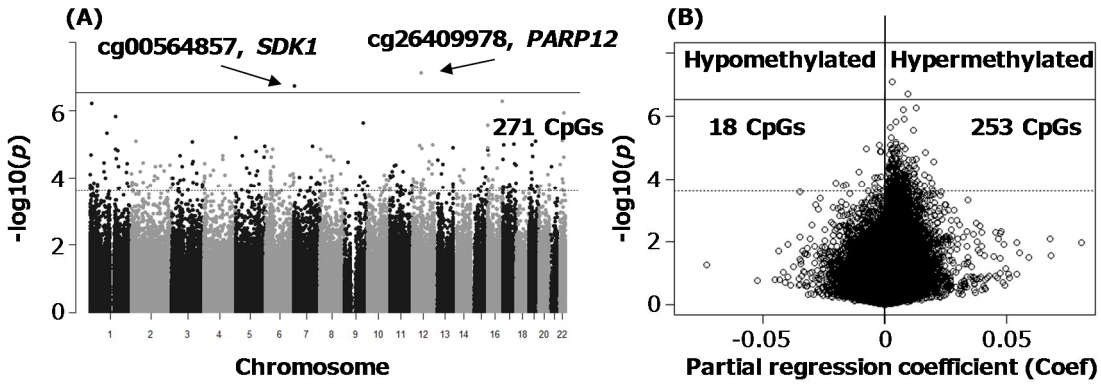

Figure 1. Manhattan (A) and Volcano (B) plots of the genome-wide associations of DNA methylation with prenatal exposure to DEHP.

Adjusted for maternal age, educational levels, pre-pregnancy BMI, smoking during pregnancy, blood sampling periods, gestational age, infant sex, and cord blood cell estimates.
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.

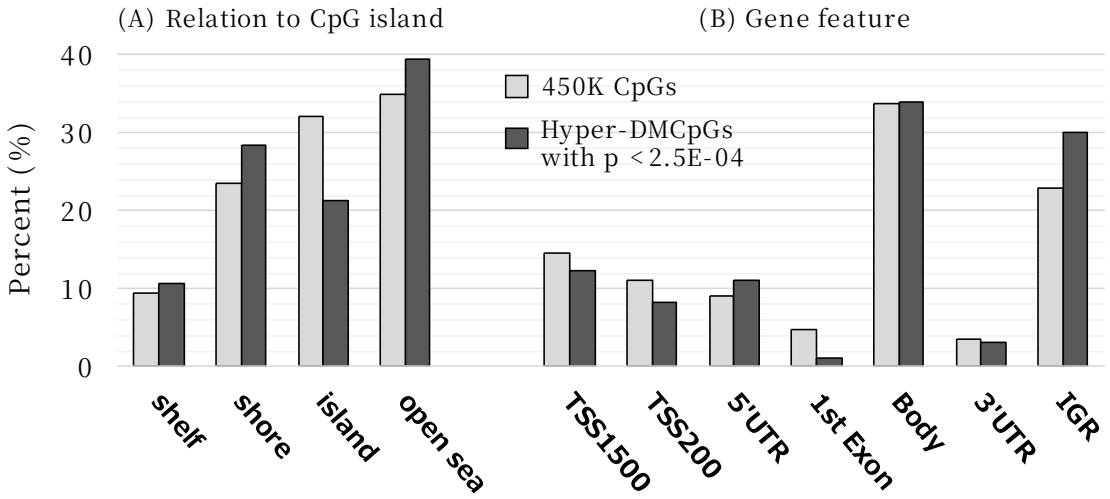

Figure 2. Location of the differentially hypermethylated CpGs (hyper-DMCpGs) with *p* <2.5E-04 (253 CpGs) compared to all CpGs on the methylation array.

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

Table 2. Direction of cord blood DNA methylation changes associated with maternal MEHP levels at differentially methylated regions identified by Solomon *et al*. (2018) 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: +; increased, –; decreased.

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

Table 3. Significantly enriched pathways (FDR <0.05) for the gene targets of 253 differentially hypermethylated CpGs (hyper-DMCpGs) 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 the hyper-DMCpGs with p <2.5E-04.

