Human Molecular Genetics, 2021, Vol. 30, No. 21 1968–1976

https://doi.org/10.1093/hmg/ddab161
Advance Access Publication Date: 21 June 2021
General Article

GENERAL ARTICLE

Genetic and *in utero* environmental contributions to DNA methylation variation in placenta

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Abstract

Genetic and prenatal environmental factors shape fetal development and cardiometabolic health in later life. A key target of genetic and prenatal environmental factors is the epigenome of the placenta, an organ that is implicated in fetal growth and diseases in later life. This study had two aims: (1) to identify and functionally characterize placental variably methylated regions (VMRs), which are regions in the epigenome with high inter-individual methylation variability; and (2) to investigate the contributions of fetal genetic loci and 12 prenatal environmental factors (maternal cardiometabolic-,psychosocial-, demographic- and obstetric-related) on methylation at each VMR. Akaike's information criterion was used to select the best model out of four models [prenatal environment only, genotype only, additive effect of genotype and prenatal environment (G + E), and their interaction effect (G × E)]. We identified 5850 VMRs in placenta. Methylation at 70% of VMRs was best explained by G × E, followed by genotype only (17.7%), and G + E (12.3%). Prenatal environment alone best explained only 0.03% of VMRs. We observed that 95.4% of G × E models and 93.9% of G + E models included maternal age, parity, delivery mode, maternal depression or gestational weight gain. VMR methylation sites and their regulatory genetic variants were enriched ($P < 0.05$) for genomic regions that have known links with regulatory functions and complex traits. This study provided a genome-wide catalog of VMRs in placenta and highlighted that variation in placental DNA methylation at loci with regulatory and trait relevance is best elucidated by integrating genetic and prenatal environmental factors, and rarely by environmental factors alone.

Introduction

The prenatal period is a critical milestone of life impacting fetal development and long-term health (1–7). The placenta, an organ that facilitates exchange of nutrients, hormone production and mitigation of adverse environmental exposures at the feto-maternal interface, is now considered crucial to understanding mechanisms in fetal development and diseases in later life (8,9). One of the molecular processes through which the placenta regulates fetal development is epigenetic mechanisms. The placenta displays a unique epigenetic profile, with genome-wide DNA methylation levels lower than other tissues (10–15), and undergoes changes in DNA methylation throughout gestation (16,17). The importance of epigenetic factors in placental function has been underscored through studies of imprinted genes (18,19) and observation of altered placental gene expression and DNA methylation in response to exogenous signals and stochastic events (20–22).

Variation in DNA methylation can be explained in whole or in part by environmental factors, genetic factors (23,24) or

Received: April 3, 2021. **Revised:** June 8, 2021. **Accepted:** June 9, 2021

integrated effects of genetic and environmental influences (25–27). Previous studies have found that prenatal environments like maternal pre-pregnancy body mass index (BMI) (28), dyslipidemia (29), blood pressure (30), organic pollutant chemicals (31), stress (32) and gestational weight gain (28) are associated with placental DNA methylation at specific loci. Moreover, recent studies have identified inherited genetic variation, especially single nucleotide polymorphisms (SNPs) that regulate DNA methylation at nearby genomic regions in placenta (13,33). However, the coordinated and independent influence of genetic and environmental factors on placental DNA methylation remains unknown.

There is a recent focus on regions in the genome with high variability in DNA methylation across individuals, called variably methylated regions (VMRs) to elucidate the integrated effect of genetic and environmental factors on DNA methylation and epigenetic mechanisms of common diseases. VMRs are attractive in mechanistic studies because they co-localize with other functional genomic features. VMRs are also less vulnerable to the influence of artifacts unrelated to methylation variance than individual DNA methylation sites (34). Analogous to common genetic variants as posited by the common disease common variant hypothesis (35), inter-individual variation in methylation at VMRs can lend an alternative insight about mechanisms of complex diseases (34). The stochastic variations exhibited in VMRs have been leveraged in prior studies to understand the role of epigenetic selection in phenotypic variation (36,37), tissue-specific function (34), genetic mechanisms (23,24,38) and environmental adaptation (39–41). Several studies have shown that analysis of VMRs enhances detection of epigenetic variability at loci enriched for functional elements (36,42–44). Notably, recent studies have found that most cord blood VMRs are best explained by interactions of nearby genetic variants with prenatal environmental factors (25,27).

