MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

DNA Methylation Profiling of Placental Villi from Karyotypically Normal Miscarriage and Recurrent Miscarriage

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Miscarriage occurs in 15% of clinical pregnancies. Although chromosomal errors are observed in >50%, causes of karyotypically normal losses are poorly understood. DNA methylation undergoes reprogramming during development and must be appropriately set to maintain a healthy pregnancy. We hypothesize that aberrant DNA methylation may cause karyotypically normal miscarriage, particularly among women experiencing recurrent miscarriage (RM). DNA methylation in first-trimester chorionic villi was assessed in chromosomally normal miscarriages from women with RM ($N = 33$) or isolated miscarriage (M; $N = 21$) and elective terminations (TA; $N = 16$). Differentially methylated candidate loci were identified using the Illumina Infinium HumanMethylation27 BeadChip array. Follow-up bisulfite pyrosequencing at promoter regions showed an increase in methylation in M compared with TA at cytochrome P450, subfamily 1A, polypeptide 2 (*CYP1A2*; $P = 0.002$) and RM compared with TA at *AXL* receptor tyrosine kinase ($P = 0.02$), and a decrease in RM and M compared with TA at defensin β 1 (*DEFB1*; $P = 0.008$). Gene ontology analysis showed an enrichment of imprinted genes ($P = 9.53 \times 10^{-10}$) and genes previously associated with RM ($P = 9.51 \times 10^{-6}$). An increase of outliers at seven imprinted loci was observed in RM (3.9%) compared with M (0%) and TA (0.9%) ($P = 0.02$), with increased average methylation at *H19/IGF2* ICR1 in M samples ($P < 0.0001$). Altered DNA methylation in the placenta at specific loci, and global dysregulation in specific cases, may contribute to or be a consequence of poor placental function in karyotypically normal miscarriage. (*Am J Pathol* 2013, 182: 2276–2284; <http://dx.doi.org/10.1016/j.ajpath.2013.02.021>)

Miscarriage, the spontaneous abortion of pregnancy before 20 weeks' gestation, affects 15% of pregnancies,¹ with higher rates as women approach menopause. Numerical and structural chromosomal errors have long been established as the primary cause of miscarriage, particularly with advancing maternal age.² However, causes of karyotypically normal miscarriages are poorly understood, and these may be particularly important in women experiencing recurrent miscarriage (RM), >50% of whom have euploid losses.³ Although several maternal factors have been implicated in the etiology of RM, including immunological, anatomical, endocrinological, and thrombophilic, half of RM cases remain unexplained.⁴ Other chromosome characteristics, such as epigenetic marks, may be error prone during early development,⁵ but the contribution of such errors to miscarriage has not been well explored.

DNA methylation is an epigenetic mark that involves the addition of a methyl group to cytosine in the context of a CpG dinucleotide. DNA methylation at the promoter regions of genes can influence the level of gene expression by affecting the accessibility of this DNA to transcription factors, histone modifiers, and transcriptional machinery.⁶ During development, changes in DNA methylation are associated with the stable differentiation of cell types from a single totipotent zygote to the embryonic and extraembryonic tissues. However, subtle environmental perturbations during embryonic development or gametogenesis can disrupt the setting of these epigenetic marks.^{5,7} Imprinted genes, those showing

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parent-of-origin differences in gene expression controlled by differentially methylated regions, are environmentally sensitive and may be particularly susceptible to disruptions in DNA methylation.⁸ Many imprinted genes are important in placental development and are frequently involved in the regulation of placental and fetal growth.⁹ Furthermore, DNA methylation may be inherently more variable in the placenta, because of its need to be responsive to a variety of signals in its function as a mediator of exchange between the fetus and mother.¹⁰

Aberrant DNA methylation, arising during embryo development or gametogenesis, has been suggested as a potential cause of pregnancy loss. Extreme DNA methylation values at several imprinted loci were more frequent in the muscle tissue of stillborns and spontaneous abortions than in induced abortions.¹¹ In addition, aberrant hemimethylation and monoallelic expression of the maternal *CBG5* gene, a component of the placental hormone human chorionic gonadotropin, was seen in trophoblasts from two RM cases and one elective termination, but not in healthy pregnancies.¹² Given the importance of imprinted genes in the placenta, they are attractive candidates to test for abnormalities associated with poor placental function. A comprehensive analysis of genome-wide and site-specific differences in DNA methylation in miscarriage and RM is needed to evaluate the frequency and nature of epigenetic errors in early pregnancy.

