Original Paper

Distinct DNA Methylomes of Human Placentas Between Pre-Eclampsia and Gestational Diabetes Mellitus

Lan Liu^{a,b} Xiang Zhang^b Can Rong^a Can Rui^b Hui Ji^b Yu-jia Qian^b Ruizhe Jia^b Lizhou Sun^c

^aNanjing Maternal and Child Health Medical Institute, Nanjing Medical University Affiliated Nanjing Maternal and Child Health Hospital, ^bDepartment of Obstetrics, Nanjing Medical University Affiliated Nanjing Maternal and Child Health Hospital, ^cDepartment of Gynecology and Obstetrics, First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Key Words

DNA methylation • Placenta • Pre-eclampsia • Gestational diabetes mellitus

Abstract

Background: The placenta acts not only as a conduit of nutrient and waste exchange between mother and developing fetus but also functions as a regulator of the intrauterine environment. Pre-eclampsia (PE) and gestational diabetes mellitus (GDM) are leading causes of complications during pregnancy. Pathophysiologies show that they are associated with one another. Epigenetics provides a link between environmental factors that have previously been linked to poor pregnancy outcomes and fetal programming. **Methods and Results:** The present study investigated genome-wide DNA methylation changes in PE and GDM compared with control subjects through DNA methylation microarray. We found that the methylation patterns of placentas from PE and GDM women were similar; 64.4% of the annotated genes with differential methylation presented concordant changes between PE and GDM patients. Significantly, the same functional processes were affected by PE and GDM, with cell adhesion and cell differentiation being the most populated clusters and including genes related to carbohydrate metabolism and lipid metabolism. **Conclusion:** Our work showed that of DNA methylation patterns in human placentas are reliably and significantly associated with PE and GDM. DNA methylation status in the human placenta can function as a marker for the intrauterine environment and potentially play a functional role in PE and GDM development.

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L. Liu and X. Zhang contributed equally to this work.

Ruizhe Jia
and Lizhou Sun

Department of Obstetrics, Nanjing Medical University Affiliated Nanjing Maternal and Child Health Hospital, Nanjing 210004 (China); and Department of Gynecology and Obstetrics, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029 (China)
E-Mail cuixianwei198615@163.com and E-Mail lizhou_sun121@hotmail.com

Introduction

Pre-eclampsia (PE) and gestational diabetes mellitus (GDM) are leading causes of complications during pregnancy with serious negative effects on the health of both the mother and her unborn child [1–3]. The pathophysiologies of both illnesses have been shown to be associated with each other [3–6]. It appears that gestational diabetes and pre-eclampsia have several mechanisms in common: altered carbohydrate metabolism causes vascular changes such as arteriosclerosis and a glomerular filtration dysfunction, which results in a predisposition to pre-eclampsia. Some authors have recently discussed gestational diabetes as a risk factor of pre-eclampsia and pre-eclampsia as a possible consequence of gestational diabetes [5]. This theory is supported by a prospective random study that the treatment of gestational diabetes mellitus reduced the rate of pre-eclampsia by 30% [7]. Other authors cite dysfunctions such as increased insulin resistance as the cause of both illnesses [3, 4, 8, 9]. Several authors therefore have reached the conclusion that the state of research is inadequate and ambiguous [4, 8–10] for the difficulty of providing support for these explanations in the form of epidemiological data.

Epigenetics constitutes an important mechanism capable of regulating gene transcription, linking early life events to adult morbidity. Epigenetics entails heritable changes in chromatin that alter gene expression without changing the DNA sequence [11, 12]. DNA methylation is the most commonly studied epigenetic processes which is subject to developmental and environmental regulation and potentially modifiable with appropriate interventions [13]. It is primarily observed in CpG dinucleotides, which are underrepresented in the genome, generally at low density, but may be enriched in the promoter regions of genes [14, 15]. Elevated DNA methylation in such regions is generally associated with gene inactivity whereas a lack of methylation indicates the potential for gene expression.

The placenta function is directly linked to fetal development, and as such, placental dysfunction is implicated in many pregnancy-associated diseases [16]. Aberrant placental gene expression has been linked to pre-eclampsia, intrauterine growth restriction, gestational diabetes mellitus and gestational trophoblastic disease [17–23]. Given the link between gene expression and promoter methylation, it is reasonable to assume that aberrant expression in placental disease is associated with a disrupted DNA methylation profile or other epigenetic change [24]. Furthermore, epigenetics may provide a link between environmental factors that have previously been linked to poor pregnancy outcomes and fetal programming [25, 26]. It is reported that the patterns of genome-wide hypermethylation in promoters are widespread in the chorioamniotic membranes of early onset pre-eclampsia [27] and DNA methylome profiling of maternal peripheral blood and placentas reveal potential fetal DNA markers [28].