The goal of this study was to determine the integrated effect of prenatal environment and genetic factors in explaining inter-individual variation of DNA methylation at VMRs in placenta. Specifically, we aimed to (1) identify and catalog VMRs in placenta, (2) determine whether genetic variation, prenatal environmental factors or the additive or interactive effect of genetic and prenatal environmental factors best explains inter-individual variability in methylation levels at each VMR and (3) determine whether VMRs in placenta are enriched for functional regulation and disease risk. Our analysis identified 5850 VMRs in the placenta. We found that the variability in methylation at the majority of VMRs in placenta is best explained by models integrating genetic variants with prenatal environments. We also observed that the DNA methylation sites harbored within VMRs are functionally relevant and have regulatory effects on gene expression. Finally, the genetic variants identified in the best models were significantly enriched for loci associated with complex diseases in genome-wide association studies (GWAS).

Results

Dataset and analysis overview

The dataset included 301 pregnant women from the (National Institute of Child Health and Human Development) NICHD Fetal Growth Studies–Singletons who provided placental samples at delivery that have been profiled for fetal genome-wide DNA methylation and genotype data (45,46). Descriptive statistics of the 12 prenatal environmental factors included in the present analysis is presented in Supplementary Material, Table S1. Briefly, women were on average 27.7 years old, delivered at 39.5 gestational weeks, had 11.7 kg weight gain during gestation, 53.4% were parous and 10.5% had pre-pregnancy obesity. A flowchart of the research is summarized in Figure 1.

Identifying VMRs in placenta

We identified 5850 VMRs in placenta [consisting of 14 022 cytosine–phosphate–guanine sites (CpGs)], each representing a region in the genome with highly variable CpG methylation, using a strategy that assigned a median absolute deviation (MAD) score >90th percentile (see Materials and Methods) as implemented in prior studies (25,27) (Supplementary Material, Table S2). The majority of VMRs contained two CpGs (Supplementary Material, Fig. S1). Consistent with expectation, methylation levels of VMR tag-CpGs (a CpG with the highest MAD score per VMR) were not correlated with each other [mean (SD) $r = 0.06\ (0.07)$, $P < 10^{-300}$). Methylation level of VMR CpGs followed a bell-shaped and unimodal distribution, as opposed to non-VMRs CpGs that displayed bimodal distribution (Supplementary Material, Fig. S2). This is analogous with the bell-shaped allelic distribution of common SNPs associated with complex traits in GWAS (47).

Best model explaining variation in placenta DNA methylation

For each VMR tag-CpG, four models, i.e. environment model (E), genotype model (G), gene–environment additive model (G + E) and gene–environment interactive model (G × E) were computed, and the model that best explained the methylation variance was selected based on the lowest Akaike's information criterion (AIC) (48). Out of the 5850 tag-CpGs, 5848 were included in analysis because no SNPs were found in our data within 1 Mb distance from two tag-CpGs. Variation in placental methylation at 70% of tag-CpGs was best explained by G × E, followed by G (17.7%), G + E (12.3%) and E (0.03%) (Fig. 2A; Supplementary Material, Tables S3–S6).

We evaluated the models using root means square error (RMSE) and delta AIC and confirmed that G × E is the best model (Fig. 2B and C). Furthermore, we obtained different sets of VMRs using MAD score cut-off values ranging from 0 to 90 percentile and investigated whether G × E remained the best model. The G × E model remained the best model for the majority of VMRs (71.6–75.8% across MAD scores), followed by G (13.9–17%), G + E (9.8–11.3%) and E (0.01–0.15%) (Supplementary Material, Fig. S3). Among the 12 prenatal environmental factors investigated, maternal age, parity, delivery mode, maternal depression or gestational weight gain were identified among 95.3% of G × E best models and 93.9% of G + E best models (Fig. 2D–F).