In this study, we evaluated changes in DNA methylation in chorionic villus samples from the products of conception of women with RM, isolated miscarriage, or elective termination. We hypothesized that placental villi of karyotypically normal miscarriages, particularly those occurring in women with RM, would exhibit aberrant DNA methylation globally and/or at specific loci. By using both microarray and targeted approaches, we assessed the following: i) changes at specific candidate loci between groups, ii) overall changes and individual outliers at imprinted loci, and iii) global alterations in DNA methylation based on long interspersed element 1 (LINE-1) sequences and all CpG sites interrogated.

Materials and Methods

Samples

Placental chorionic villus samples were obtained anonymously from miscarriage samples evaluated through the Embryopathology Laboratory at the BC Children's and Women's Hospital (Vancouver, BC, Canada). Cases were composed of karyotypically normal miscarriage samples from women with a history of recurrent miscarriage (RM; $N = 33$) and women with a single miscarriage (M; $N = 21$). RM was defined as three or more consecutive miscarriages. As part of a routine clinical workup, all miscarriage specimens with culture failure or in which the karyotype was 46,XX, were further assessed with comparative genome hybridization. First-trimester chromosomally normal control samples were separately ascertained from anonymous 8- to

12-week elective terminations (TAs; $N = 16$). Chromosome constitution of TA samples was assessed with multiple ligation-dependent probe amplification. Table 1 describes and compares the demographics for each study group.

The collection of the samples for this study was approved by the University of British Columbia (Vancouver, BC, Canada) Clinical Ethics Review Board.

Array-Based Quantification of DNA Methylation

DNA from placental chorionic villus samples was purified using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and 750 ng of DNA for each sample was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research Corporation, Irvine, CA). A subset of 10 RM and 10 TA villi samples was run independently on the Infinium HumanMethylation27 BeadChip array (Illumina Inc., San Diego, CA). This array interrogates DNA methylation at 27,578 CpG sites throughout the genome and is enriched for sites within gene promoters. Samples and arrays were prepared as per the manufacturer's specifications (Illumina Inc.). Data were normalized to background intensity levels using GenomeStudio software version 2011 (Illumina Inc.). Probes on the X and Y chromosomes ($N = 1092$) and with bad detection P values ($P > 0.01$ in any sample; $N = 424$) were omitted, leaving a total of 26,062 probes for analysis. The array data from this study are publicly available at National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number 16704108).

Targeted DNA Methylation

Candidate CpG sites from four significant Infinium array probes, seven imprinted genes (Table 2), and LINE-1 sequences were assayed using bisulfite pyrosequencing. This was performed on a PyroMark MD machine using the Pyro-Gold SQA reagent kit (Qiagen). Primers were designed using PSQ Assay Design Software version 1.0.6 or were selected from published studies (Table 2). Each 15- μ L PCR reaction contained 1 $\times$ PCR Buffer (Qiagen), 3 mmol/L Gibco dNTPs (Invitrogen, Carlsbad, CA), 0.9 U HotStart TaqDNA polymerase (Qiagen), 6 μ mol/L each of the forward and reverse primers, and approximately 15 ng of bisulfite-converted DNA. Cycling conditions were as follows: 95°C for 15 minutes, 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds (40 cycles), with a final extension of 72°C for 10 minutes. PCR cycling conditions for LINE-1 primers were those from the commercially available LINE-1 assay (Qiagen). DNA methylation of LINE-1 sequences has been used extensively in the literature as a proxy for global methylation, because they are CG rich and distributed widely throughout the genome.¹⁸

Statistical Analysis

A Student's two-tailed t -test was used to compare study group demographics, including maternal age, gestational

Table 1 Comparison of Demographics for the RM, M, and TA Study Groups

Variable	RM group (N = 33)	M group (N = 21)	TA group (N = 16)	P value
Maternal age (years)*	33.7 ± 5.0	31.1 ± 8.7	NA	0.17
Fetal male/female ratio	17:16	11:10	9:7	0.95 [†]
Gestational age (weeks)*	9.5 ± 2.4	12.6 ± 3.2	First trimester	0.001
Gestations	4 (3-9)	1 (1-4)	NA	<0.0001
Parity	0 (0-2)	0 (0-2)	NA	0.48
Miscarriages	3 (3-9)	1 (1-1)	NA	<0.0001

Data are given as median (range) unless otherwise indicated. The Student's two-tailed *t*-test was used to statistically compare groups for each variable, unless otherwise denoted.

