Insufficient gestational weight gain associated with higher DNA methylation in cord blood cells

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Abstract

Background Gestational weight gain (GWG) is one of the crucial factors affecting fetal growth as well as the in utero environment, influencing fetal cell programming during development. We reported previously that GWG affected the occurrence of outlying CpG methylation values of placental DNA in a U-shaped manner, with the occurrence of outlying values from adequate GWG subjects positioned at the bottom of the curve. In the present study, we aimed to elucidate the effects of GWG on the infant epigenome by the view that the influence of insufficient GWG on infant DNA methylation may turn in some other direction at the borderline of the optimal weight gain.

Method We collected cord blood from 60 subjects with uncomplicated term delivery whose mean pre-pregnancy body mass index and mean GWG was 19.8 ± 1.9 and 8.1 ± 4.3 kg, respectively. Cord blood DNA was underwent analysis using the Infinium MethylationEPIC BeadChip to profile genome-wide methylation status.

Results GWG was continuously associated with cord blood DNA methylation at five CpG loci significantly (multiple test corrected p-value, 0.043) in the lower than upper limit of the recommended GWG group ($n = 51$). The significant association between DNA methylation levels and GWG was disappeared when added 9 subjects who gained weight more than upper limit of recommendation during pregnancy. The methylation plot of the five loci plateaued or traced a U-curve near the border of the upper limit of the GWG recommendation. Cord blood DNA methylation of these five were all negatively associated with GWG. Validation using deep targeted bisulfite sequencing reproduced negative correlations between GWG and methylation levels within one of the five targets; at the upstream of LINC01816. This region has been annotated as a promoter or an enhancer depending on blood cell types based on their epigenetic marks.

Conclusions It is known that demethylation at enhancer region in genome is one of the features during late fetal development. We found that insufficient GWG showed higher methylation status in some enhancer-candidate loci in cord blood cells, which may indicate incomplete demethylation during in utero development.

Background

The perinatal environment affects fetal growth in addition to lifelong health—a concept well-known as the Developmental Origins of Health and Disease (DOHaD) theory [1]. Epigenetic regulation is thought to be one of the molecular mechanisms of cell memory established in perinatal environments because the methylation of cytosine at CpG sites changes in response to extracellular stimuli and is maintained after cell divisions in daughter cells. Maternal smoking, one of the deleterious prenatal environments, robustly affects the fetal epigenome by changing the DNA methylation status of specific genes [2], with such effects on DNA methylation still observed in grown children at 17 years of age [3]. This points toward the long-term effects of perinatal environments on epigenetic regulation. In humans, the status of maternal

nutrition is evaluated indirectly by maternal pre-pregnancy body mass index (BMI) and/or gestational weight gain (GWG). Maternal pre-pregnancy obesity and excessive GWG are often associated with adverse health outcomes in children [4]. Childhood adiposity was associated with total GWG [5] or GWG during the third trimester of pregnancy [6]. Other contributors that cause insufficient GWG [7] lead to fetal growth restriction; moreover, low birth weight is related to a high incidence of type 2 diabetes [8] and cardiovascular disease [9] later in life. These results indicate that the intrauterine environment may program the phenotype through epigenetic regulation. Although the reports that focused on maternal lean effects is less than the ones on maternal obesity, it is well-known that the maternal undernourishment during pregnancy caused by famine increased the newborn morbidity [10] and altered DNA methylation later in life [11].

Large cohort study examined continuous GWG effects on infant epigenetics across whole subjects and showed that GWG did not associate cord blood DNA methylation values [6, 12]. Whereas, in Japan, pre-pregnancy BMI of a half of mothers are lower than 20.0 [13]. That is smaller than European cohorts in which mean reported pre-pregnancy BMI is from 22.8 to 27.7 [14]. It makes us to consider the possibility that insufficient GWG has more severe effects in babies born from lean pre-pregnancy mothers. Japanese mothers gain less weight during pregnancy compared to European cohorts. The mean GWG in Avon Longitudinal Study of Parents and Children (ALSPAC) cohort was 12.5 ± 4.8 . The Japanese mean GWG was 10.11 ± 3.96 and 10.2 ± 3.9 in 69,126 and 61,960 normal weight pre-pregnancy mothers, respectively [15, 16].