We evaluated overlaps between the SNPs associated with VMR tag-CpGs in our best models (1035 SNPs in G, 720 SNPs in G + E and 4091 SNPs in G × E) and previously identified methylation quantitative trait loci (meQTLs) in placenta (33) and in blood at birth, childhood, adolescence, pregnancy and middle age (49). Only 18 SNPs overlapped with meQTLs in placenta (Supplementary Material, Table S7), whereas 43.9% VMR-associated SNPs from G model, 49.7% from G + E model and 43.1% from G × E model overlapped with meQTLs in blood (Supplementary Material, Table S8).

Regulatory and phenotypic annotations and enrichment of pathways

VMR CpGs were significantly enriched for CpG islands, shore regions, 5′ untranslated region (UTR), promoters, introns, transcription factor-binding sites (TFBS), chromatin marks (Supplementary Material, Table S9) and DNase I hypersensitive sites in

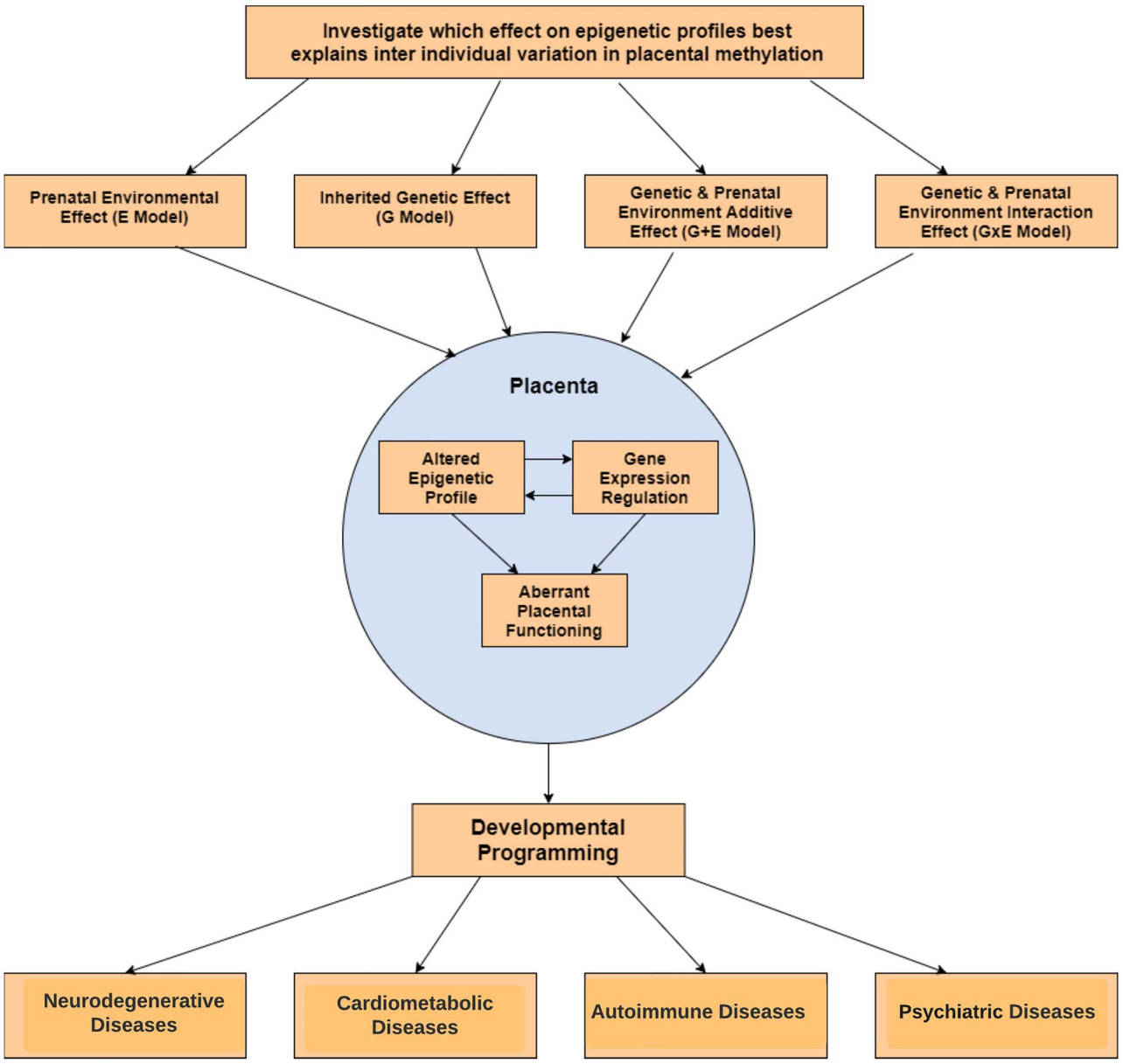

Figure 1. Research flow chart.

several tissues and cells (Supplementary Material, Table S10). In addition, VMR CpGs in the best G + E and G × E models were enriched for exons ($10^{-300} < P < 6.57 \times 10^{-9}$) and VMR CpGs in G + E were enriched for 5′ UTRs ($P = 3.86 \times 10^{-7}$) (Supplementary Material, Figs S4–S7). VMR CpGs in the G, G + E and G × E best models were significantly enriched for trait-associated methylation loci cataloged by the epigenome-wide association studies (EWAS) atlas (50) (Supplementary Material, Table S11). SNPs associated with the CpGs in the best models were also enriched for disease or trait-associated loci from the GWAS catalog (51) (Supplementary Material, Table S12).

Genes annotating the VMR CpGs in these best models have been implicated in previous GWAS for cardiometabolic, autoimmune, psychological and neurodegenerative disorders (Supplementary Material, Table S13). To gain further biological insights on genes annotating VMR CpGs in best G, G + E and G × E models, we investigated enrichment in canonical pathways utilizing the web-based platform functional mapping and annotation of genetic associations (FUMA) (52). Several pathways implicated in diverse molecular functions were overrepresented (Supplementary Material, Table S14).

Discussion

We identified genomic regions with high inter-individual variability in DNA methylation (VMRs) in placenta and provided evidence for genetic and environmental effects in explaining methylation variability at VMRs. We identified 5850 VMRs and found that methylation variation at more than two-thirds of VMRs was best explained by the interactive or additive effects of genotype and prenatal environmental factors, and very rarely by prenatal environmental factors alone. We also found that VMRs in placenta were enriched for regulatory genomic regions and for genomic regions previously associated with complex diseases, suggesting their functional and etiologic relevance.