*Data are given as means ± SD.

[†]Fisher's exact probability test.

M, single miscarriage; NA, not available; RM, recurrent miscarriage; TA, elective termination.

age, and number of gestations, parity, and miscarriages. The Fisher's exact probability test was used to compare the male/female ratio among the study groups.

For the 26,062 quality Infinium array probes, M-value conversion¹⁹ and color channel normalization were performed using R Statistical Software, version 2.12.0 (The R Project for Statistical Computing, Auckland, New Zealand). Significance Analysis of Microarrays (Stanford University, Stanford, CA) was used on M-values to select significant candidate CpG sites. A false-discovery rate (FDR) of <0.05 was used in conjunction with an absolute difference ($\Delta \beta$) of >0.05 average β values between the RM and TA groups (Supplemental Figure S1). Within the candidate probe list, probes containing single-nucleotide polymorphisms and/or showing bimodal distribution of β values ($N = 3$) or cross hybridizing to multiple locations within the genome ($N = 0$) were eliminated from analysis (Supplemental Table S1).

Gene ontology analysis was completed using ErmineJ version 2.1.21 (<http://www.chibi.ubc.ca/ermineJ>).²⁰ A gene score resampling method was done using $\Delta \beta$ values as the probe score for each of the 26,062 Infinium array probes. The best score for gene replicates was used, with 10,000 iterations to obtain corrected *P* values. In addition to standard biological processes gene ontology groups, custom gene sets included in this analysis were genes previously associated with RM,^{21,22} and imprinted genes,²³ which were further subdivided into maternally and paternally expressed imprinted genes.

Average DNA methylation of target regions, assessed by bisulfite pyrosequencing, was compared between groups using the nonparametric Mann-Whitney ($k = 2$) and Kruskal-Wallis ($k = 3$) tests. The post hoc Dunn's multiple comparisons test was used to further assess which pairwise comparisons were contributing to significance in the Kruskal-Wallis analysis. Correction for multiple comparisons was done using the Benjamini Hochberg FDR method.²⁴ The relationship between DNA methylation (%) by pyrosequencing and Infinium average β values or gestational age was evaluated using linear correlation. A Fisher's exact test was used to compare the number of outliers for DNA methylation at imprinted genes between the RM, M, and TA groups. Principle component analysis (PCA) using DNA methylation (%) values for all first-trimester placental

samples ($N = 70$) at the 12 targeted loci assessed by bisulfite pyrosequencing in this study (Table 2), including LINE-1 sequences, was done to identify outlier samples.

GraphPad Prism software version 4.0 (GraphPad Software, Inc., La Jolla, CA), VassarStats software (<http://www.vassarstats.net>; Vassar College, Poughkeepsie, NY), and R Statistical Software, version 2.12.0 (The R Project for Statistical Computing), were used for statistical analyses and graphing.

Results

Array-Based Quantification of DNA Methylation

The Illumina Infinium HumanMethylation27 BeadChip array quantifies DNA methylation of CpG sites within the proximal promoter regions of almost 15,000 genes throughout the genome. By using a criterion of an FDR <0.05 and $\Delta \beta$ >0.05, 14 differentially methylated candidate CpG sites were identified from the comparison of 10 RM and 10 TA placental samples. Three of these were omitted because of the presence of a single-nucleotide polymorphism in the probe binding region and/or bimodal distribution of β values (suggestive of being influenced by a single-nucleotide polymorphism). Of the remaining 11 candidates, 5 were more highly methylated in cases compared with controls, whereas six were less methylated (Supplemental Table S1).