The proportion of low birth weight infants who were born weighing less than 2500 grams in Japan was 3rd highest within 45 Organization for Economic Cooperation and Development (OECD) countries [17]. We previously reported that inadequate GWG is related to the frequency of outlying DNA methylation values at non-specific CpG sites in chorionic villi, even when newborn birth weights are normal. The number of CpG sites that showed outlying methylation values in each sample increased as GWG drifted outside the range of adequate gain [18]. Therefore, we hypothesized that the effects of continuous GWG change differ between insufficient and excessive GWG and GWG may affect fetal epigenome independently of birth weight. In the present study, we aimed to verify the effects of insufficient GWG of relatively lean mothers on infants DNA methylation.

Results

2.1. Sample characteristics

The characteristics of the 60 mother-infant pairs examined in this study are presented in Table 1. There were no pre-pregnancy overweight or obese women (BMI > 25) enrolled in our study. The mothers experienced no complications during pregnancy and newborns were all full-term births. Morisaki et al. estimated optimal and acceptable range of GWG for lowest probability of adverse outcomes such as complicated delivery, preeclampsia, and preterm delivery with each pre-pregnancy BMI category in 104,700 Japanese women [13]. According to the study of Morisaki et al., we considered upper limit of

GWG recommendations of the Japanese Ministry of Health, Labour and Welfare (JMHLW) guidelines are theoretical: women with a pre-pregnancy BMI between 18.5 and 25 should gain approximately 7–12 kg and those less than 18.5 should gain 9–12 kg body weight during the gestational period. To see insufficient GWG effects compared to optimal GWG, we extracted samples whose GWG were equal to or less than the upper limit of recommended GWG; 12 kg ($n = 51$). The distribution of GWG of 51 subjects is shown in Figure S1 (6.8 ± 3.4 kg). Figure S2 shows that the results of the estimation of cell type composition differed between individuals. Therefore, we considered the composition of each cell type as confounders in the linear regression model described in Methods.

2.2. Continuous GWG in the equal and less than upper limit of recommendation was associated with infant cord blood methylation

We hypothesized that GWG effects on infant epigenome might plot U-shape or reach plateau at a certain weight. Therefore, to see insufficient GWG effects, we extracted subjects whose GWG were insufficient or in optimal range as referring upper limit of recommendation (ULO; under upper limit of optimal range). In these subjects, GWG was significantly associated with cord blood methylation at five CpG sites with an FDR-adjusted P-value < 0.05 . No CpG site was significant after Bonferroni correction adjusted the P-value < 0.05 (Figure 1A and Figure S3, Table 2). Meanwhile, the regression analysis of methylation values against continuous GWG among all 60 175 subjects, which includes 9 subjects whose GWG exceeded optimal range, resulted in no significant association after multiple test correction (Figure 1B).

The methylation status were negatively associated with continuous GWG at all these significant five CpGs in 51 ULO subjects (Figure 1C and Table 2). Assessment of the methylation values of these five CpG sites in all 60 subjects revealed that the methylation plot reached a plateau or traced a U-curve near the border of the upper limit of the GWG recommendations (Figure 2). Meanwhile, non-linear categorical analysis showed that both insufficient and excessive GWG ($n = 22$ and 9, respectively) did not exhibit any significant differences when compared to the adequate ($n = 29$) group (Figure S4). These results indicated that insufficient GWG may gradually affect the fetal development as detectable range by DNA methylation levels and those effects may be reversed in excessive GWG. Within the five significant CpG sites, cg00599163 exists at the promoter site of LINC01806 and the methylation levels at this site in cord blood has been reported to be negatively associated with gestational age (GA) [19] (Table 2). The four other sites were in intergenic regions or gene body sites; out of these four, cg10370704 is in an intragenic enhancer chromatin state in several types of blood cells according to NIH Roadmap Epigenomics Mapping Consortium [20] (Figure S5).