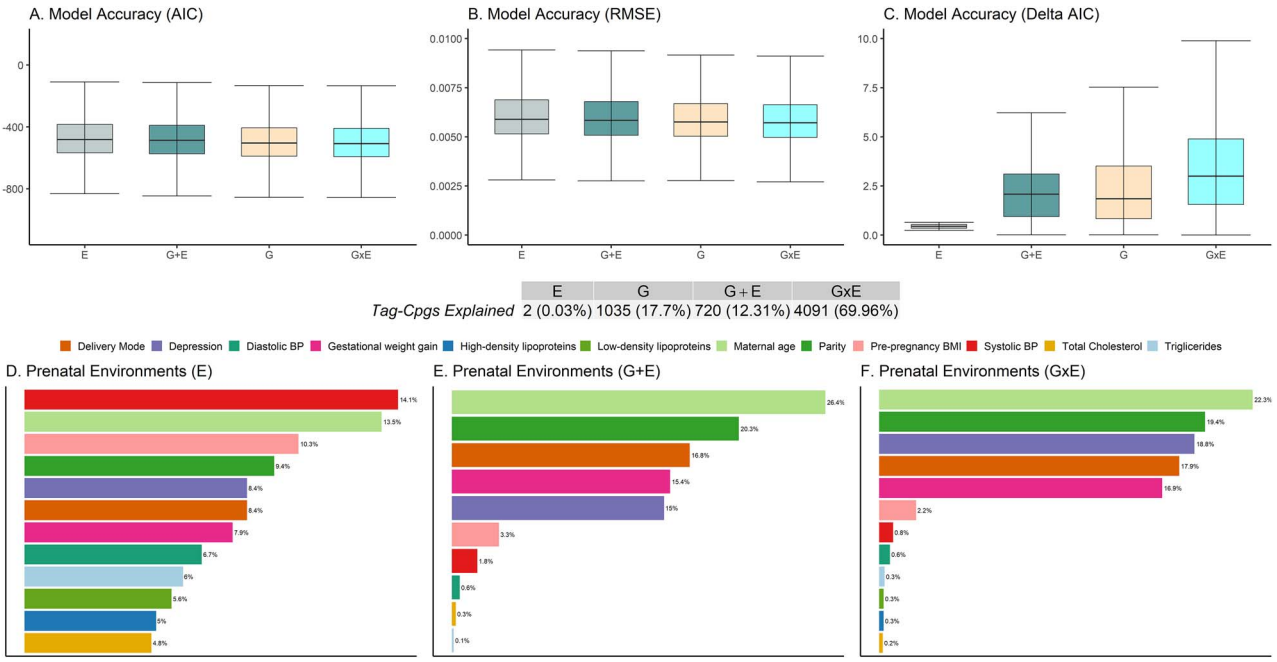

Figure 2. Identification of models and their associated prenatal environments that best explain the inter-individual variation of DNA methylation in placenta. (**A–C**) Shows the range of AIC, RMSE and delta AIC values for four different models analyzing 5848 VMR tag-CpGs. The boxes are colored by model names and are arranged by median values. (**D–F**) Shows the degree of association of 12 different prenatal environments in explaining the variation in placenta DNA methylation in combined effects with genetics (E, F) as compared with all nominally significant prenatal environments without the genetic factor (D).

There are similarities and differences between findings of our study of VMRs in placenta and previous studies in cord blood (25,27). Like our findings, the cord blood studies found that variance in methylation at most VMRs is best explained by interactions of genetic and prenatal environmental factors. Moreover, in our as well as the cord blood studies, prenatal environmental factors alone are very rarely best at explaining variability in VMRs. On the other hand, the G × E model was best at explaining methylation variation for a strikingly larger proportion of VMRs in placenta from our data (~70%) than cord blood from the previous studies (~41%) (25,27). Although this may partly be due to study differences in distribution of prenatal and other sociodemographic factors, the unique DNA methylation profile of the placenta (10–15) may be another important source of difference. In line with the latter, the overlap of VMR CpGs between placenta in our study and that of cord blood was just 2.5% (25,27), suggesting potential tissue specificity.

Previous research (36) has indicated that VMRs harbor genes linked to development and morphogenesis (e.g. BMP7 and POU3F2) (36). In agreement with prior reports, our pathway analysis results indicate that genes in close proximities to placental VMRs regulate important developmental processes such as neurogenesis, mitosis and immune system. In addition, VMRs have been found to be enriched in various functional genomic features such as enhancers, CpG island shores, 3′ UTR, indicating their potential functional roles in transcription regulation (34,53). It is worth noting that placental VMRs exhibited some unique functional characteristics not observed in cord blood VMRs (25,27). Placental VMRs had the highest enrichment for CpG island regions, which are implicated in basic cellular function and development (54). Placental VMRs also showed widespread co-localization with TFBS such as Pol2 and EZH2, which have been linked to several placental pathologies such as pre-eclampsia (55–57). Lastly, our finding that the placental VMRs and the SNPs associated with the VMRs were enriched for EWAS and GWAS loci suggests their potential in advancing insights about the pathobiology of complex diseases.

Our study has limitations. The map of placental VMRs identified is not likely to be comprehensive, because the methylation array used covers only <2% of CpGs in the human genome and has biased representation of gene promoters and CpG islands (58,59). Future work utilizing sequencing approaches can provide a better map of VMRs in placenta. Second, we did not replicate the findings in an independent dataset because pregnancy cohorts with multi-omics data on placenta are uncommon. Last, the analysis included SNPs in *cis* regions from the VMRs and a limited number of environmental factors. Although this is consistent in scope with prior studies in cord blood (25,27), future studies incorporating *trans*-acting SNPs and other environmental factors may refine our findings.