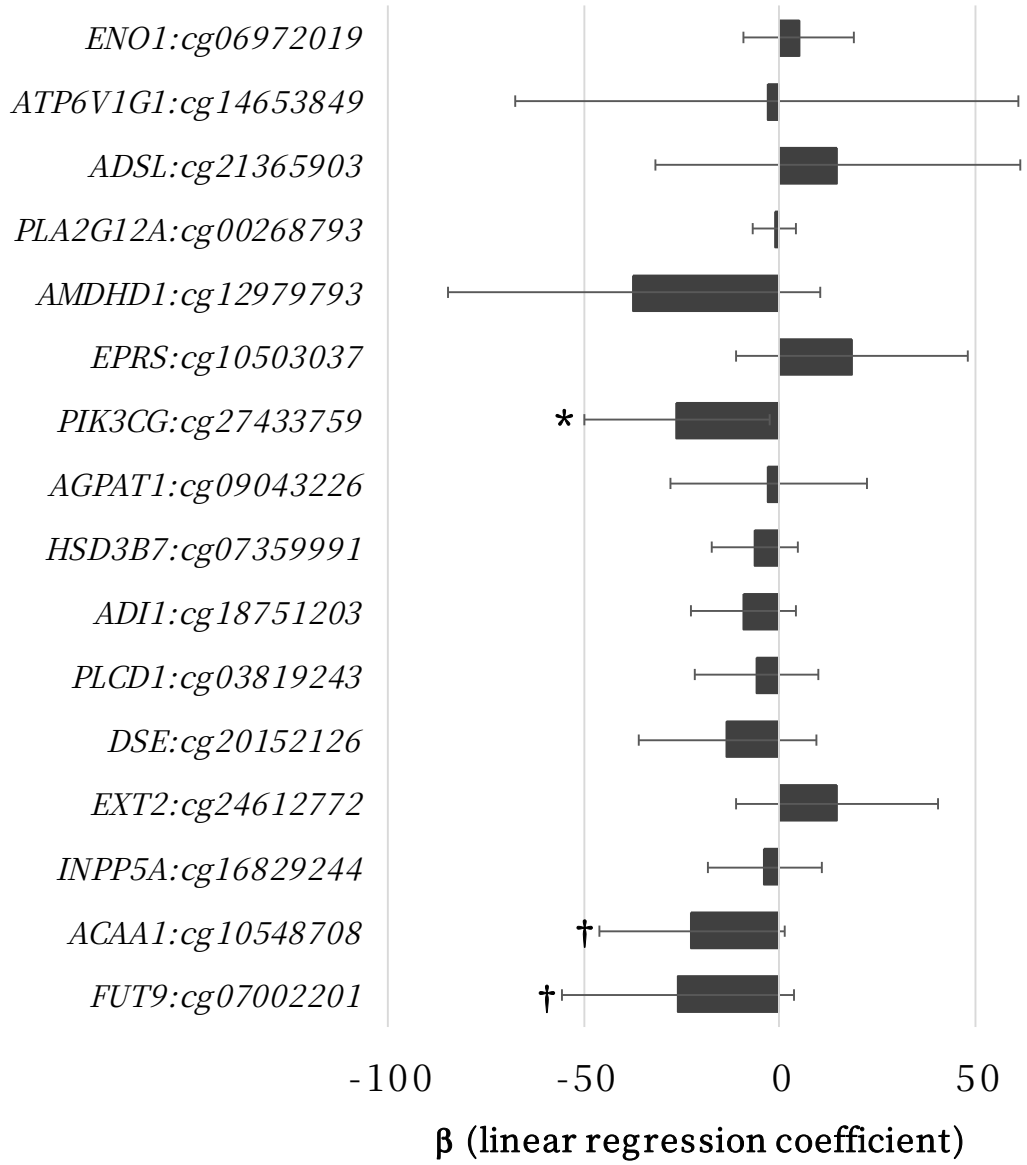

Figure 3. Linear regression coefficients (β) of ponderal index at birth in relation to methylation levels at CpGs positively associated with MEHP with *p*-value <2.5E-04, mapped to the genes involved in metabolic pathways. Error bars indicate 95% confidential interval (CI). Adjusted for maternal age, educational levels, parity, pre-pregnancy BMI, smoking during pregnancy, gestational age, infant sex.

†*P* <0.1, **P* <0.05

Table 4. Mediation analysis examining the association between prenatal exposure to DEHP and ponderal index at birth through differential methylation at CpGs by PROCESS (Hyaes, 2013)

	β[a] (95% CI)
Total effect of maternal MEHP on ponderal index at birth	-0.56 (-1.02, -0.11)*

Methylation at CpG	Direct effect	Indirect effect		Mediation % (Indirect/Total)
	β[b] (95% CI)	β[c] (Bca CI)	Sobel *p*-value	
PIK3CG:cg27433759	-0.48 (-0.95, -0.02)*	-0.08 (-0.23, -0.00)	0.16	14.3
ACAA1:cg10548708	-0.49 (-0.96, -0.01)*	-0.08 (-0.23, 0.03)	0.24	14.3
FUT9:cg07002201	-0.51 (-0.97, -0.05)*	-0.05 (-0.27, 0.01)	0.27	8.9
Average_3CpGs[d]	-0.39 (-0.87, 0.08)	-0.17 (-0.37, -0.05)	0.04*	30.4

[a]Coeffieicnt represents total effect estimate for DEHP exposure in the model: ponderal index = MEHP levels + covariates

[b]Coeffieicnt represents direct effect estimate for DEHP exposure in the model: ponderal index = MEHP levels + DNA methylation levels at CpGs + covariates

[c]Coeffieicnt represents indirect effect estimate for MEHP on ponderal index through differential methylation at CpG, equals (total effect) – (direct effect).

[d]Average of methylation levels at three CpGs; cg27433759, cg10548708, and cg07002201

Covariates; maternal age, parity, educational levels, pre-pregnancy BMI, smoking during pregnancy, blood sampling periods, gestational age, and infant sex

Bca CI, bias-corrected and accelerated CI

*p < 0.05