2.3. Neither pre-pregnancy BMI nor birth weight associated with infant cord blood DNA methylation in our subjects

Regression analysis against pre-pregnancy BMI was also analyzed. An association between continuous pre-pregnancy BMI and DNA methylation in cord blood was not found at any CpG site (Figure S6A). Non-linear pre-pregnancy BMI distinguishing samples between lean ($\text{BMI} < 18.5$; $n = 17$) and normal ($\text{BMI} \geq 18.5$ to ≤ 25 ; $n = 43$) also showed no association with DNA methylation (Figure S6B). None of our samples were collected from obese women ($\text{BMI} > 25$). The effects of pre-pregnancy BMI were not associated with methylation status in our samples. Simultaneously, there was no association between cord blood DNA methylation sites and birth weight in our samples (Figure S6C).

2.4. The CpG sites associated with GWG were also GA-related methylation sites

GA at birth is known to be associated with methylation levels at several CpG sites in cord blood [19, 21, 22]. Therefore, we conducted additional regression analysis on GA at birth. Continuous GA was significantly associated with methylation levels at two CpG sites (Figure S6D) located very close to one another (30 bp distance) on the promoter of the PLCH1 gene (Table 3). One of these two, cg11932158, was reported to be associated with GA at birth in two different cohorts [19, 22] on which a DNA methylation assay using a 450K array (i.e., a previous version of the array) was performed. Another cg21262198 was assayed with a newly designed probe in the EPIC array employed in this study; therefore, information regarding the association between the DNA methylation of cord blood cells and GA may not exist yet. However, it has been suggested that the promoter region of the PLCH1 gene, including the cg21262198 locus, might be associated with GA even after 37 weeks. GWG was not correlated with GA ($P = 0.339$) in our samples. Thus, we added GA as a covariate to an association study with continuous GWG and methylation, in which the minimum FDR-adjusted p value slightly elevated to 0.055 that was confirmed at 16 CpG sites. The above five CpG sites were also included in these 16 as top minimum nominal p values (Table 4). Meanwhile, an additional 11 CpG sites showed the same FDR-adjusted p value after considering GA as a covariate. Methylation at these 11 CpG sites also showed a negative association with continuous GWG, except for one of the CpG sites:

cg00445959 was in the enhancer chromatin modifications and was reported to be associated with last menstrual period-estimated GA [19]. These results indicated that lower GWG may slow fetal epigenetic development separately from GA.

2.5. Validation of methylation values at 5 CpG sites that associated with GWG in ULO

We validated GWG-associated methylation at the 5 CpG sites using an alternative methylation-measurement method to BeadChip namely deep targeted bisulfite sequencing. For the validation, we again converted unmethylated cytosines into uracils by treating subject's genome DNA with bisulfite. Then targeted PCR was performed as described in method. Deep sequencing of bisulfite PCR amplicons using next generation sequencer enabled us to detect 1% methylation difference with minimum 6 reads to

maximum 153 reads (supplementary table 2). As a result, within 20 CpGs that was included in PCR amplicons for 5 targeted CpGs, only 2 CpG sites which is adjacent to cg00599163 were significantly correlated with GWG in ULO negatively, although correlation of methylation at cg00599163 with GWG was not significant by validation analysis (Figure 3). Significant negative correlation between methylation at each two CpG site and GWG were also confirmed within whole subjects when added 9 subjects who gained weight more than upper limit of recommendation during pregnancy in validation experiments (supplementary Figure 7). Regarding to the region around cg00599163, we acquired 4998 ± 2391 reads (min. 2384 to Max. 15108) and the half of them on the middle 3 CpGs including cg00599163 and the most 5' and 3' CpGs, respectively. The distribution of methylation percentage of the cg00599163 in whole subjects was 81.4 to 96.5 % in deep targeted bisulfite sequencing which was wider than b value distribution which was 0.88 to 0.99 with BeadChips. The other 4 CpG sites other than cg00599163 were not significantly correlated in negative direction with GWG. It was the same as for neighborhood CpGs of each 4 CpG site (Supplementary table 2).