The critical role of placenta in responding to intrauterine environment and the environment can influence the pattern of DNA methylation. The present study investigated genome-wide DNA methylation changes of pre-eclampsia and gestational diabetes mellitus to examine their association in human placentas. We found that the overall methylation patterns of placentas from PE and GDM women were similar, 64.4% (3348/5195) of the annotated genes presented concordant methylation changes. Additionally, several genes previously associated with carbohydrate metabolism with concordant methylation alterations were identified. This study will be helpful in exploring gestational diabetes mellitus and pre-eclampsia's having several mechanisms in common from the perspective of epigenetics.

Material and Methods

Human Samples

Human placental tissues were collected at the Maternal and Child Health Hospital (Nanjing, Jiangsu, China) with the approval of Nanjing Medical University, Human Research Ethics Committee. Patients were fully informed of the study and signed informed consent forms of their own accord. Sometimes these

puerperas were in an extremely dangerous situation (such as in a state of unconsciousness) and required immediate surgery. In this situation, consent to collect placental tissues was obtained from a family member instead of the patient. After that, we sought the patients' medical records with the consent of the patients. Placental biopsies were obtained during Cesarean section (CS) from both normal patients (n= 30) and those with pre-eclampsia (n= 27) or gestational diabetes mellitus (n= 28). Pre-eclampsia was defined as a blood pressure of $\geq 140/90$ mmHg taken twice, 6 hours apart, with proteinuria of $\geq 2+$ or ≥ 300 mg in a 24-hour collection. Women were diagnosed with GDM when a 75 g oral glucose tolerance test (OGTT) revealed either a fasting venous plasma glucose level of 5.1 mmol/L glucose and/or at 2 hours a plasma glucose level > 8.1 mmol/L glucose. Pre-eclampsia and gestational diabetes mellitus patients were control-matched with uncomplicated term pregnancies with normotensive subjects and normal glucose tolerance (NGT). Information on maternal reproductive data, delivery characteristics, and infant outcomes were collected from maternal medical records and presented in Table 1.

Placentas were prepared within 10–15 minutes of delivery. Tissues were dissected and repeatedly rinsed until free from blood in chilled PBS (4 °C). Placental lobules were always taken from the central region of the placenta, and the basal plate and chorionic surface were removed with the villous tissue obtained from the middle cross section. Dissected tissue was immediately snap frozen in liquid nitrogen and stored at -80 °C until ready for DNA extraction.

DNA extraction and modification

DNA was extracted from the placenta samples using the QIAmp tissue kit (Qiagen, Hilden, Germany) following manufacturer's protocols. Purified DNA was quantified with the NanoDrop ND1000 spectrophotometer, and 1 μ g of placental DNA was bisulfite modified using the EZ DNA ethylation Kit D5008 (Zymogen, # D5001).

DNA methylation microarray

DNA from placenta samples was fragmented by sonication to 300-1000 bp average size as determined on agarose gels. Enrichment of the methylated double-stranded DNA fraction by MIRA was performed as described previously [29, 30]. The labeling of amplicons, microarray hybridization, and scanning were performed according to the NimbleGen protocol. NimbleGen tiling arrays were used for hybridization (385 K Human CpG Island plus Promoter arrays). The single array design covers all 28,226 University of California, Santa Cruz (UCSC) Genome Browser-annotated CpG islands and the promoter regions for all RefSeq genes. The promoter region covered is 1 kb (-800 to + 200 relative to the transcription start sites). For all the samples, the MIRA-enriched DNA was compared with the input DNA. We conducted three technical replicates for each enrichment and hybridization, after which we averaged the log ratios for each replicate to produce one value per individual per locus.

Microarray Data Preprocessing

The Illumina GenomeStudio Software (version 2011.1) was used to assess quality and extract the DNA methylation signals from scanned arrays. Methylation data were extracted as raw signals with no background subtraction, and the data were normalized to control probes present on the array. Log 2 ratio data were converted into P-value scores using Student's *t*-test. Probes were selected as positive if their P-value scores were above 2 ($P < 0.05$). In addition, the multivariate characteristics (or Mahalanobis distance based on fitted mean vector and variance-covariance matrix) of array control probes supplied by NimbleGen were used to diagnose problems such as poor bisulfite conversion or color-specific problems; none were identified.