Four of these candidates were selected for confirmatory follow-up based on functional relevance of the associated gene, including cytochrome P450, subfamily 1A, polypeptide 2 (*CYP1A2*) cg04968473, defensin β 1 (*DEFB1*) cg24292612, adenomatous polyposis coli (*APC*) cg20311501, and *AXL* cg14892768. These sites were further assessed in the larger sample populations of 33 RM, 21 M, and 16 TA samples, with bisulfite pyrosequencing. The correlation between the Infinium β values and percentage methylation measured by pyrosequencing was highly significant ($P < 0.0001$ for all sites) (Supplemental Figure S2). There was a significant difference in methylation at the *CYP1A2* promoter region between groups ($P = 0.002$) (Figure 1A), which post hoc analysis identified as a significant increase in average methylation in M (64.4%) compared with TA (50.6%; $P < 0.01$); although

Table 2 Primers Used for the Assessment of DNA Methylation by Bisulfite Pyrosequencing

Gene/region	Primers	Reference (if applicable)
<i>APC</i>	F: 5'-TTTTTTGTTTGTGGGGATTG-3' R: Biotin/5'-AATCCRACAACACCTCCATTCTAT-3' S: 5'-TTTGTGGGGATTGG-3'	13
<i>PLAGL1</i>	F: Biotin/5'-GAYGGGTGAATGATAAATGGTAGATG-3' R: 5'-TCRACRCAACCATCCTCTTAACCTAC-3' S: 5'-ACRCAACCATCCTCTTA-3'	14
<i>SGCE</i>	F: 5'-TGGTGTGTGTGTYGAAGAAATTTGATTG-3' R: Biotin/5'-CAAACRCRATCTCCACTAAATAC-3' S: 5'-TGTGTGTGTYGAAGAAATTTGAT-3'	15
<i>H19/IGF2 ICR1</i>	F: Biotin/5'-ACAATACAACTCACACATCACAAC-3' R: 5'-TGAGTGTTTTATTTTATAGATGATTTT-3' S: 5'-GTGGTTTGGGTGATT-3'	16
<i>CDKN1C</i>	F: Biotin/5'-TATTATATTATGTTAATTGTGGTTGGG-3' R: 5'-CAACAAACACTAATACACACTAATA-3' S: 5'-AACACTAATACACACTAATACTAAA-3'	NA
<i>KvDMR1</i>	F: 5'-TTAGTTTTTTGYGTGATGTGTTTATTA-3' R: Biotin/5'-CCCACAAACCTCCACACC-3' S: 5'-TTGYGTGATGTGTTTATTA-3'	14
<i>MEG3</i>	F: Biotin/5'-GGTTTATATTGGGAATTAGTTATGT-3' R: 5'-CCCCCAAATTCCTATAACAAATTA-3' S: 5'-AATACTTTTTCCCTAC-3'	NA
<i>SNRPN</i>	F: Biotin/5'-TATGTTTATAGGYGGGGATGTGTG-3' R: 5'-AAAAACCACCRACACAACCTAACCTTAC-3' S: 5'-CAAATACRTCAAACATCT-3'	14
<i>AXL</i>	F: 5'-TTTGAGGAAAGTTTGGTATTTATG-3' R: Biotin/5'-CACTCACCCCTAAAAACCAT-3' S: 5'-TAGGATGGGTAGGGT-3'	NA
<i>CYP1A2</i>	F: 5'-TGGGGATTTGGGTTGAAAATTAG-3' R: Biotin/5'-AAACTTCTTTCCCACTACACACATAA-3' S: 5'-GATTTGGGTTGAAAATTA-3'	NA
<i>DEFB1</i>	F: 5'-GGATTTTAGGAATTGGGGAGA-3' R: Biotin/5'-CCTTAACCTATAACACCTCCCTTCA-3' S: 5'-AGGTTTTTAGAGGTTGGA-3'	NA
<i>LINE-1</i>	F: 5'-TTTTGAGTTAGGTGTGGGATATA-3' R: Biotin/5'-AAAATCAAAAAATTCCTTTC-3' S: 5'-AGTTAGGTGTGGGATATAGT-3'	17

F, forward; NA, not available; R, reverse; S, sequencing.

the RM group was also increased relative to the TA group, this was not significant. *DEFB1* showed a difference in methylation between groups ($P = 0.008$) (Figure 1B), in which both RM (9.3%) and M (7.9%) had marginally decreased methylation compared with TA (11.3%, $P < 0.05$). The result for *APC* was not confirmed in this larger sample set (Figure 1C). Finally, altered methylation was observed at the *AXL* promoter ($P = 0.02$) (Figure 1D), with an increase in RM (59.1%) compared with TA (52.0%; $P < 0.05$) in post hoc analysis.