Discussion

In this study, we revealed that a significant association between continuous GWG and cord blood DNA methylation at 5 CpG sites in the “under upper limit of optimal range” (ULO) group using BeadChip. The methylation regression curve of these five identified CpG sites reached a plateau or inverted in the excessive GWG group. This may suggest that the effects of GWG on the fetal epigenome are not linear through all category GWG subjects. The methylation of the 5 CpG sites were all associated negatively with GWG. The validation of results by an alternative method, i.e. deep targeted bisulfite sequencing, showed significant negative correlations between continuous GWG and cord blood DNA methylation at only 1 region around one of the above 5 CpG sites. The discrepancy of the methylation values between BeadChip and bisulfite sequencing is reported [23]. Meyer revealed that the beta value indicating 0.9 to 1.0 by BeadChip type 2 probes, that is the case with our identified 5 CpG probes in this study, were not linearly correlated to the methylation percentages in bisulfite sequencing results [23]. It may be the reason why we failed to reproduce the significant correlations between GWG and methylation values when determined by deep targeted bisulfite sequencing. Besides, bisulfite conversion and PCR amplification may have triggered biases in our validation method [24]. Eventually, the region in which methylation significantly correlated to GWG at CpG site level was only the upstream of *LINC01806* in validation analysis. This region is annotated as a promoter in most of blood cells based on the results of epigenetic analysis by NIH roadmap project (supplementary figure 7) [20]. Meanwhile, in monocytes and neutrophils from peripheral blood and B cells from cord blood, this region is annotated as a bivalent promoter. The promoters where 4th and 27th lysine of histones H3 were both tri-methylated in bivalent way are a feature of developmental genes [25]. Although the function of *LINC01806* is not well-known, according to Expression Atlas, thyroid gland has been confirmed to highly express *LINC01806* and induction of pluripotent stem cells from fibroblast of dermis upregulated expression of *LINC01806* with 5.7-log2 fold change [26]. It suggests that *LINC01806* works in T cells and/or is involved in stemness. Hence, it may be possible to detect significant GWG-associated methylation changes in other genes than *LINC01806* if we

examined different tissues from cord blood. In addition, in bulk mononuclear cells and natural killer cells from blood, this region is annotated as an active enhancer. It has been reported that negative association between DNA methylation and GA is predominant in cord blood cells [19]. Further, daily sampling during late fetal development in mice revealed that the feature of the 5mC dynamics is that enhancers predominantly lose 5mC during fetal development [27]. Similarly, a study comparing DNA methylation changes in four different human fetal tissues during the first and second trimesters revealed that loss of DNA methylation during human development was enriched at enhancer regions in all four tissues [28]. Regulation of enhancer accessibility through DNA demethylation is key steps for cell-lineage development [29]. The five identified CpG sites by BeadChips were all negatively associated with GWG. These results indicate that lower GWG may delay epigenetic development. Thus, we removed GA effects from the analysis. Though GA slightly affected the results, they still indicated that GWG tended to associate with DNA methylation independently of GA. These results may suggest that lower GWG may affect programmed epigenetic development independently from GA. The pre-pregnancy BMI- [6, 14], birth weight- [21, 22], and GA- [19, 22] associated methylation changes in cord blood cells have been reported from cohort studies. In our samples, GWG did not show significant correlation with birth weight ($p = 0.128$). Moreover, there was no significant association between DNA methylation and continuous birth weight in our samples. This indicate that GWG may establish the infant epigenome regardless of growth. Furthermore, pre-pregnancy BMI was not associated with DNA methylation levels in cord blood cells. GA-related methylation changes were detected at two sites in our samples, although our subjects were all born after 37 weeks. GWG-associated methylation sites and GA-associated sites did not overlap in our samples. As for the effects of GWG on the infant epigenome, the Avon Longitudinal Study of Parents and Children (ALSPAC) and the Accessible Resource for Integrated Epigenomics Studies (ARIES) [6], as well as the Norwegian Mother and Child Cohort Study (MoBa) [12], reported no association between newborn blood DNA methylation and continuous GWG from 673 and 729 mother-newborn pairs, respectively. These two studies assessed continuous GWG effects throughout insufficient to excessive all together. This may explain why GWG effects were not observed in these studies.