In conclusion, we constructed the first genome-wide catalog of VMRs in placenta and determined whether genotypes or prenatal environmental factors or their interaction best explains methylation variations at each VMR. Given our finding that the majority of VMRs are best explained by gene–environment interactions, incorporating genetic as well as prenatal environmental factors can give better insight about epigenetic mechanisms underlying developmental and later life phenotypes.

Materials and Methods

Study population and dataset

The study population of the present study was the 'Eunice Kennedy Shriver' NICHD Fetal Growth Studies–Singletons. The NICHD Fetal Growth Studies–Singletons is a prospective longitudinal cohort of 2802 pregnant woman without major pre-existing medical conditions from four self-identified race/ethnic

groups (i.e. non-Hispanic White, non-Hispanic Black, Hispanic and Asian or Pacific Islander) recruited from 12 clinic sites in the USA and followed through delivery. Details about the study design and data collection methods have been previously reported (45,46). The inclusion criteria were age 18–40 years, viable singleton pregnancy and planning to give birth at the participating health facilities. Exclusion criteria included previous history of poor obstetric outcomes, pre-existing chronic medical and psychiatric conditions, smoking in the previous 6 months or use of illicit drugs during the previous 12 months, and consumption of ≥1 alcohol drink daily. As part of the study, 312 women provided placenta samples at delivery. Placental samples were obtained within 1 h of delivery, and biopsies measuring $0.5 \times 0.5 \times 0.5$ cm were taken directly below the fetal surface of the placenta. Samples were placed in RNALater and frozen for molecular analysis. The study was approved by institutional review boards at NICHD and each of the participating clinical sites. Written informed consent was obtained from all study participants.

Placental DNA methylation

DNA from placental biopsies was extracted and methylation was measured using Illumina's Infinium Human Methylation450 Beadchip (Illumina Inc., San Diego, CA). Quality control filters applied on methylation probes and samples has been previously described (33,60). A total of 301 samples and 409 101 CpGs that passed quality control were included in the present study.

Placental genotyping

Placental DNA samples were genotyped using HumanOmni2.5 Beadchips (Illumina Inc.), followed by initial data processing using Illumina's Genome Studio, as previously described (33). Standard GWAS quality control filters were applied on the genotype data as previously described (60), and the remaining 301 samples and 1 337 250 autosomal SNPs were included in the present study.

Prenatal environmental factors

Cardiometabolic-, psychosocial-, demographic- and obstetric-related maternal prenatal factors were included as prenatal environmental explanatory factors for DNA methylation in placenta. These include maternal depression status measured using the Edinburgh Postnatal Depression Scale; pre-pregnancy BMI (continuous in kg/m^2); total gestational weight gain (continuous in kg); change in systolic and diastolic blood pressure between 1st and 3rd trimester measurements (continuous in mmHg); first trimester plasma total cholesterol (high as ≥200 mg/dl versus normal as <200 mg/dl); first trimester plasma triglycerides (high as ≥150 mg/dl versus normal <150 mg/dl); first trimester plasma high-density lipoprotein cholesterol (low as 50 mg/dl versus normal as >50 mg/dl); first trimester plasma low-density lipoprotein cholesterol (high as ≥100 mg/dl versus normal as <100 mg/dl); parity (nulliparous, multiparous); mode of delivery (cesarean after labor, cesarean without labor, outlet vacuum and spontaneous vaginal); and maternal age (continuous in years).

Identification of VMRs

The VMR detection approach adapted in this study has been described in a previous study (44). First, for each of the 409 101 CpGs, an MAD score was calculated as the median of the absolute deviation of each individual's methylation beta value from the CpG's median methylation beta value. Using a MAD score cut-off greater than the 90th percentile to represent the most variable CpGs, 40 910 CpGs were selected. Next, each candidate VMR was defined based on a cluster of two or more CpGs that were ≤1 kb apart in genomic distance. In each VMR, the CpG with the highest MAD score was defined to be a VMR tag-CpG.