Gene Ontology (GO) and pathway analysis

We next used Gene Ontology analysis to investigate if any common functional trends are associated with the genes exhibiting differences from the PE and GDM groups compared with the control group. Those loci exhibiting a significance value of less than $p < 0.05$ from the Student's *t*-test were selected to include only those loci likely to demonstrate a true DNA-methylation difference between groups. We then mapped all gene candidates to GO terms in the database (<http://www.geneontology.org/>), calculating gene numbers for each term. Gene networks and canonical pathways representing key genes were identified using the curated IPA (Ingenuity Pathway Analysis) database according to KEGG, Biocarta, and Reatome, as previously

Table 1. Clinical characteristics of PE, GDM and control women. ^ap<0.05 when compared with control group. ^bp<0.001 when compared with control group. BMI, body mass index; BP, blood pressure; SD, standard deviation

Clinical Features	PE n = 27	GDM n = 28	Control n = 30
Maternal age (mean ± SD)	30.1 ± 2.7	30.8 ± 1.4	29.7 ± 1.8
Maternal BMI (mean ± SD)	32 ± 2.9 ^a	31.3 ± 0.6 ^a	26.3 ± 1.9
Week gestation (mean ± SD)	35.7 ± 0.9 ^a	36.2 ± 0.8 ^a	36.4 ± 0.5
Mode of delivery CS(%)	100 %	100 %	100 %
Birth weight (g) (mean ± SD)	2112.0 ± 454.3 ^b	3920.5 ± 54.3 ^a	3620.5 ± 26.9
BP Systolic (mmHg)	151.3 ± 12.1 ^a	127.8 ± 7.1	117.6 ± 5.6
BP Diastolic (mmHg)	95.2 ± 5.6 ^a	80.3 ± 5.4	78.6 ± 2.1
Proteinuria (g/24 h)	5.3 ± 2.5	N/A	N/A
OGTT fasting	4.9 ± 0.5	5.9 ± 0.3	4.7 ± 0.2
1h	6.8 ± 1.2	10.5 ± 0.3 ^b	5.3 ± 0.3
2h	5.0 ± 0.7	8.6 ± 0.4 ^b	4.9 ± 0.5

described [31]. Those loci exhibiting a significance value of less than $p < 0.05$ from the Student's *t*-test were selected to include only those loci likely to demonstrate a true DNA-methylation difference between groups. The threshold of significance was defined by the P-value and FDR.

Validation of the microarray finding

We further investigated samples from 85 placentas by bisulfite sequencing PCR (BSP), including 30 normal placentas, 28 placentas from women with GDM and 27 placentas from women with PE. Primers were designed with MethPrimer using the loci nominated from microarray analyses. The four pair primers were as follow: Resistin (Forward: TATTGTTTGTGTTAGGGGTTTTTTT, Reverse: AAAAAACAAC TACTACTTACCCTCTC, 163 bp), RBP4 (Forward: TGTATAAGTGGATTTTGA AGGTAGA, Reverse: ACCCTAACTCAATTACCC TACTCTC, 226 bp), GLUT3 (Forward: TAAGGGAAATGTTTATTTTGTGAG, Reverse: CATAAAACAACCA CCAATAACAAC, 192 bp), PPAR α (Forward: GGTTTAGTGGTGTGTTTGTGTA GAT, Reverse: TCAAACTCCTAACTTCAAATAATCC, 165 bp).

PCR amplifications were performed with a standard hot-start PCR protocol in 25 μ l volume reactions containing 3 μ l of sodium-bisulfite-treated DNA, 1 mM primers, and a master mix containing hot-start Taq polymerase (Sigma). PCR conditions were as follows: 95 °C for 10 min, 40 cycles (95 °C/40 s; 50 °C/40 s; 72 °C/40 s), and then 72 °C for 7 min. All PCR reactions were checked on a 1.5% agarose gel to ensure successful amplification. For BSP clone sequence analysis, the PCR products were subcloned into a pMD-19-T vector (Takara Bio, Inc., Japan). Ten to twenty clones were selected for sequencing to identify methylation differences between PE, GDM and control groups.

Statistical analysis.

The statistical significance of the methylation datasets was performed using a one-way ANOVA corrected for multiple comparisons followed by the Student's *t*-test with a significance of $p < 0.05$. Data from the bisulfite sequencing PCR experiments were analyzed with the Student's unpaired or paired *t*-tests where appropriate. Values are presented as mean $\pm$ standard deviation.