Demethylation along with increases in GWG may indicate an increase in the activation of enhancers and/or promoters in the adequate GWG group compared to the insufficient GWG group. The transcription factors and enzymes of the ten-eleven translocation (TET) family which catalyze the stepwise oxidation of 5-methylcytosine are known to cooperatively demethylate enhancers [30]. Several nutritional factors are known to be modulators of TET activity. Treatment with vitamin C leads to a significant increase in the levels of oxidized methylcytosine, consistent with its function in stimulating the catalytic activity of TET enzymes [31-33]. Meanwhile, maternal vitamin C deficiency leads to incomplete loss of DNA methylation in the female mice embryonic germline cells presumably through inactivation of TET1 [34]. In addition, the BCAA (branched-chain amino acid) – BCAT1 (BCAA transaminase 1) – α KG (α ketoglutarate) axis also engages in TET activity. Reduction of α KG eventually induces DNA hypermethylation [35, 36]. Mitsuya et al. showed that oxidation of 5mC was attenuated in placentas from pre-pregnancy obese women compared to normal women. They also revealed that pre-pregnancy or first trimester BMI was negatively correlated with placental α KG levels in obese women and the amount of vitamin C and α KG in

placentas showed a very clear positive relationship [37]. This suggests that these two nutrients correlate with DNA methylation during pregnancy. Vitamin C levels in maternal serum is decreased in several pregnancy complications such as gestational diabetes mellitus [38] and insufficient infant growth [39], as well as in the cord blood of low birth weight babies [40]. Insufficient GWG could be accompanied by caloric and protein undernourishment and multiple micronutrient deficiencies [41]. Low levels of vitamin C as well as restricted or imbalanced protein intake, which attenuate α KG synthesis, might underlie the lack of 5mC oxidation in insufficient GWG. Whereas, excessive GWG may also produce deficiency in some nutrients which involve in TET activity [37]. Therefore, these bipolar GWG may alter the infant DNA methylation in Ushaped manner.

As the limitation of our study, we did not record about severity of morning sickness. It might be the consequences of nausea and vomiting during pregnancy that some subjects gained almost 0 kg during gestation. It is known that the serum high levels of GDF15 is related to vomiting [42, 43]. However cytokine is not supposed to transmit placenta [44]. Thus, weight loss and/or malnutrition during early pregnancy because of morning sickness may affect infant epigenome more sensitive than GWG. Further studies are needed to elucidate this possibility. Next, there is no subject enrolled who were obese before pregnancy and there were only nine subjects with GWG greater than the JMHLW-recommendations. In Japan, half of all pregnant women had pre-pregnant BMIs less than 20 and mean GWG is around 10.2 kg in pre-pregnancy normal weight women [13, 15, 16]. Therefore, maternal characteristics of our subjects were distinct from European cohorts [14]. The recruitment of obese subjects from different areas may yield stronger correlations regarding adequate physiological DNA demethylation in cord blood cells. Further, we did not collect maternal weight gain data by each period during pregnancy. Those data could help explanation of particular GWG temporal effects on infant epigenome in accordance with stage of development.

Conclusions

This study indicated that the effects of GWG on the infant epigenome were not linear throughout all blocks of GWG. Further, insufficient GWG showed higher methylation status at some CpG sites compared to optimal GWG. A part of these sites were the same genomic loci where DNA demethylation was observed in cord blood cells along with increased gestational age. It may indicate that insufficient GWG delay development in infant epigenetics through repressing demethylation of the specific genomic regions such as enhancers.

Methods

5.1. Ethics Statement

This study complies with the principles of the Declaration of Helsinki in its current version. Mandatory patient information and informed consent forms were approved by the Ethics Committee of the National Center for Child Health and Development (approval number; 406) and Keio University School of Medicine

(approval numbers; 20100154, 20110321, 20150103, and 20150168). Written informed consent was obtained from all subjects.