Statistical analysis

We performed multiple linear regression using the lm function in R 4.0 (https://www.r-project.org/). To identify the best model that explains the highest variability of each VMR tag-CpG ($n = 5850$) representing their corresponding VMR ($n = 5850$), we analyzed four models. The four models were environment model (E), genotype model (G), gene–environment additive model (G + E) and gene–environment interactive model (G × E). For each model evaluated at a particular VMR tag-CpG, the outcome variable (Y) was the methylation values (β values) and the explanatory variables depending on the model being evaluated were *cis*-genotypes (G model: SNPs 1 Mb up- and downstream from the VMR tag-CpG), 12 prenatal environmental factors (E model) or a combination of both in an additive (G + E model) or multiplicative manner (G × E model). The genotypes were coded as 0, 1 or 2, representing the number of minor alleles. For computational efficiency, analyses were performed on a pruned set of 204 571 uncorrelated SNPs after linkage disequilibrium (LD) pruning as implemented in PLINK (i.e. r^2 threshold of 0.2, and a sliding window of 50 SNPs by skipping five SNPs between consecutive windows) (61). No evidence of multicollinearity was observed among the environmental factors. Linear regression was performed for each model with adjustment for the top four genotype principal components (PCs), four methylation PCs, maternal education status (high school or below, above high school), maternal employment status (employed, unemployed), maternal medical insurance status (has insurance, no insurance), ethnicity and fetal sex (C). Specifically, given some random noise (ε), the regression equations for each model at ith VMR tag-CpG can be specified as follows:

$$\text{G Model}: Y_i = (cis\text{-SNP})_j + C + \varepsilon,$$

where $i = 1, 2, \ldots, N$ (VMR tag-CpGs); $j = 1, 2, \ldots, M$(*cis*-SNPs)

$$\text{E Model}: Y_i = (\text{Environment})_k + C + \varepsilon,$$

where $i = 1, 2, \ldots, N$(VMR tag-CpGs); $k = 1, 2, \ldots, S$ (Environments)

$$\text{G + E Model}: Y_i = (cis\text{-SNP})_j + (\text{Environment})_k + C + \varepsilon,$$

where $i = 1, 2, \ldots, N$ (VMR tag-CpGs); $j = 1, 2, \ldots, M$ (*cis*-SNPs); $k = 1, 2, \ldots, S$ (Environments)

$$\text{G} \times \text{E Model}: Y_i = (cis - \text{SNP})_j + (\text{Environment})_k + (cis\text{-SNP})_j \times (\text{Environment})_k + C + \varepsilon,$$

where $i = 1, 2, \ldots, N$ (VMR tag − CpGs); $j = 1, 2, \ldots, M$ (*cis* − SNPs); $k = 1, 2, \ldots, S$(Environments).

To evaluate fit of each model under a given VMR tag-CpG, we calculated three metrics AIC (48), Akaike's deltas (62) (Delta AIC) and RMSE. AIC was calculated using the AIC function in R.

Delta AIC for each VMR tag-CpG was calculated as the difference between the AIC of the best model and the AIC of the next best model. RMSE is the difference between the value predicted by a model and the observed value and was calculated using the rmse function of the Metrics v0.1.4 package in R. The model with highest Delta AIC margin depicts a good separation in prediction capability of the model as compared with other models, and a model with lower RMSE values depicts a better fit to the observed data.

For each VMR tag-CpG, a model was chosen as the best model if it had the lowest AIC, lowest RMSE and highest Delta AIC value among all four models. For each model to determine the statistical significance of association of a particular *cis*-SNP, environment, or their additive and interactive effect with methylation values of the respective VMR tag-CpG, *P*-values were calculated using the summary function in R. To account for multiple testing, *P*-values were adjusted for false discovery rate (FDR) using Benjamini–Hochberg procedure (63) [using p.adjust(method = 'BH') function in R]. All significant associations were determined based on FDR-adjusted *P*-values <0.05.s

