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Brief Report

Maternal genome-wide DNA methylation profiling in gestational diabetes shows distinctive disease-associated changes relative to matched healthy pregnancies

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Abbreviations

GDM Gestational diabetes

KEGG Kyoto encyclopedia of genes and genomes

T2DM Type 2 diabetes mellitus

QUIN Quinolinic acid

SNP Single-nucleotide polymorphism

SWAN Subset-quantile within array normalization

Supplemental Material

[Supplementary Icon] Supplemental data for this article can be accessed on the publisher's website.

Abstract

Several recent reports have described associations between gestational diabetes (GDM) and changes to the epigenomic landscape where the DNA samples were derived from either cord or placental sources. We employed genome-wide 450Karray

analysis to determine changes to the epigenome in a unique cohort of maternal blood DNA from 11 pregnant women prior to GDM development relative to matched controls. Hierarchical clustering segregated the samples into two distinct clusters comprising GDM and healthy pregnancies. Screening identified 100 CpGs with a mean β-value difference of ≥0.2 between cases and controls. Using stringent criteria, 5 CpGs (within *COPS8*, *PIK3R5*, *HAAO*, *CCDC124,* and *C5orf34* genes) demonstrated potentials to be clinical biomarkers as revealed by differential methylation in 8 of 11 women who developed GDM relative to matched controls. We identified, for the first time, maternal methylation changes *prior* to the onset of GDM that may prove useful as biomarkers for early therapeutic intervention.

Keywords: gestational diabetes, epigenetics, fetal programming, biomarker, 450K array

Introduction

Gestational diabetes (GDM) is a pregnancy-specific endocrine disorder with a prevalence of 3.5-14%.[1] Due to the worldwide obesity epidemic and recently modified diagnostic criteria, GDM is increasingly prevalent.[2] It occurs because of a mismatch between insulin production and requirement, leading to maternal hyperglycemia. Since glucose is able to cross the placenta, whereas insulin is not, the fetus is also exposed to hyperglycemic conditions. Women with GDM are at increased risk of caesarean section and stillbirth compared with healthy women.[3, 4] They are also more likely to develop type 2 diabetes (T2DM), dyslipidemia, and cardiovascular disease in later life,[5-7] while their offspring have an increased long-term risk of obesity and diabetes.[2]

Epigenetic modifications, which may be causal of or associated with changes in gene expression, offer significant promise for understanding the underlying mechanisms of GDM. Indeed, and as an example, epigenetic changes in T2DM have been reported in genes involved in metabolism.[8-13] Since maternal epigenetic modifications are known to contribute to fetal programming,[14] recent studies have investigated the role of epigenetic alterations in offspring exposed to maternal hyperglycemia and found positive associations.[15-19] Furthermore, previous studies suggest that epigenetic

modifications may play a role in the pathogenesis of GDM.[20, 21]

Epigenetic research in GDM has largely used targeted (candidate gene) approaches.[15, 16, 18, 19] To date, only two studies have utilized genome-wide methodology[17, 22] and in these cases investigators examined placenta and cord blood samples from GDM pregnancies. Differentially methylated genes were identified between GDM and healthy pregnancies,[17, 22] which provide evidence for the involvement of these genes and/or their differential methylation in GDM. However, there have been no genome-wide studies examining methylation differences between *maternal* tissue samples from GDM and healthy pregnancies. We decided to focus on maternal epigenetic profiles, as they would facilitate the assessment of the *in utero* environment and allow identification of predictive biomarkers that would enable targeted intervention to high risk groups.

On the basis of the current literature, we hypothesized the presence of pre-existing epigenetic markers in women who subsequently go on to develop GDM. In this study, and for the first time in this disease, we interrogated genome-wide DNA methylation in peripheral blood samples collected from women *prior to* the development of GDM and relative to matched healthy controls that did not develop GDM. Using this discovery

cohort, our aim was to identify candidate genes with future promise as potential biomarkers for the prediction of GDM in early pregnancy.

