

Short Communication

Extreme Methylation Values of Imprinted Genes in Human Abortions and Stillbirths

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Imprinted genes play an important role in fetal and placental development. Using quantitative bisulfite pyrosequencing assays, we determined the DNA methylation levels at two paternally methylated (*H19* and *MEG3*) and four maternally methylated (*LIT1*, *NESP55*, *PEG3*, and *SNRPN*) imprinted regions in fetal muscle samples from abortions and stillbirths. Two of 55 (4%) spontaneous abortions and 10 of 57 (18%) stillbirths displayed hypermethylation in multiple genes. Interestingly, none of 34 induced abortions had extreme methylation values in multiple genes. All but two abortions/stillbirths with multiple methylation abnormalities were male, indicating that the male embryo may be more susceptible to excess methylation. Hypermethylation of multiple imprinted genes is consistent with stochastic failures of the mechanism, which normally protects the hypomethylated allele from *de novo* methylation after fertilization. Two of six informative abortions/stillbirths with *H19* hypermethylation revealed significant biallelic expression of the autocrine growth factor *IGF2*. In two other cases hypermethylation of *MEG3* was associated with transcriptional down-regulation. We propose that primary epimutations resulting in inappropriate methylation and expression patterns of imprinted genes may contribute to both normal human variation and disease, in particular spontaneous pregnancy loss. (*Am J Pathol* 2010, 176:1084–1090; DOI: 10.2353/ajpath.2010.090764)

Pregnancy loss is a common medical problem. Approximately 25% of reproductive-aged women attempting pregnancy have spontaneous abortions, and at least 10% of all clinical pregnancies are lost.^{1,2} The predominant cause of pregnancy loss in the first trimester are aneuploidies, mainly attributable to chromosome errors during maternal meiosis.^{3,4} In addition, multiple other etiologies have been described, including single gene defects (ie, thrombophilic mutations), endocrine and immunological factors, uterine anatomical abnormalities, environmental exposures, and infections. Despite modern diagnostics of chromosomal and nonchromosomal factors, a large proportion of cases remains unexplained.

Epigenetic changes of the genetic information are not produced by changes in the DNA sequence itself, but by reversible modifications of DNA and chromatin structure. The most thoroughly studied epigenetic modification is methylation of CpG dinucleotides in *cis*-regulatory sequences, which is associated with specific histone modifications leading to a condensed chromatin structure and transcriptional repression.^{5,6} Genomic imprinting is a parent-specific epigenetic modification in which allele-specific expression depends on male versus female germline transmission. Most imprinted genes display differentially methylated regions (DMRs), which are thought to function as imprinting control centers.^{7,8}

Parent-specific methylation patterns (genomic imprints) are established during gametogenesis. In the zygote and early embryo genome-wide demethylation waves erase most of these germline patterns, followed by *de novo* methylation and establishment of somatic methylation patterns around the time of implantation.^{9–11} Only 100 to 200 imprinted genes among our approximately 25,000 genes escape this methylation reprogramming after fertilization and maintain their parent-specific methylation and activity. Many imprinted genes are active in

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the placenta and/or fetus and play an important role in resource acquisition of the fetus.^{12,13}

Animal studies have shown that genome-wide alterations of methylation reprogramming lead to developmental arrest and embryo loss in the preimplantation period.¹⁴ More subtle reprogramming defects in individual genes may be compatible with further development and cause abnormal phenotypes.^{15,16} It has been estimated that such primary epimutations occur at a much (one or two orders of magnitude) higher rate than somatic DNA mutations.^{17,18} Most epimutations may be stochastic errors during establishment and/or maintenance of the appropriate methylation patterns, however environmental and genetic factors are also known to affect the regulation of methylation reprogramming.^{14,19,20} It is well known that epimutations affecting methylation imprints can cause Beckwith-Wiedemann syndrome (BWS) and other imprinting disorders.^{19,21} However, the pathological significance of epimutations is not restricted to these rare syndromes.²²⁻²⁴ Here, we have analyzed the methylation patterns of two paternally and four maternally methylated genes in human abortions and stillbirths.

Materials and Methods

Tissue Samples

Muscle samples were obtained from aborted fetuses and stillbirths who underwent diagnostic examination at the Department of Pediatric Pathology of Mainz University Medical Center. Use of "excess" tissue materials for scientific analyses was approved by the local ethics committee (Aerztekammer Rheinland-Pfalz, decision no. 837.103.04 [4261]). Gestational age of each fetus was determined by foot length measurements (in mm) and last menstrual period. The sex was identified by examination of the external genitalia. Biopsies from the *Musculus quadriceps* were dissected within 24 hours to 48 hours after abortion and stored at -80°C until further analysis. The DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany) was used for genomic DNA isolation. Total RNAs were isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany).

Of the 146 studied cases, 55 were spontaneous abortions (from 12 to 20 weeks gestation), 57 stillbirths (from 21 to 42 weeks gestation), and 34 induced abortions (from 12 to 24 weeks gestation). Placenta and fetus of all cases were analyzed pathomorphologically by an experienced pediatric pathologist. Of the 112 spontaneous pregnancy losses (spontaneous abortions and stillbirths), 63 had a normal karyotype and 49 an unknown karyotype. Because we wished to study epigenetic causes of pregnancy loss, we focused on chromosomally normal cases. Twenty-four (21%) spontaneous pregnancy losses displayed an abnormal phenotype; no fetal abnormalities were detected in the majority of cases. The 34 induced abortions included eight cases with unbalanced karyotypes; the remaining 26 cases (without known chromosome abnormalities) also exhibited different malforma-

tions, including skeletal dysplasias, heart defects, brain malformations, and/or neural tube defects.