Results

Maternal and neonatal characteristics

Table 1 describes the maternal and infant characteristics of the randomly generated training and testing datasets. All recruited women belonged to the same age group, however, the body mass index of patients from PE and GDM groups were significantly different from control group. The duration of gestation was significantly lower in the control group when compared with the pre-eclampsia group and the gestational diabetes mellitus group. The systolic and diastolic blood pressure of pre-eclampsia women was significantly higher than normotensive women. Further, baby weight was significantly higher in the gestational diabetes mellitus group when compared with the control group. GDM and PE women had a

Table 2. Distribution of differentially methylated regions in placentas DNA from PE and GDM. Differential methylation analysis threshold included probes with detection P value < 0.001. ^aPercentage of DMR from the average number of array probes detected per PE sample at p < 0.001 (185,577). ^bPercentage of DMR from the average number of array probes detected per GDM sample at p < 0.001 (185,554). *Probes showing gain or loss of DNA methylation in PE and GDM samples comparison to controls. #DM probes excluding those located at X and Y-chromosomes. &Probes associated with annotated genes. †Probes associated with annotated genes showing concordant changes in methylation in both PE and GDM samples

Gene name	PE	GDM
Differentially methylated regions	8191 ^a	10424 ^b
Increased methylation*	3394	4021
Located in Autosomal CHR#	272	279
Annotated genes&	1738	2021
Co-variation†	1532	
Decreased methylation*	4797	6403
Located in Autosomal CHR#	431	269
Annotated genes&	2140	2644
Co-variation†	1816

Fig. 1. (A) Comparison of the fractions of consistent and inconsistent methylated loci in PE and GDM samples. (B) The breakdown of the Methyl-seq regions into specific annotated genomic elements and binding locations. Coordinates for promoters and other genic elements were calculated using the transcription start sites and gene annotations from the Known Genes file, compiled by the UCSC Genome Browser. (C) Chromosome location distribution of differentially methylated regions in PE and GDM samples. GDM, hypomethylated and hypomethylated regions of GDM placentas. PE-up, hypomethylated and hypomethylated regions of PE placentas.

significantly greater BMI at their first antenatal visit (12 weeks gestation) when compared with the control group. Although fasting OGTT levels were not significantly different between groups, both 1-hour and 2-hour OGTT readings were significantly higher for GDM women. It should be noted that we did not exclude the influence of BMI, whether it has some effects is still unknown.

Table 3. Differentially methylated autosomal genes that co-varied in placentas DNA from PE and GDM