Results

Our initial data analyses focused on comparison of our data in antenatal samples with the two recent genome-wide studies that investigated cord blood and placental tissue samples.[17, 22] We compared our data with those of Finer et al.[22] and Ruchat et al.[17] separately due to the different approaches used for data processing by each study (**Figure 1**). Using the filtering criteria shown in step 1A of **Figure 1**, comparison of our data with those of Finer et al.[22] identified 4,755 differentially methylated CpGs (representing 2,236 genes) where the mean β-value difference between the GDM and healthy groups was >0.05 and statistically significant (P<0.05). In contrast, comparison with the data of Ruchat et al.[17] (step 1B of **Figure 1**) identified 1,035 CpGs (representing 633 candidate genes). We also performed the same comparison after applying multiple testing adjustment using the false discovery rates, which showed no overlap of our data with these two studies.

As shown in **Figure 2A**, by comparing the 2,236 genes identified as differentially methylated in our study with those reported by Finer et al.,[22] two genes were common

between maternal blood, umbilical cord, and placenta: Hook Microtubule-Tethering Protein 2 (*HOOK2*) and Retinol Dehydrogenase 12 (*RDH12*). Conversely, and as summarized by the Venn diagram in **Figure 2B**, there were no genes common to all three tissue types when we compared our data with that of Ruchat et al.[17]

The 4,755 CpGs initially identified as differentially methylated were then subjected to further filtering (steps 2 and 3, **Figure 1**). Using this approach, we identified 100 unique CpGs (comprising 66 genes) that were differentially methylated between GDM and healthy pregnancies (the full annotated list is shown in **Table S1**). None of these CpGs have an annotated single-nucleotide polymorphism (SNP) in the probe. Closer examination of the 100 CpGs revealed that the majority (53%) were hypomethylated in GDM relative to healthy pregnancies. The observed differences in mean β-value showed a maximum difference of 0.38. The frequency and DNA methylation of these differentially methylated CpG sites in relation to their genomic location and CpG islands are shown in **Figure S1**. Of the differentially methylated CpGs, 45% were associated with a CpG island, shelf, or shore (**Figure S1C)**.

Hierarchical clustering was performed to determine whether the methylation patterns in these 100 CpGs can be used to distinguish between GDM and healthy pregnancies.

The heatmap in **Figure 3** illustrates that there are distinctive methylation patterns between GDM and healthy pregnancies, which segregate samples into two distinct groups comprising those from GDM and healthy populations. The slide type did not cause the clustering; therefore, our results were not due to batch effects. Calculation of the genomic inflation factor before and after normalization steps showed that removal of SNP containing probes and subset-quantile within array normalization (SWAN) by the *minfi* package reduced the genomic inflation.[23-25] Pre-normalization λ was estimated to be 1.189 (standard error of the estimation = 9.461×10^{-5}); after normalization, the estimated λ was reduced to 1.132 (standard error of the estimation = 7.461×10^{-5}). The remaining genomic inflation suggests that mild confounding stratification factors remain unaccounted for in the data.

Enrichment of gene ontology terms and biological pathways within the 66 genes associated with differentially methylated CpGs were assessed using DAVID online software[26] and identified 11 overrepresented pathways, with the top three (ranked by *P*-value) involved in cell adhesion molecules, type 1 diabetes mellitus, and keratin pathways. However, enrichment of these pathways was not statistically significant following adjustment for false discovery rates (**Table S2**).

Finally, we examined the absolute β-value differences across all 11 matched pairs. Using this stringent criteria, in 5 of the 100 CpGs identified, at least 8 of the 11 GDM pregnancies showed β-value differences of >0.2 relative to matched controls. The 5 CpGs comprised of 5 genes (*COPS8*, *PIK3R5*, *HAAO*, *C5orf34,* and *CCDC124*) and their functions are shown in **Table 1**.

Discussion

We describe for the first time, genome-wide DNA methylation changes in maternal blood *prior* to the diagnosis of GDM. We identified 2 differentially methylated genes that shared identity with genes previously described in studies that interrogated placenta and umbilical cord blood samples and, in these cases, using the same array platforms.[17, 22] Furthermore, using stringent filtering criteria, we identified 100 unique CpGs that segregated GDM and healthy pregnancies into distinct groups upon hierarchical clustering.