Previous studies have shown that gestational age has a strong influence on DNA methylation at many sites throughout the genome in placental villi.²⁵ Unsupervised clustering showed that the TA samples do not cluster separately and are, thus, of similar nature and gestational age as the RM samples (Supplemental Figure S3). However, because gestational age is not known for the remainder of the follow-up group of TAs, the influence of gestational age was

considered in further analyses. By using the RM and M samples ($N = 54$), we identified a significant positive correlation between gestational age and DNA methylation at the *CYP1A2* promoter region ($r = 0.58$, $P < 0.0001$) (Supplemental Figure S4). It is, thus, possible that the observed increase in methylation in the RM and M groups compared with the TA group (Figure 1A) may be confounded by differences in gestational age. There was no significant correlation between maternal age and DNA methylation at any of the assayed regions (data not shown).

ErmineJ gene ontology analysis, of the 26,062 Infinium array probes, was used to assess whether certain gene ontologies were enriched among those sites showing the largest $\Delta \beta$ between the RM and TA groups. In other words, this analysis is not based on the small subset of probes we identified as candidates, but on the distribution of differences between the two groups relative to the gene content on the array. There was a highly significant enrichment of

Figure 1 Box plots comparing DNA methylation (%) at the promoter regions of *CYP1A2* ($P = 0.002$) (A), *DEFB1* ($P = 0.008$) (B), *APC* ($P = 0.18$) (C), and *AXL* ($P = 0.02$) (D) genes between the RM ($N = 33$), M ($N = 21$), and TA ($N = 16$) groups. The box plot whiskers indicate $1.5\times$ the interquartile range, whereas the open circles are outlier values. The horizontal bars with asterisk indicate which comparisons were statistically significant in post hoc pairwise analysis. $*P < 0.05$, $**P < 0.01$. M, single miscarriage; RM, recurrent miscarriage; TA, elective termination.

imprinted genes ($P = 9.53 \times 10^{-10}$) and genes previously associated with RM ($P = 9.51 \times 10^{-6}$) (Supplemental Table S2). When subcategorizing the imprinted genes by parental origin of expression, maternally expressed genes (paternally methylated) were more significantly enriched ($P = 1.90 \times 10^{-9}$) than paternally expressed genes ($P = 7.98 \times 10^{-6}$). Notably, there was a high percentage of gene ontology biological processes involved in immune response among those identified as significantly enriched in this data set (18.4%).

DNA Methylation at Imprinted Genes

DNA methylation was assessed at imprinted loci using bisulfite pyrosequencing (Figure 2), because of the previously reported association with pregnancy loss¹¹ and the observed enrichment in the ErmineJ gene ontology analysis. These seven loci, including maternally methylated *PLAGL1*, *SGCE*, *KvDMR1*, and *SNRPN* and paternally methylated *H19/IGF2* ICR1, *CDKN1C*, and *MEG3*, were selected because they have been previously demonstrated to maintain their imprints in placenta.^{14,26} After correction for multiple comparisons, there was significantly increased average methylation in M (51.7%) compared with RM (48.2%) and TA (47.8%) at the *H19/IGF2* ICR1 ($P < 0.0001$) (Supplemental Figure S5). In addition, there was no correlation between DNA methylation at any of the seven imprinted loci and gestational age (Supplemental Figure S6).

Because we may expect only a subset of miscarriages to be attributed to changes in DNA methylation as the result of the heterogeneous etiology, we sought to identify individual samples that display values outside of the normal range.

It was previously demonstrated that spontaneous abortion and stillbirth were associated with an increased number of outliers, defined as $>1.5\times$ the interquartile range, for DNA methylation at imprinted loci.¹¹ By using this criterion, we observed a significant increase in the number of outliers for DNA methylation in the RM group (3.9%) compared with the M group (0.0%) and TA group (0.9%, $P = 0.02$) (Table 3).