Chromosome	Peak average start site	Peak average end site	log ₂ (GDM/Control)	P-value	log ₂ (PE/ Control)	P-value	Gene name
Methylated regions both down-regulated in placentas DNA from PE and GDM(Both > 3 folds)							
chr7	104696526	104697491	-4	5.3E-05	-4.26	2.3E-05	SRPK2
chr5	76283637	76284586	-3	7.4E-03	-4.12	3.4E-03	CRHBP
chr12	91345874	91346517	-3.37	9.8E-16	-4.08	4.8E-16	CLLU10S
chr11	55757093	55757994	-3.88	2.8E-02	-4.06	1.8E-02	OR5T2
chr13	45577339	45577921	-3.4	4.2E-03	-4.06	3.2E-03	CPB2
chr12	7487837	7488584	-4.05	2.3E-02	-4.05	2.5E-02	CD163L1
chr12	101036295	101037102	-3.28	1.5E-05	-3.86	2.5E-05	NUP37
chr3	162421057	162421765	-3.04	2.3E-03	-3.75	5.3E-03	RBP4
chr1	246880827	246881562	-3.43	4.9E-02	-3.73	3.9E-02	OR2T27
chr2	167700164	167700866	-3.28	2.7E-03	-3.7	2.7E-03	CMYA3
chr12	53809144	53809873	-3.21	4.5E-03	-3.66	4.5E-03	OR9K2
chr2	204509011	204509714	-4.26	1.1E-09	-3.63	5.3E-04	Resistin
chr6	99969288	99969995	-3.22	5.3E-05	-3.62	7.4E-03	C6orf111
chr3	161599340	161600476	-3.29	7.4E-03	-3.59	9.8E-10	IFT80
chr6	28199585	28200288	-5.32	9.8E-13	-3.57	2.8E-02	ZNF435
chr11	18018686	18019564	-3.74	2.8E-02	-3.45	4.2E-03	TPH1
chr6	26127898	26128552	-3.59	3.2E-03	-3.41	3.5E-02	PPARα
chr11	5835631	5836283	-3.32	2.5E-02	-3.36	2.5E-05	OR52E8
chr6	27399778	27400622	-3.08	4.5E-05	-3.36	5.8E-03	FKSG83
chr2	113451639	113452228	-3.12	5.3E-03	-3.34	4.9E-03	IL1F9
chr6	64339091	64340035	-3.78	4.9E-02	-3.33	2.7E-03	PTP4A1
chr6	50124184	50125095	-3.75	4.2E-03	-3.3	2.9E-03	DEFB112
chr13	48854948	48855791	-3.16	5.3E-05	-3.3	4.3E-05	CAB39L
chr2	132099568	132100319	-3.37	7.4E-03	-3.28	5.3E-05	FKSG30
chr3	115494779	115495265	-3.53	9.8E-16	-3.27	7.4E-04	VSIG9
chr15	40288057	40288571	-3.7	2.8E-02	-3.24	9.8E-16	VPS39
chr1	78887319	78888224	-4.15	4.2E-03	-3.19	2.8E-02	IFI44
chr7	113346120	113346824	-3.33	2.5E-02	-3.19	4.2E-03	PPP1R3A
chr16	30364923	30365414	-3.22	2.5E-05	-3.18	2.5E-02	SEPHS2
chr14	23982151	23982604	-3.81	5.2E-03	-3.16	2.5E-05	C14orf124
chr4	177427177	177427991	-3.01	4.9E-02	-3.15	5.3E-04	ASB5
chr4	177427177	177427991	-3.01	2.7E-03	-3.15	4.9E-02	ASB5
chr11	6147947	6148797	-3.6	4.5E-03	-3.14	2.7E-03	OR52B2
chr18	22699776	22700480	-3.4	1.1E-09	-3.12	3.5E-03	AQP4
chr4	2200579	2201234	-3.08	1.1E-12	-3.12	1.3E-09	POLN
chr12	47162495	47163207	-3.05	3.7E-06	-3.12	3.7E-06	C12orf54
chr18	50408694	50409566	-4.21	3.3E-05	-3.11	1.3E-05	C18orf26
chr14	64002798	64003646	-3.82	1.3E-06	-3.11	1.4E-07	AKAP5
chr1	196874047	196874821	-3.51	2.4E-03	-3.1	2.7E-04	PTPRC
chr11	62813206	62814061	-4.15	2.7E-04	-3.09	1.5E-04	SLC22A10
chr1	156954378	156954990	-3.53	3.7E-04	-3.08	2.4E-04	OR6K3
chr3	166278846	166279697	-3.26	1.4E-04	-3.08	1.4E-04	SI
chr14	22634682	22635284	-3.02	1.5E-06	-3.07	3.5E-05	ACIN1
chr3	44728343	44728813	-3.23	1.4E-06	-3.05	2.4E-05	ZNF502
chr11	4563831	4564388	-2.62	5.4E-05	-3.05	8.4E-05	OR5212
chr7	133461864	133462545	-3.43	1.5E-05	-3.04	2.5E-06	FLJ32786
chr11	55362024	55362958	-3.15	1.3E-07	-3.04	1.4E-06	OR5D16
chr10	69126045	69126688	-4.09	2.5E-05	-3.02	1.5E-04	CTNNA3
chr12	10174035	10174905	-3.37	1.7E-07	-3.02	3.7E-06	CLEC7A
chr1	157675789	157676325	-3.76	3.3E-05	-3.01	1.3E-06	OR10J1
chr8	69511912	69512817	-3.63	1.7E-07	-3	1.3E-05	C8orf34
Methylated regions both up-regulated in placentas DNA from PE and GDM(Both > 4 folds)							
chr16	2154227	2156176	4.68	2.7E-05	4.03	3.7E-06	TRAF7
chr8	1720479	1721321	4.4	1.3E-05	4.08	3.3E-04	C8orf61
chr5	140241262	140242338	3.66	2.7E-04	4.17	1.5E-04	PCDHA13
chr2	72227038	72227749	4.37	3.3E-04	4.2	3.4E-05	GLUT3
chr22	18969719	18970878	4.16	1.4E-04	4.22	1.4E-04	LOC643069
chr10	135000492	135001242	4.18	5.4E-05	4.27	1.5E-05	DRD1IP
chr11	1891292	1893649	4.2	1.4E-05	4.28	2.4E-05	TNNT3
chr8	86753857	86755646	4.54	6.4E-05	4.34	8.4E-05	REXO1L3P
chr7	72051109	72051768	4.02	1.5E-06	4.38	2.5E-06	POM121
chr21	13974048	13975959	4.46	1.5E-06	4.38	1.4E-06	LOC441956
chr8	86908165	86910007	4.63	2.5E-03	4.45	2.6E-03	REXO1L1
chr2	853943	855102	5.07	2.7E-03	4.62	2.7E-03	LOC642837
chr11	1849454	1850493	4.36	2.7E-06	4.68	3.7E-06	LSP1
chr15	18823777	18824438	4.79	3.5E-05	4.68	3.3E-05	LOC646122
chr8	86871871	86873718	4.67	2.3E-07	4.69	1.3E-07	REXO1L1
chr10	126906574	126907323	4.11	2.4E-03	4.88	2.7E-03	LOC642622
chr3	140246279	140247062	4.24	1.5E-04	4.9	2.5E-04	FLJ46210
chr8	86741932	86743791	4.43	1.7E-06	4.91	3.7E-06	LOC653760
chr15	33172135	33172784	4.54	4.3E-05	4.92	3.3E-05	LOC441722
chr8	86884061	86885908	4.97	1.3E-07	4.94	5.3E-07	REXO1L2P
chr19	3788785	3789439	4.92	2.4E-03	4.98	2.9E-03	KIAA1086
chr12	129937787	129938451	4.73	2.9E-04	5.06	2.5E-04	LOC390367
chr8	86760698	86762541	4.86	3.4E-05	5.07	3.6E-04	REXO1L1
chr6	101018081	101018925	4.96	1.4E-04	5.07	3.7E-06	SIM1
chr21	44030904	44031563	4.91	5.5E-05	5.14	3.3E-05	D21S2056E
chr6	150327197	150328184	4.61	2.4E-05	5.22	1.3E-07	ULBP1
chr8	6411613	6412267	4.82	8.4E-05	5.27	2.4E-03	ANGPT2
chr1	1254852	1255501	5.1	2.5E-06	5.32	2.5E-04	TAS1R3
chr19	618079	618738	5.13	1.4E-06	5.43	3.4E-04	RNF126
chr17	78606128	78606792	3.88	1.5E-04	5.47	3.7E-06	B3GNTL1
chr16	87759722	87760446	5.46	2.4E-04	5.49	3.4E-04	LOC400558
chr18	75188453	75189122	5.56	2.5E-03	5.57	2.5E-03	ATP9B
chr17	77870432	77871156	5.89	2.7E-03	5.85	2.7E-03	CD7
chr6	31026748	31027714	4.95	1.1E-19	6.26	1.1E-19	DPCR1