The strength of our study, in contrast to previous studies, is that we carefully matched each GDM pregnancy to a healthy one to ensure the samples were comparable.[17, 22] Furthermore, as all samples were taken prior to development of pregnancy complications, there was limited sampling bias.

We were able to compare our data to those from two recent genome-wide studies in GDM using cord blood and placenta tissue.[17, 22] Comparative analysis with Finer et al.[22] showed that *HOOK2* and *RDH12* were common to maternal blood, placenta and cord blood. *HOOK2* codes for a linker protein that mediates binding to organelles and is responsible for morphogenesis of cilia and endocytosis.[27, 28,29] *RDH12* encodes a retinal reductase, which also plays a role in the metabolism of short-chain aldehydes.[27, 30] In terms of KEGG orthology, it is involved in metabolic pathways as well as retinal metabolism.[31] These two genes, therefore, may represent important candidates for further study.

The disparity of candidate genes when comparisons are made to the previous studies might reflect the different data filtering criteria used by Ruchat et al. [17] and Finer et al.[22] Using the Finer et al. criteria, many of the differentially methylated CpGs are likely to have β-value differences <0.2, which could be difficult to reproduce either by alternative methodologies, such as pyrosequencing or in replication studies using independent patient cohorts. Moreover, we used a distinct patient population to the other two studies. We used samples from women *prior* to the development of their GDM, while both Ruchat et al.[17] and Finer et al.[22] used samples from women with

established GDM. Furthermore, we used maternal blood samples, rather than placenta and cord blood samples. These disparities may have contributed to the differences in the absolute numbers of CpGs/genes identified.

Further analysis of our cohort identified 100 independent CpGs (comprising 66 genes), which were found to cluster GDM and healthy pregnancies separately. Reassuringly, these CpGs have no annotated SNPs in the probe. Enrichment of gene ontology terms and biological pathways of these 66 genes showed enrichment for genes involved in cell adhesion, type 1 diabetes mellitus, and keratin pathways.[26, 32] Although the enrichment was not statistically significant following adjustment for false discovery rates, these are promising candidates, which are worth examining to elucidate the biological mechanisms behind GDM. In future work, it will be important to verify, in larger independent cohorts, the candidates identified herein and to determine the impact of differential methylation. This may in the future improve the understanding of GDM pathogenesis and aid in the development of therapy.

The design of this pilot study was to generate a list of genes of interest using a relatively small number of samples. In order to avoid type II errors (false negatives), we used uncorrected *P*-values to identify potential candidates in the preliminary

screening. We then applied more stringent methodology (steps 2-4 of Figure 1) to identify candidate genes. A potential limitation of our study is the possibility of genomic inflation. Mild confounding stratification factors, such as changes in composition of blood during the pregnancy, the time of blood sampling, and parity, may have inflated the data. Therefore, we further validated the array data using an independent method with pyrosequencing in order to confirm our findings.

On closer inspection, 8 of 11 women who subsequently developed GDM showed differential methylation at 5 CpGs (consisting of *COPS8*, *PIK3R5*, *HAAO*, *CCDC124,* and *C5orf34* genes) relative to matched controls. *COPS8* encodes a regulator of multiple signaling pathways.[27, 33] It is involved in protein binding and negative regulation of cell proliferation.[33, 34] The PIK3R5 protein has important roles in cell growth, proliferation, motility, differentiation, survival, and intracellular trafficking.[27, 35-37] The HAAO protein catalyzes the synthesis of quinolinic acid (QUIN). Increased cerebral levels of QUIN may participate in the pathogenesis of neurologic and inflammatory disorders, which may be mediated by HAAO.[27, 38] This unique epigenetic signature may form the basis of future biomarker studies using a larger validation cohort. The CCDC124 protein is involved in cell cycle and division.[39] *C5orf34* encodes for a protein that is highly conserved across species; however, its function remains

uncharacterised.[27]

In summary, for the first time, using a genome-wide approach in maternal blood, we have identified maternal methylation changes *prior* to the diagnosis of GDM. As a discovery-based study, our findings may prove useful towards developing simple biomarkers for predicting GDM, thus facilitating intervention strategies in the early antenatal period to improve the health of the mother and baby, both during pregnancy and in the long-term.