Global Measures of DNA Methylation

To assess whether there was overall dysregulation of DNA methylation in any sample, or groups as a whole, two global measures, using representative dispersed sequences, were used: the average of the 26,062 Infinium array probes and the average methylation at a consensus LINE-1 sequences. There were no significant differences in average methylation observed between groups, after correction for multiple comparisons (Figure 3). To investigate individual samples, PCA was used to identify those that show distinct patterns of DNA methylation at the 12 targeted loci within this study, including LINE-1 sequences. In a PCA plot comparing the two primary principle components, attributing 44% of the variance within the data set, outlier samples were identified (Figure 4). The identified outlier samples show altered DNA methylation at multiple loci (Table 4), defined as >1 SD or <1 SD from the mean, measured in all first-trimester chorionic villi ($N = 70$), although no consistent pattern of dysregulation was evident.

Discussion

In this study, we assessed DNA methylation globally and at targeted loci in placental samples from first-trimester RM, M, and TA pregnancies. This was used to address whether there were differences between these groups and whether a subset of pregnancies showed distinct epigenetic changes. By using both candidacy and gene ontology approaches, several differences in DNA methylation were associated with RM and/or isolated miscarriage. Two candidate CpG sites, near the promoters of *DEFB1* and *AXL*, were

Figure 2 Box plots of DNA methylation (%) at seven imprinted loci for all first-trimester placental samples ($N = 70$). The box plot whiskers indicate $1.5\times$ the interquartile range, whereas outlier values are denoted for each group: RM, recurrent miscarriage (circle); TA, elective termination (triangle). No outliers were observed for the M group, single miscarriage (square).

Table 3 Comparison of the Number of Outliers (Defined as $>1.5 \times$ the Interquartile Range) for DNA Methylation at Seven Imprinted Loci between the RM, M, and TA Groups ($P = 0.02$)

Gene/region	Location	Methylated allele	Average methylation (%)	RM group (N = 33)	M group (N = 21)	TA group (N = 16)
<i>PLAGL1</i>	6q24.2	M	53.7	3	0	0
<i>SGCE</i>	7q12.3	M	48.1	1	0	0
<i>KvDMR1</i>	11p15.5	M	61.4	0	0	0
<i>SNRPN</i>	15q11.2	M	48.7	1	0	0
<i>H19/IGF2</i> ICR1	11p15.5	P	49.2	2	0	1
<i>CDKN1C</i>	11p15.5	P	24.8	1	0	0
<i>MEG3</i>	14q32.3	P	36.3	1	0	0

Data for number (percentage) of outliers are as follows: RM (recurrent miscarriage) group, 9 (3.9%) of 231; M (single miscarriage) group, 0 (0%) of 147; and TA (elective termination) group, 1 (0.9%) of 112.

M, maternal; P, paternal.

identified as differentially methylated between RM and TA, whereas *DEFB1* and *CYP1A2* were differentially methylated between M and TA. Because a subset of the RM and TA groups did not cluster separately using Infinium array profiles, it does not appear that mode of fetal demise is associated with gross changes in cell composition or epigenetic gene regulation in the placenta. The gene ontology analysis of differential methylation on the Infinium array showed an enrichment of genes previously associated with RM, imprinted loci, and immunological pathways. In addition, there were more outliers for DNA methylation at seven imprinted loci among RM placentas. Although we did

not observe an overall trend of altered global DNA methylation in any group, specific placental samples in each of the three comparison groups showed altered methylation at multiple loci.

The *CYP1A2* gene promoter region showed an increase in DNA methylation in the M placental samples compared with TA. However, the contribution of gestational age on the observed association cannot be addressed, because information was not available for the TA samples. There is a strong positive correlation between DNA methylation at this site and gestational age in RM and M samples; therefore, the association would need to be independently validated in a well-characterized population. *CYP1A2* is of particular interest, given its role in caffeine and drug metabolism. High *CYP1A* activity, a combination of both isoforms 1 and 2, in first-trimester placenta correlates strongly with maternal age and is associated with maternal smoking and alcohol consumption.²⁷ Maternal intake of caffeine, in conjunction with genetically altered metabolic activity of *CYP1A2*, has been associated with both karyotypically normal miscarriage and RM.^{28,29} Together, these findings raise the possibility that altered expression of *CYP1A2* may be reflective of genetic and environmental influences that contribute to risk for miscarriage.