Fig. 2. Illustrative CpG methylation levels for GDM and PE in chromosomes 10 and 16.

Fig. 3. Gene ontology analysis of annotated genes showing differential methylation clustering by biological process: (A) functional categorization of PE, (B) functional categorization of GDM.

DNA methylation patterning in human placentas from PE and GDM patients

DNA methylation profiles were obtained on 85 human placenta samples using the NimbleGen DNA Methylation Analysis. We first compared the methylation profiles between PE and GDM to investigate whether placentas could represent a good source from which to profile methylation changes. We detected 8191 loci with differential methylation in placentas from PE patients and 10,424 loci in placentas from GDM cases (Table 2). Further analysis showed that the overall methylation patterns of PE and GDM were similar, with more than 64.4% (3348/5195) of the total autosomal annotated genes reported exhibiting consistent methylation (Fig. 1A).

Fig. 4. Pathway analysis of annotated genes with methylation changes in PE and GDM patients compared with control: (A) functional categorization of annotated genes from PE group, (B) functional categorization of annotated genes from GDM group.

We assigned those regions exhibiting consistent methylation into distinct categories of genomic elements and then analyzed the Methyl-seq data in each sample according to each type of functional element. The annotated genomic elements are (1) promoters, (2) exons, (3) introns, and (4) intergenic regions. In Fig. 1B, we present the degree of DNA methylation for each genomic element in each sample. We observed that regions that include exons and intergenic region domains are more highly unmethylated (less than 16.8% of regions methylated), whereas regions including those occupied promoters and introns are more highly methylated (from 24.5% to more than 45.4% of the sites are methylated, Fig. 1B). Thus, different genomic functional elements vary in their degree of DNA methylation.

Further analysis revealed that these regions with differential methylation are spread throughout the genome and cover all chromosomes (see Fig. 1C). It is remarkable to see that chromosome 1 comprised the largest component of 10.5% with 1118 (PE) and 827 (GDM). There were only 127 (PE) and 118 (GDM) regions on chromosome 13 (1.3%). Illustrative CpG methylation levels for single chromosomes (chr 10 and chr 16) are shown in Fig. 2. As shown in Table 3, 52 hypomethylated regions constituted a significantly greater proportion of these regions; both changed more than threefold in PE and GDM patients, and 37 hypomethylated regions were present at a significantly higher level in PE and GDM patients (abundance changed more than fourfold). All regions involved showed statistically significant changes ($p < 0.05$).