Materials and Methods

Patients

Peripheral blood samples were obtained from women prospectively recruited at the University Hospital of North Midlands, UK, between 12-16 weeks gestation, prior to the diagnosis of any pregnancy complications as part of the EFFECT-M study.[40] At the end of pregnancy, we identified 11 women who had GDM and individually matched each one with a healthy woman who had a normal pregnancy. They were matched in terms of age, body mass index, ethnicity, smoking status, medications and folate supplementation (**Table S3**). The study was approved by the West Midlands (Black Country) Research Ethics Committee (REC reference no. 08/H1204/121).

Genome-wide DNA methylation profiling

We performed genome-wide analysis of DNA methylation using the Illumina HumanMethylation450 BeadChip (450K) array, which examines over 480,000 individual CpG sites. We first extracted genomic DNA from blood samples collected into potassium EDTA using standard phenol/chloroform procedures. Next, samples were sodium bisulfite converted [41] and hybridized to arrays according to Illumina recommended protocols that we have previously described.[42] Methylation at individual CpGs is reported as a methylation β-value, which is a quantitative measure of methylation for each CpG site with range between 0 (no methylation) to 1 (completely methylated).

Validation by sodium bisulfite pyrosequencing

A technical validation between array β-values and methylation levels was determined by sodium bisulfite pyrosequencing in all 22 samples. To increase template quantity for pyrosequencing assays, whole genome amplification of bisulfite-converted DNA followed by touchdown PCR were performed as previously described.[42] A PyroMark Q24 instrument was used to run pyrosequencing assays according to the manufacturer's instructions (Qiagen). Analyses of Pyrograms were conducted on the

PyroMark Q24 software (v 2.0.6., build 20; Qiagen). Seven CpGs representing 5 genes were chosen to provide a range of β-values. These demonstrated a strong positive correlation between β-values and percentage methylation by bisulphite sequencing (Spearman's r = 0.92, **Figure S2**).

Data analysis

Each array passed quality control assessment based on the performance of internal array controls. Initial processing, probe type correction and assessment of array data was conducted using the *minfi* package and SWAN.[23, 24] Probes with known SNPs were removed. All CpGs for which one or more of the 22 samples displayed detection *P*-values > 0.05 (indicating an unreliable site) or presented with missing β-values were excluded. The genomic inflation factor (λ, the ratio of the median of the observed distribution of the test statistic to the expected median) was calculated using the *estlambda* function of GenABEL.[25]

We filtered the data using criteria shown in **Figure 1** to identify differentially methylated sites between GDM and healthy pregnancies. In step the first analysis, we elected to use a minimum β-value difference of 0.05, in part to permit comparisons with a recent report describing DNA methylation in placenta and umbilical cord blood

from GDM pregnancies also using the 450K array platform (step 1, Figure 1).[22] The genes identified as differentially methylated were obtained from the supplementary data of this particular publication. We also compared our data with a separate cohort of placenta and umbilical cord blood samples from GDM pregnancies.[17] We obtained their list of differentially methylated genes through personal communication with the corresponding author of the publication. Further filtering steps were applied to facilitate a more stringent analysis. To reduce the number of non-variable sites to improve the statistical power of subsequent analyses, we removed all sites with β-values ≥0.8 and ≤0.2 in all 22 samples (step 2, Figure 1). This is an approach that has been used by our group as well as by others.[41-44] As described previously by our group, we consider it a more robust methodology to remove from the data set CpGs that failed in any one of the samples, instead of eliminating specific failed CpGs from specific samples.[42] We retained only those CpGs that had a mean β-value difference of ≥0.2 (step 3, Figure 1). Finally we examined the absolute β-values in each matched pairs. We used a cut-off of ≥0.2 mean β-values difference to identify CpGs with considerable methylation differences.