5.2. Sample collection

The perinatal data were collected from medical records. Full-term cord blood samples with gestational ages (GAs) of 37 to 41 weeks were obtained from cesarean sections or vaginal deliveries and stored at – 80 °C. Tissue DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Samples of genomic DNA (1.5 mg) were bisulfite converted using an Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA, USA). After determining the concentration of bisulfite-converted DNA, 300 ng of each bisulfite-converted DNA underwent analysis using an Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA) to profile methylation status for approximately 850 000 CpG loci.

5.3. DNA methylation profiling and statistical considerations

Data was obtained from the iScan system (Illumina) as idat files and then processed using the minfi and ChAMP packages ("<https://bioconductor.org/biocLite.R>") in R3.4.0. Background correction was performed using NOOB methods within the minfi package as specified by the software authors [45]. Corrected data were normalized by BMIQ within the ChAMP package (ver. 2.8.9) as specified by the software authors [46].

The manifest file was annotated using "IlluminaHumanMethylationEPICanno.ilm10b2.hg19". We removed 5861 probes having detection P-values > 0.01 in at least one sample and 8332 probes with a beadcount < 3 in at least 5% of samples and retained only the CpG detection probes. Furthermore, we filtered 17 228 probes located on either the X or Y chromosomes, 48 multi-hit probes [47], and 78 422 SNP-related probes [48]. This filtration resulted in 754 028 autosomal probes from 60 samples. Cell composition estimation for cord blood was performed with installed "FlowSorted.CordBlood.450k" packages [49]. "Bcell", "CD4T", "CD8T", "Gran", "Mono", "Neu", and "nRBC" were estimated for each sample as implemented in the minfi package. These estimates were then added to the regression model described above as separate covariates [50]. Next, we considered the variance inflation factor (VIF) between cell types and removed "Gran" fractions from covariates when VIF showed 55.3. Other cell-type compositions were added to the regression model as separate covariates. We conducted epigenome-wide association studies (EWAS) using a linear regression model. This was applied to each CpG site individually and the results were then corrected for multiple testing by controlling the expected proportion of false-positives among all discoveries (FDR) using the Benjamini-Hochberg method. The genomic inflation factor (λ , lambda) and quantilequantile (Q-Q) plots were used to compare the genome-wide distribution of P-values with the expected null distribution.

5.4. Deep targeted bisulfite sequencing

Bisulfite PCR was performed as described previously [18, 51] using bisulfite-PCR primers designed by the MethPrimer website [52]. Samples of genomic DNA (500 ng) were bisulfite converted using an EZ DNA methylation-Gold (Zymo). The list of primers and the genomic interval (hg19) of the amplicon were described in supplementary table 1. PCR was performed by TaKaRa Ex Taq® (TaKaRa, Shiga, Japan). Deep amplicon sequencing was performed as described previously [53]. The obtained amplicons for 5 targeted regions from the same subject's sample were pooled and subjected to adaptor ligation and indexing (6 cycles) using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). The resulting libraries were subjected to paired-end sequencing (151 bp × 2) on a MiSeq platform (Illumina). MiSeq Reporter Software version was 2.3.32. Bisulfite-treated sequencing reads were aligned by a paired-end alignment method for a unique best hit to the human reference genome (hg19) using the Bismark program [54], and adapter trimming and quality control was performed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) as described previously [55]. Percent methylation scores were obtained from bismark.cov files. Samtools version 1.6 and WashU EpiGenome Browser v46.2 [20] were used for the sequence analysis procedures.

List Of Abbreviations

GWG: Gestational weight gain

EWAS: Epigenome wide association study

GA: Gestational age

BMI: Body mass index

JMHLW: Japanese Ministry of Health, Labour and Welfare

ULO: Under upper limit of optimal range TET: Enzymes of the ten–eleven translocation αKG: α-ketoglutarate

Declarations

Ethics approval and consent to participate

The study was approved by Keio University School of Medicine Ethics Committee (nos. 20100154, 20110321, 20150103, and 20150168) and the institutional review board of National Research Institute for Child Health and Development (no. 406) and was carried out in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki and its later amendments. Written, informed consent was obtained from all subjects.

Consent for publication Not applicable.