At the promoter of *DEFB1* (or hBD-1), there was an incremental decrease in methylation in RM and M placental villi compared with TA. *DEFB1* encodes for an antimicrobial peptide involved in the innate immune response, which is expressed from placental tissues.³⁰ Increased placental expression of *DEFB1* was observed in HIV-positive women,³¹ and a trend toward increased levels was observed in women with preterm premature rupture of membranes and chorioamnionitis.³² The placenta provides an immunological barrier between the mother and fetus, protecting the genetically distinct fetus from the maternal immune system. Furthermore, the process of implantation is mediated by the immune system, and it has been suggested that miscarriage may be a process of an exaggerated inflammatory response by the mother.³³ There is conflicting evidence as to whether infection during pregnancy is associated with RM³⁴; however, gene expression studies,^{21,35} and the observed enrichment of gene ontology groups

Figure 3 Comparison of measures of global DNA methylation using representative dispersed sequences: average methylation at LINE-1 consensus sequences ($P = 0.03$) between RM ($N = 33$) and M ($N = 21$) (A) and TA ($N = 16$) (B) groups and Infinium array probe average ($P = 0.19$) between RM ($N = 10$) and TA ($N = 10$). The box plot whiskers indicate $1.5 \times$ the interquartile range, whereas the open circles are outlier values. M, single miscarriage; RM, recurrent miscarriage; TA, elective termination.

Figure 4 PCA plot of component 1 versus component 2 (44% of variance) for DNA methylation (%) at 12 targeted loci among RM (*N* = 33, blue), M (*N* = 21, green), and TA (*N* = 16, black) placental samples. Red arrows represent the vectors for each of the 12 assays, and circles are outliers. M, single miscarriage; RM, recurrent miscarriage; TA, elective termination.

involved in immune response in our gene ontology analysis, support some role of immune function in risk for RM.

CpG sites within the promoter regions of *APC* and *AXL*, two putative imprinted genes, were also identified in the candidate analysis.^{26,36} In follow-up studies, only *AXL* showed a consistent increase in methylation in the RM compared with the TA samples. The paternally methylated *AXL* functions to promote cell proliferation, although its role in the placenta has not been studied. Interestingly, a knockout of three tyrosine kinases in mice, including *Axl*, resulted in lupus erythematosus and recurrent fetal loss.³⁷

A threshold mechanism has been proposed, suggesting that an accumulation of aberrant DNA methylation at developmentally important loci, such as imprinted genes, past a tolerated threshold, may result in the miscarriage of pregnancy.¹¹ Supporting this hypothesis, Plushch et al¹¹

identified outliers in 4.6% and 1.0% of muscle samples from spontaneous abortions and induced abortions, respectively, at six imprinted genes. By using the same definition of outlier DNA methylation, but placental samples, we report similar differences between the RM (3.9%) and TA (0.9%) groups at seven imprinted genes (four of six from the Plushch et al¹¹ study), although we observed no outliers in the M group. Because the previous study did not stratify cases based on pregnancy history, it is possible these represent a similar subset of patients at increased risk for miscarriage.

By using *ErmineJ* gene ontology analysis, we additionally observed an enrichment of imprinted genes in those sites showing greater differences in β values between the RM and TA groups. Deletion of a single gene, *Trim28*, in the oocytes of female mice resulted in RM with no live-borns, and the corresponding fetuses showed widespread loss of DNA methylation at imprinted loci, particularly those that are paternally methylated.³⁸ Interestingly, we also observed a stronger enrichment of paternally imprinted (maternally expressed) genes in the gene ontology analysis. However, there was no apparent increase in the number of outliers at paternally methylated imprinted loci using a targeted approach (Table 3); however, this may be the result of the few outliers identified in this study and the limited analysis of only three paternally methylated genes. It has been hypothesized that maternal protein complexes within the oocyte provide protection of germline differentially methylated regions near imprinted loci, before embryonic transcription initiates.³⁸ Therefore, dysregulation of these maternal effect genes, either genetically or environmentally, may contribute to risk for RM, with corresponding DNA methylation abnormalities in the embryo. These types of genes may be the potential candidates for future studies in women with RM, with no liveborns and evidence for dysregulation of methylation at imprinted genes in these miscarriages.