Hierarchical clustering was performed utilizing Genesis software (v1.7.6) using Euclidian distance and average linkage criteria.[45] Enrichment of gene ontology terms

and biological pathways within the genes associated with differentially methylated CpGs were assessed using DAVID online software. [26, 32]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Table 1. Annotation for the 5 genes differentially methylated in 8 of 11 matched pairs, as determined by genome-wide DNA methylation analysis. *The official gene symbol, gene name and stated function were retrieved from the NCBI Gene database (accessed September 2015). **The absolute β-value difference range is the minimum to the maximum value of the individual absolute β-value differences for each differentially methylated CpG.

Gene symbol*	**Absolute β-value difference range****	**Gene name***	**Functional summary**
COPS8	0.05-0.84	Constitutive photomorphogenic homolog subunit 8	Regulator of multiple signaling pathways
PIK3R5	0.02-0.82	Phosphoinositide-3-kinase, regulatory subunit 5	Cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking
HAAO	0.02-0.77	3-hydroxyanthranilate	Catalyses the

		3,4-dioxygenase	synthesis of quinolinic acid (QUIN), which is an excitotoxin that may participate in the pathogenesis of neurologic and inflammatory disorders
CCDC124	0.01-0.79	Coiled-coil domain containing 124	Cell cycle, cell division
C5orf34	0.01-0.77	Chromosome 5 open reading frame 34	Unknown, but sequence is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, and zebrafish

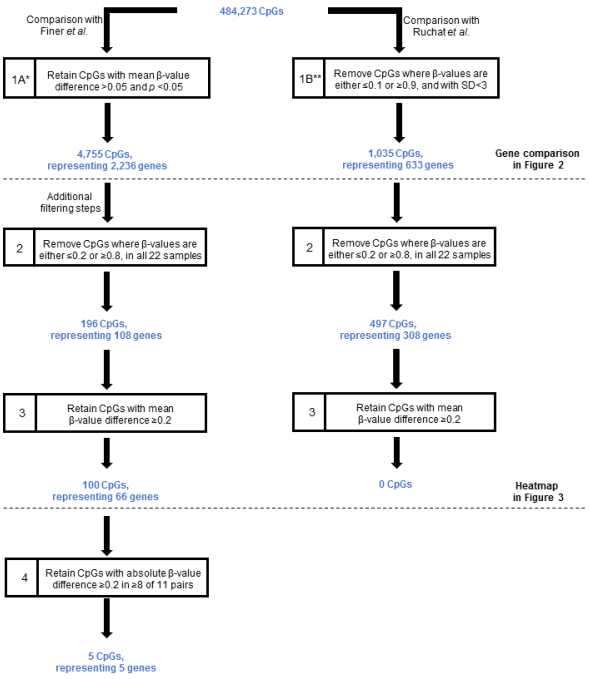

Figure 1.

Figure 1. Filtering criteria for the identification of CpGs differentially methylated between GDM and normal pregnancies. The starting number of CpGs (484,273) was derived through the removal of CpGs with high detection values (p >0.05) and those with missing β-values in any one of the 22 samples, as described in the Materials and Methods. Horizontal line denotes additional filtering steps. *According to Finer *et al*. criteria.[22] **According to Ruchat *et al*. criteria.[17] GDM, gestational diabetes. SD, standard deviation.