Availability of data and materials

The data set supporting the results of this article are available in the Gene Expression

Omnibus repository (GSE122288, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122288>). Methylation data and analytical conditions are available upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

TK conceived the study. TK and KM designed the study. YK and SK performed the experiments and acquired the data. TK analyzed the data. YK conducted medical record collection. KH contributed reagents. KM and MT contributed materials. TK wrote the paper. KH edited the manuscript and provided intellectual input. KM and KH conceived and supervised the project. All authors read and approved the final manuscript.

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Tables

Table 1. Subject characteristics (N = 60)

Characteristic	Number	%
Maternal age, years		
30-35	28	46.7
36-40	26	43.3
41-45	6	10.0
Parity		
0	43	71.7
≥1	17	28.3
Gestational age at delivery, weeks		
37-40	56	93.3
>40	4	6.7
Infant birth weight, g		
<2500	8	13.3
2500-3499	52	86.7
Infant sex		
Male	32	53.3
Female	28	46.7
Gestational weight gain, kg		
(*JMWHLW border line)		
<7; insufficient	22	36.7
≥7 to ≤12; adequate	29	48.3
>12; excessive	9	15.0
Pre-pregnancy BMI		
<18.5	17	28.3
≥18.5 to ≤25	43	71.7
>25	0	0
Caesarian section		
Yes	20	33.3
No	40	66.7
Maternal smoking		
Yes	0	0
No	60	100
Race		
Japanese	60	100

*JMWHLW ; The Japan Ministry of Health, Labour and Welfare guidelines

Table 2. The 5 CpG sites that were significantly associated with continuous GWG in ULO after adjustment for cell counts

TargetID	coe	P val	Genic region	Gene Name	Chr	Position	Predicted	450K	Bohlin <i>et al.</i>
		(FDR-adjusted)	(Nearest TSS)				Enhancer		(MoBa) [31]
cg00599163	-0.0056	1.04.E-07 (0.043)	promoter-TSS	<i>LINC01806</i>	2	162100495	Yes	Yes	Yes*
cg19516245	-0.0034	1.14.E-07 (0.043)	Intergenic	<i>ZBTB10</i>	8	81353432		No	
			(-44,422 bp)						
cg16530016	-0.0064	2.09.E-07 (0.043)	GeneBody	<i>RAMP3</i>	7	45215683		No	
			intron 1 of 2						
cg03962019	-0.0029	2.47.E-07 (0.043)	Intergenic	<i>FOXO6</i>	1	41807865		Yes	
			(-19,738 bp)						
cg10370704	-0.0033	2.82.E-07 (0.043)	GeneBody	<i>CABIN1</i>	22	24500452	Yes	No	
			intron 26 of 36						

* The association was observed only in ultrasound- based GA

Table 3. The 2 CpG sites that were significantly associated with continuous GA after adjustment for cell counts

TargetID	coe	P val	Genic region	Gene Name	Chr	Position	450K	Bohlin <i>et al.</i> (MoBa) [31]		Simpkin <i>et al.</i>
		(FDR-adjusted)								(Aries) [33]
								Ultrasound_based	LMP_based	
cg11932158	-0.043	1.73.E-08 (0.013)	promoter-TSS200	<i>PLCH1</i>	3	155422129	Yes	Yes	Yes	Yes
cg21262198	-0.036	1.02.E-07 (0.038)	promoter-TSS200	<i>PLCH1</i>	3	155422159	No	-	-	-

Table 4. The 16 CpG sites with the top FDR-adjusted P value that were associated with continuous GWG in ULO after adjustment for cell counts and GA