Concordant variation of differentially methylated loci in human placentas from PE and GDM patients

Differential methylation analysis applying the Illumina Custom model was performed after normalization to control probes present in the array and using the control group as reference. We identified 3394 probes in placentas from PE patients and 4021 in placentas

Fig. 5. Site-specific CpG-methylation analysis in selected genes. The percentage of DNA methylation was assessed with bisulfite sequencing for (A) resistin, (B) GLUT3, (C) RBP4 and (D) PPAR α in 27 placentas with PE, 28 placentas with GDM, and 30 normal placentas. P-values (indicated as “*”) were calculated using the Student’s *t*-test. Locations for the differentially methylated regions targeted by bisulfite sequencing assays (regions bounded by vertical lines) are indicated in the box above the plots.

from GDM patients with increased methylation, whereas 4797 probes in placentas from PE patients and 6403 in placentas from GDM patients showed decreased methylation in PE (Table 2). We further refined our analysis to the fraction of autosomal probes showing differential methylation and an association with annotated genes, which corresponded to a total of 3878 genes in placentas from PE patients (1738 with increased and 2140 with decreased methylation) and 4656 annotated genes in GDM patients (2012 with increased and 2644 with decreased methylation, Table 2). Strikingly, 64.4% (3348/5195) of the annotated genes with different methylation presented concordant changes in methylation between PE and GDM patients (1532 loci with increased methylation and 1816 with decreased methylation, respectively, Table 2).

Gene Ontology and Pathway Analysis

A gene-ontological term and canonical pathways investigation were designed to determine whether any common functional trends are associated with the genes exhibiting differences from PE and GDM groups compared with the control group. The top 15 GO categories for each diagnostic group can be observed in Fig. 3, which lists all significant GO categories with a *p* < 0.05. GO categories detected by this analysis include genes involved in the epigenetic regulation of transcription and development. The results showed that the

same functional groups were affected in PE and GDM patients, with cell adhesion and cell division being the more populated clusters and including genes related to carbohydrate metabolism and lipid metabolism, molecular pathways largely implicated in PE and GDM in common.

Ingenuity Pathway Analysis was used to identify pathways and gene networks represented among the sets of protein-coding mRNAs identified in the VSD gene expression signature. In our survey of existing data, annotated genes that exhibited differences from GDM groups compared with the control group mainly involved the following pathways: (1) cell adhesion molecules (CAMs), (2) nicotinate and nicotinamide, (3) metabolism selenoamino acid metabolism, (4) cytokine-cytokine receptor interaction, (5) Wnt signaling pathway, (6) arachidonic acid metabolism, (7) glycolysis/gluconeogenesis, (8) toll-like receptor signaling pathway, (9) calcium signaling pathway, and (10) leukocyte transendothelial migration. The annotated genes exhibiting differences from GDM groups compared with the control group mainly involved the following pathways: (1) cell adhesion molecules (CAMs), (2) GnRH signaling pathway, (3) selenoamino acid metabolism, (4) MAPK signaling pathway, (5) Wnt signaling pathway, (6) arachidonic acid metabolism, (7) apoptosis, (8) toll-like receptor signaling pathway, (9) calcium signaling pathway, and (10) leukocyte transendothelial migration (Fig. 4).

Site-Specific CpG-Methylation Analysis in Selected Genes

The above analyses suggest that a number of methylation changes in placentas are shared between PE and GDM, positioning these genes (Table 2) that co-vary among tissues as candidates for biomarker discovery. To determine the potential association of these co-varying genes between PE and GDM, we tested four genes' loci that have been confirmed as participating in carbohydrate metabolism or lipid metabolism to further verify the microarray approach. We quantitatively measured site-specific CpG methylation upstream of resistin ($n = 15$), GLUT3 (glucose transporter 3, $n = 15$), RBP4 (retinol binding protein 4, $n = 15$), PPAR α (peroxisome proliferator-activated receptor alpha, $n = 15$). We focused primarily on surrounding CpG positions located in or near genomic regions corresponding to specific microarray probes.

Our site-specific CpG analyses show good consistency with data obtained from microarray analysis although the absolute differences observed are generally small. Two examples are shown in Fig. 5 for regions upstream of the genes' resistin and RBP4. Microarray analysis predicted these regions to be hypomethylated in PE and GDM samples. Fig. 5B shows BSP data confirming GLUT3 hypermethylation both in PE and GDM samples compared with controls. BSP data also verified PPAR α hypomethylation in samples from women with GDM. However, there is no significant methylation difference between PE groups and control groups (Fig. 5D).

Discussion

Placental function is directly linked to fetal development and health, and as such, placental dysfunction is implicated in many pregnancy-associated diseases [18]. Accordingly, aberrant placental gene expression has been linked to pre-eclampsia, intrauterine growth restriction, gestational diabetes mellitus and gestational trophoblastic disease [19-25]. In this study, we performed a microarray-based epigenomic scan using human CpG-island plus Promoter microarrays and observed placenta-associated DNA methylation differences in numerous loci. Many genes that have been functionally linked to PE and GDM etiology were included. Consistent with increasing evidence, gestational diabetes mellitus and pre-eclampsia have several mechanisms in common. We identified epigenetic changes in loci-associated pathophysiologies of both illnesses with similar methylation patterns.