There are several limitations to this study. First, there was incomplete information on obstetrical history and clinical investigations of the women with RM. A well-defined population would allow comparisons of DNA methylation levels with specific clinical features. Obtaining exact gestational

Table 4 Variability of DNA Methylation Observed at 12 Loci among Samples Identified in the PCA as Outliers

PCA plot no.	Sample	Gestational age (weeks)	Karyotype	DNA methylation range											
				<i>PLAGL1</i>	<i>SGCE</i>	<i>KvDMR1</i>	<i>SNRPN</i>	<i>H19/IGF2</i>	<i>ICR1</i>	<i>CDKN1C</i>	<i>MEG3</i>	<i>CYP1A2</i>	<i>DEFB1</i>	<i>APC</i>	<i>AXL</i>
8	RM27	13.6	46,XY	++	+	+	++	N		N	+	+	N	N	N
16	RM43	11.1	46,XX	N	N	+	N	+		--	++	N	++	--	++
35	M2	13.5	46,XY	+	N	+	N	N		N	+	N	N	+	N
36	M4	6	46,XX	N	--	N	N	N		--	+	+	+	--	++
39	M18	10	46,XX	++	N	++	N	N		N	N	N	N	+	N
59	TA6	NA	46,XX	N	N	N	N	N		--	+	+	+	--	++
61	TA9	NA	46,XX	N	N	N	--	N		--	+	N	+	--	++

N, a normal range of methylation [within 1 SD of the mean in all first-trimester placental samples (*N* = 70)]; NA, not applicable; +, >1 SD from the mean; ++, >2 SDs from the mean; --, <1 SD from the mean; ---, <2 SDs from the mean.

ages for the TA cohort would also improve the study power, allowing statistical correction for this covariate. Assessment of gene expression corresponding to the observed DNA methylation changes would also strengthen the findings; however, because of the complex nature of sample collection from spontaneous or scheduled abortions, the duration for placental tissue degradation is extensive, and this is detrimental to the integrity of placental RNA.¹³ Also, a criticism has been that DNA methylation may not be a stable epigenetic mark at imprinted loci in the placenta³⁹; to address this, we specifically targeted sites that show maintenance of DNA methylation throughout gestation.¹⁴ Furthermore, the observed frequency of outliers at imprinted loci was similar between first-trimester placenta and fetal somatic tissue.¹¹

The observed changes in DNA methylation between the RM, M, and TA groups appear to be limited to specific loci, because global DNA methylation was not altered using either representative dispersed sequence assay. This contrasts with a recent study that found decreased average methylation of all genomic CpG sites and altered expression of DNA methyltransferases in placental villi of early pregnancy losses, although with no correction for gestational age.⁴⁰ The targeted differences, combined with findings of the gene ontology analysis, suggest that changes in placental DNA methylation of genes involved in environmental adaptation, immune response, and imprinted genes may contribute to the etiology of RM. However, it is difficult to determine whether these changes are causal or a consequence of placental adaptation to an unhealthy embryo. Evidence suggesting that aberrant establishment or maintenance of DNA methylation in the embryo may contribute to miscarriage is mounting.^{11,38,40} Studies from mouse suggest that altered DNA methylation in the embryo may impair implantation and normal growth.⁴⁰ Alternatively, the demise of pregnancy is marked by declining progesterone and altered uterine immune cell composition,⁴¹ suggesting that apoptosis of placental cells and/or a cellular response to the termination of pregnancy is possible and may be reflected by changes in DNA methylation. Future studies with a larger, well-characterized sample population will allow for a more comprehensive assessment of small changes in DNA methylation between groups.

Several samples showed distinct patterns of altered DNA methylation, not only at imprinted loci, but at several of the 12 targeted loci investigated. A more extensive genomic analysis of these dysregulated samples may further elucidate the nature of these altered patterns. In addition, the clinical relevance of these findings will need to be determined, elucidating whether these differences in DNA methylation are more common in placentas associated with adverse pregnancy outcomes.

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Supplemental Data

Supplemental materials for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.02.021>.

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