TargetID	coe	P val (FDR-adjusted)	Genic region (Nearest TSS)	Gene Name	Chr	Position	chromHMM Enhancer	450K	Bohlin <i>et al.</i> (MoBa) [31]
cg19516245	-0.003	1.89E-07 (0.056)	Intergenic (-44,422 bp)	<i>ZBTB10</i>	8	81353432		-	
cg00599163	-0.005	2.30E-07 (0.056)	promoter-TSS	<i>LINC01806</i>	2	162100495	Yes	Yes	Yes*
cg03962019	-0.003	2.49E-07 (0.056)	Intergenic (-19,738 bp)	<i>FOXO6</i>	1	41807865		Yes	
cg16530016	-0.006	3.71E-07 (0.056)	intron 1 of 2	<i>RAMP3</i>	7	45215683		-	
cg00445959	-0.006	5.93E-07 (0.056)	intron 1 of 23	<i>XPO6</i>	16	28220268	Yes	Yes	Yes§
cg10370704	-0.003	6.04E-07 (0.056)	intron 26 of 36	<i>SUSD2</i>	22	24500452	Yes	-	
cg03806175	-0.003	6.06E-07 (0.056)	intron 56 of 70	<i>LOC101927550</i>	7	98577593		Yes	
cg17714110	-0.001	6.08E-07 (0.056)	Intergenic (-2,610 bp)	<i>FOXN3</i>	14	90088104	Yes	-	
cg24629380	-0.001	9.10E-07 (0.056)	intron 4 of 7	<i>VANGL1</i>	1	116214059	Yes	Yes	
cg26970265	-0.001	9.11E-07 (0.056)	promoter-TSS	<i>TTC3-AS1</i>	21	38566282		-	
cg04032453	-0.001	9.12E-07 (0.056)	intron 3 of 11	<i>TBC1D16</i>	17	77960186		-	
cg21398491	-0.002	9.44E-07 (0.056)	Intergenic (+ 670,924 bp)	<i>KCNJ3</i>	2	156226017		-	
cg08538468	0.001	9.95E-07 (0.056)	promoter-TSS	<i>SH3GL2</i>	9	17579028		-	
cg21428460	-0.012	1.08E-06 (0.056)	promoter-TSS	<i>C11orf96</i>	11	43963367		-	
cg00949980	-0.003	1.16E-06 (0.056)	intron 1 of 3	<i>SUMO3</i>	21	46237117		Yes	
cg23702903	-0.004	1.18E-06 (0.056)	exon 15 of 20	<i>GAA</i>	17	78061443		-	

The association was observed only in ultrasound- based GA (*) and last menstrual period-estimated GA (§), respectively, in Bohlin *et al*'s study.

Figures

A Continuous GWG within upper limit of recommendation (n = 51)

B Continuous GWG of whole subjects (n = 60)

C Significantly associated 5 CpG sites

Figure 1

EWAS analysis with continuous GWG in ULO and whole subjects The results of epigenome-wide association studies (EWAS) of infant cord blood DNA methylation with continuous GWG (kg) of ULO (n = 51) (A), and of whole subjects (n = 60) (B). The bottom (blue) and the top (red) lines indicate the FDR-

adjusted and Bonferroni-adjusted P-value threshold (0.05) for genome-wide significance, respectively. C. DNA methylation of five significantly associated CpG sites with continuous GWG in ULO (n = 51) is plotted according to GWG (kg). “r” and “p” indicates correlation coefficient and p-value, respectively, of Pearson correlation test between GWG and DNA methylation values.

Figure 2

Methylation Plots of 5 CpG sites within whole subjects. The associations of methylation at 5 CpG sites with GWG were examined in whole subjects that includes the subjects who gained their weight more than upper limit of optimal range during pregnancy. Vertical axis shows DNA methylation value in cord blood. Horizontal axis shows GWG (kg). Dashed line indicates the upper limit of optimal range of GWG. The associations turned to the other directions around dashed line. “r” and “p” indicates correlation coefficient and p-value, respectively, of Pearson correlation test between GWG and DNA methylation values.

Figure 3

Methylation Plots of CpG sites in upstream of LINC01806 within ULO subjects. Validation of results from BeadChips were performed by deep targeted bisulfite sequencing. Five CpG sites included in a targeted PCR for cg00599163 were indicated together with the loci of LINC01806 gene and a CpG island. The methylation at two CpG sites; chr2.162100399 and chr2.162100444 were significantly correlated with GWG in ULO. In scatter plots, vertical and horizontal axis show DNA methylation percentage in cord blood and GWG (kg), respectively. “r” and “p” indicates correlation coefficient and p-value, respectively, of Pearson correlation test between GWG and DNA methylation values.

Supplementary Files

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