We identified 3394 probes in placentas from PE patients and 4021 in placentas from GDM patients with increased methylation whereas 4797 probes in placentas from PE

patients and 6403 in placentas from GDM patients showed decreased methylation in PE (Table 2). We further refined our analysis to the fraction of autosomal probes showing differential methylation and associated with annotated genes, which corresponded to a total of 3878 genes in placentas from PE patients and 4656 annotated genes in GDM patients. Strikingly, 64.4% (3348/5195) of the annotated genes with different methylation presented concordant changes in methylation between PE and GDM patients (Table 2). Consistent with previous reports, although PE and GDM have distinctive clinical and pathophysiologic characteristics, they share some important similarities: both conditions develop during pregnancy, and the clinical syndromes disappear after delivery. Many risk factors such as obesity, elevated blood pressure, dyslipidemia, insulin resistance, and hyperglycemia are associated with both PE and GDM; and patients with a history of PE or GDM have increased risk of developing cardiovascular disease compared to women without such a history [1-22].

Several interesting GO categories are highlighted by our analysis, including several involved in various epigenetic processes, transcription, and development. The top 15 GO categories for each diagnostic group can be observed in Fig. 2, which lists all significant GO categories with a $p < 0.05$. Our results show that the same functional groups were affected in PE and GDM patients, with cell adhesion and cell differentiation being the more populated clusters (Fig. 2); including genes related to carbohydrate metabolism and lipid metabolism, the molecular pathways of PE and GDM are largely implicated in common. Our findings may represent alterations to such pathways related to PE and GDM, potentially linking the two pathological processes with carbohydrate metabolism and lipid metabolism pathways. Additional studies are warranted to expand on these examinations in later life endpoints to clarify the biological mechanisms at play.

It is important to note that we cannot definitively determine if these altered profiles of DNA methylation are a response of the placenta to the intrauterine environment and an independent association between these specific profiles of DNA methylation. Ongoing research is now revealing that gene-regulating methylation events may occur in regions outside of CpG islands such as the region of the regulated gene [32]. More comprehensive approaches including more inclusive arrays and genome-wide sequencing will be required to fully identify all regions contributing to PE and GDM.

We focused our investigation on methylation patterning because of the highly stable nature of DNA methylation marks. Previous studies have shown that labor induces altered expression of genes in the human placenta [33], and although the methylation status is stable, the corresponding gene expression may not be [34]. Therefore, we believe that examination of methylation may reflect changes occurring over the course of in utero development and not only occurring in the final moments of pregnancy and delivery. We used a relatively unbiased, genome-wide method to identify loci whose methylation status in the placenta was most associated with PE and GDM.

Microarray analyses suggest that a number of methylation changes in the placenta are shared between PE and GDM, positioning these genes (Table 2) that co-varied among tissues as candidates for biomarker discovery. To determine the potential association of those genes co-varying between PE and GDM, we tested four gene loci that have been confirmed as participating in carbohydrate metabolism and lipid metabolism to further verify the microarray approach. We quantitatively measured site-specific CpG methylation upstream of resistin, GLUT3, RBP4 and PPAR α . GLUT3 as a high-affinity transporter has been implicated in the extraction of glucose from the fetal circulatory system for storage as glycogen in the placental endothelial cells [35]. GLUT3 upregulation in maternal diabetes is consistent with elevated glycogen depots in the placental endothelium in this condition. Aberrant GLUT3 expression was therefore suggested as contributing to fetal hyperglycemia in maternal diabetes [36]. It may likewise play a role in integrating environmental signals, resulting in altered placental function and GDM. Increased circulating RBP4 levels have been reported in several metabolic complications including obesity, insulin resistance, polycystic ovary syndrome and cardiovascular disease [37]. RBP4 is a physiological constituent of amniotic fluid [38]. Thus, DNA methylation of RBP4 target genes may affect the maternal-

fetal interface, resulting in PE and GDM.

In conclusion, we report genome-wide alterations in DNA methylation associated with PE and GDM pathology in placentas. Although there are many differences in pathogenesis, we detected similar methylation patterns in both tissues. Significantly, we identified a set of genes whose methylation coordinately varies in PE and GDM placentas, with the majority of these loci previously being implicated in both pathologies. This study will be helpful in exploring gestational diabetes mellitus and pre-eclampsia's having several mechanisms in common from the perspective of epigenetics.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (No. 81200442), the Natural Science Foundation of Jiangsu Province, China (No. BK2011106); the Scientific and Technological Personnel Start Projects Foundation of the Nanjing Health Bureau, Jiangsu Province, China (No. QYK11141); and the Selected Member of Nanjing Health Youth Talent Training Project (QRX11209).

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