Functional annotation

CpGs within VMRs and tag-CpGs in different best models were annotated based on their overlap with different functional regions such as CpG islands, gene-centric locations, TFBS and 15-state chromatin marks. CpG island annotation file was obtained from (HumanMethylation450v1.2ManifestFile); chIP-seq narrow peaks TFBS annotation file was obtained from (The ENCODE project Consortium 2012) using the online data repository (http://zwdzwd.github.io/InfiniumAnnotation#current) (64) containing 171 transcription factors; core15-state ChromHMM annotation file for 127 samples was obtained from the Roadmap epigenomics (65) using the online data repository (http://zwdzwd.github.io/InfiniumAnnotation#current) (64). Gene-centric annotations were performed using the R package annotatr (66). The SNPs associated with VMR tag-CpGs in different best models were also annotated based on their overlap with different functional regions such as gene-centric locations, TFBS and 15-state chromatin marks. For gene-centric locations, annotatr (66) was used, for core15-state chromatin marks, ENCODE core15-state ChromHMM (65) annotation file was used, and for TFBS annotation, the data was downloaded from (ftp://ccg.epfl.ch/snp2tfbs/mapped_files/annotated/) (67), which consisted of data on 195 different transcription factors.

Functional enrichment analysis

Enrichment analysis was performed using a hypergeometric test based on the number of CpGs within VMRs or VMR tag-CpGs that overlapped with different functional regions or chromatin states as compared with other CpGs on the 450 k array. Similarly, for SNPs associated with VMR tag-CpGs, enrichment analysis was performed using a hypergeometric test based on the number of SNPs that overlapped with different functional regions or chromatin states as compared with all other LD-pruned SNPs ($r^2 \geq 0.2$) that were not associated with the VMR tag-CpGs in the best models. A CpG or SNP was determined to be enriched for a region or state if the test *P*-values were significant at 5% level. Additional online software like FUMA (52) (https://fuma.ctglab.nl/) was used to perform enrichment analysis based on the nearest gene sets obtained from VMR tag-CpGs in different best models, and eFORGE (68) (https://eforge.altiusinstitute.org/) was used for enrichment analysis of CpGs within VMRs on DNase I hypersensitive sites in various cell types, cell lines and tissues. Briefly, the online platform of FUMA and eFORGE takes a list of genes and CpGs and annotates them in a biological context. To determine enrichment for complex diseases of CpGs in different best models, data containing disease associations of all EWAS variants from published studies was downloaded from the EWAS atlas (50) (https://bigd.big.ac.cn/ewas). To determine enrichment for complex diseases of SNPs associated with CpGs in best models, data containing disease associations of all GWAS variants from published studies was downloaded from the GWAS catalog (51) of the National Human Genome Research Institute (https://www.ebi.ac.uk/gwas/docs/related-resources). Lastly, we evaluated overlap between the SNPs associated with the CpGs within VMRs and known blood meQTLs at five different life stages (birth, childhood, adolescence, during pregnancy and middle age) using the ARIES meQTL database (49) (http://mqtldb.org/search.htm).

Supplementary Material

Supplementary Material is available at *HMG* online.

Acknowledgements

The authors wish to thank research teams at all participating clinical centers (which include Christina Care Health Systems; Columbia University; Fountain Valley Hospital, California; Long Beach Memorial Medical Center; New York Hospital, Queens; Northwestern University; University of Alabama at Birmingham; University of California, Irvine; Medical University of South Carolina; Saint Peters University Hospital; Tufts University; and Women and Infants Hospital of Rhode Island). The authors also acknowledge the Wadsworth Center, clinical trials and surveys corp. and the EMMES Corporations in providing data and imaging support. This work utilized the computational resources of the National Institutes of Health high performance computing Biowulf cluster (http://hpc.nih.gov).

Conflict of Interest statement. None declared.

Funding

The Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, including American Recovery and Reinvestment Act funding via contract numbers (HHSN275200800013C, HHSN275200800002I, HHSN27500006, HHSN275200800003IC, HHSN275200800014C, HHSN275200800012C, HHSN275200800028C, HHSN275201000009C, HHSN27500008). Additional support was obtained from the National Institute on Minority Health and Health Disparities and the National Institute of Diabetes and Digestive and Kidney Diseases.

Data Availability

The placental DNA methylation data are available through dbGaP with accession number phs001717.v1.p1.

Clinical Trial Registration

ClinicalTrials.gov, NCT00912132.

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