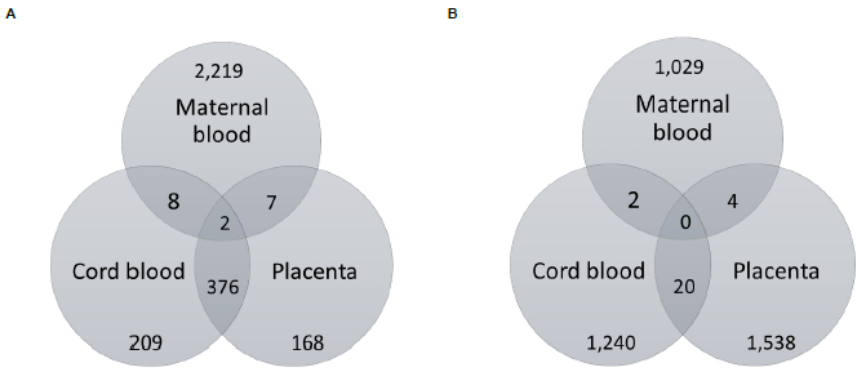

Figure 2.

Figure 2. Venn diagrams illustrating comparison of genes differentially methylated in GDM using maternal blood with those identified in cord blood and placenta of GDM affected pregnancies from the cohorts of (A) Finer *et al*.[22] and (B) Ruchat *et al*.[17], respectively. The genes from our dataset that were common with the other study are shown in dark gray shading. Genes identified as differentially methylated in Finer *et al*.[22] were obtained from Supplementary file 2 of the published article, while the list of differentially methylated genes identified by Ruchat *et al*.[17] was kindly provided through personal communication with the corresponding author of Ruchat *et al*.[17]

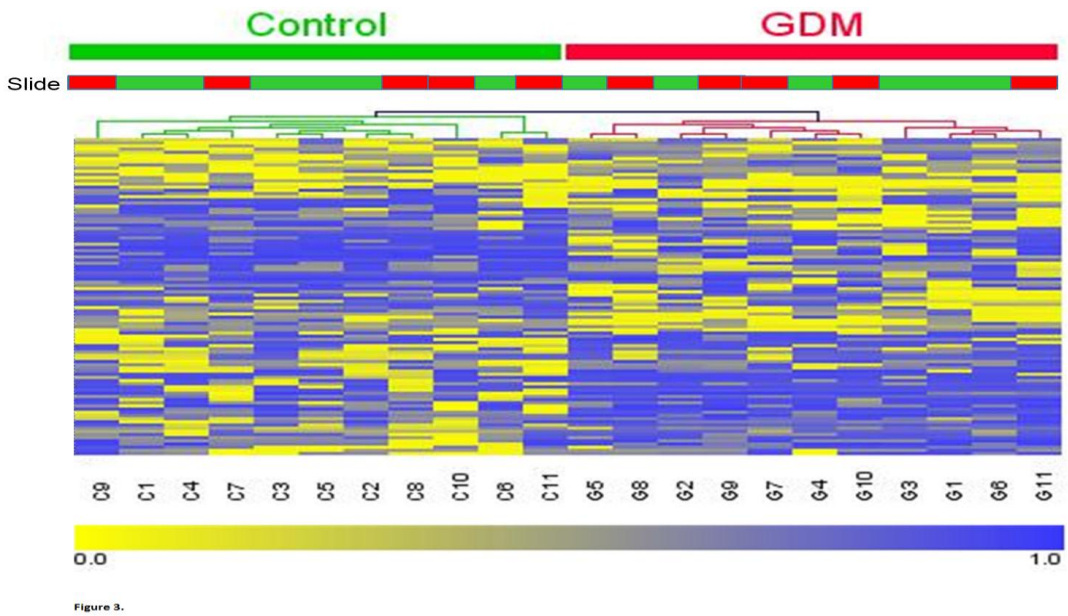

Figure 3. Heatmap and dendrograms showing clustering[45] for the 100 CpGs identified as differentially methylated (mean difference in β-values >0.2) between GDM and healthy pregnancies. DNA methylation across the 100 sites in each of the samples was analysed by hierarchical clustering using the Euclidean distance and average linkage criteria. Each row represents an individual CpG site and each column a different sample. Healthy controls and GDM samples are shown by the green and red bars, respectively. Slide type is also shown with slide 1 in green and slide 2 in red. Color gradation from yellow to blue represents low to high DNA methylation respectively, with β-values ranging from 0 (no methylation; yellow) to 1 (complete methylation; blue). GDM, gestational diabetes.