Gene-Specific Methylation Analyses

The DMRs of two paternally methylated (*H19* and *MEG3*) and four maternally methylated (*LIT1*, *NESP55*, *PEG3*, and *SNRPN*) genes that can cause developmental defects when abnormally regulated in animal models and/or humans were analyzed by bisulfite pyrosequencing. Bisulfite treatment of genomic DNA was performed with the EpiTect Bisulfite Kit (Qiagen). Bisulfite pyrosequencing was performed on a PSQ96MA Pyrosequencing System (Biotage, Uppsala, Sweden) with the PyroGold SQA reagent kit (Biotage). Polymerase chain reaction (PCR) and sequencing primers for bisulfite pyrosequencing (Table 1) were designed using the Pyrosequencing Assay Design Software (Biotage). The Pyro Q-CpG software (Biotage) was used for data analysis. To demonstrate the reliability of our quantitative methylation assays, we performed duplicate tests for a subset of fetal muscle samples. The mean methylation difference between duplicate measurements was 2.6% for *H19* (13 samples tested), 2.3% for *MEG3* (16 samples), 2.4% for *LIT1* (13 samples), 2.4% for *NESP55* (12 samples), 1.9% for *PEG3* (12 samples), and 2.0% for *SNRPN* (29 samples).

Expression Analysis

cDNAs were synthesized with random primers and SuperScript III reverse transcriptase (Invitrogen) from total RNAs of six samples that were heterozygous for a transcribed SNP, rs10770125, in *IGF2*. PCR products for quantification of allele-specific expression by pyrosequencing (QUASEP)²⁵ were generated from cDNA using FastStart TaqDNA Polymerase (Roche, Mannheim, Germany) and normal hot start PCR (Table 2). Pyrosequencing was performed on a PSQ96MA Pyrosequencing System with the PyroGold SQA reagent kit. The PSQ96MA 2.1.1 software (Biotage) was used for data analysis. Quantitative real-time RT-PCR for *MEG3* (Table 2) was performed on an Applied Biosystems (Darmstadt, Germany) 7500 Fast Real-Time PCR system using standard protocols. Relative quantification was performed with the Relative Expression Software Tool (REST, version 2.0.7),²⁶ using BRD1 (QuantiTect Primer QT00196924, Qiagen) and ERCC3 (QT00080276) as endogenous controls.

Statistical Methods

The methylation level, measured in percentage of maximal possible methylation of six representative imprinted genes, was obtained from 146 fetal muscle samples. Quantitative data were analyzed with SPSS version 17.0.1. Box plots were generated using the default parameters of SPSS. The bottom and the top of the box indicate the 25th and 75th percentile, respectively. The T bars extend from the boxes to at most 1.5 times the

Table 1. Genes and Primers for Bisulfite Pyrosequencing

Gene	Primer	Sequence	Amplicon length (bp)	Localization (bp)	Number of CpGs
<i>H19</i>	Outer forward	5'-TTTTTGGTAGGTATAGAGTT-3'	231	Chromosome 11 1977,647-1977,878	4
	Outer reverse	5'-AAACCATAACACTAAAACCC-3'			
	Nested forward	5'-TGTATAGTATATGGGTATTTTGGAGGTTT-3'			
	Nested reverse*	5'-TCCTATAAATATCCTATTCCTCAAATAACC-3'			
<i>MEG3</i>	Sequencing	5'-TGGTTGTAGTGTGGGAAT-3'	220	Chromosome 14 100,361,907-100,362,129	3
	Forward	5'-GATTTTTTTTATATATTTGTGTTTG-3'			
	Reverse*	5'-CTCATTTCTCTAAAAATAAATAACC-3'			
<i>LIT1</i>	Sequencing	5'-GTGTTTGAATTTATTTTGTGTTGG-3'	122	Chromosome 11 2677,751-2677,873	2
	Forward	5'-AATTAGTAGGTGGGGGG-3'			
	Reverse*	5'-CTAAAAAATCCCTAAAAATC-3'			
<i>NESP55</i>	Sequencing	5'-GGGGGTAGTYGGAG-3'	343	Chromosome 20 56,848,506-56,848,849	3
	Outer forward	5'-TTTTTTATTTTATAGGGTGTATTT-3'			
	Outer reverse	5'-AAAATAAAATACTTAAACACCAC-3'			
	Nested forward	5'-TTTTTGTAGAGTTAGAGGGTAGGT-3'			
	Nested reverse*	5'-AAAAAAACAACCTCAAATCTACC-3'			
<i>PEG3</i>	Sequencing	5'-GTGTTTAAAGAGGATGGAT-3'	153	Chromosome 19 62,043,756-62,043,909	4
	Forward	5'-GGGTAGAGTTTGGGTAGTTG-3'			
	Reverse*	5'-CTCACCTCACCTCAATACTAC-3'			
<i>SNRPN</i>	Sequencing	5'-TGTTATTTTGGGTTGGT-3'	237	Chromosome 15 22,751,105-22,751,342	2 × 3 = 6 3 3
	Forward*	5'-AGGGAGTTGGGATTTTGTATT-3'			
	Reverse	5'-CCCAAATATCTCTTAAAAAAAC-3'			
	Sequencing 1	5'-ACACAATAACCTTACCC-3'			
	Sequencing 2	5'-CCAACCTACCTCTAC-3'			

This table provides the primer sequences for bisulfite pyrosequencing of the six studied genes, the length and chromosomal localization (ensembl 54, May 2009) of the amplified segments, and the number of CpG sites in the amplicons. Biotinylated primers are indicated by an asterisk.

height of the box. Outliers are samples that do not lie within these T bars, extreme outliers have values more extreme than three times the box length away from the median. The χ^2 test was used to compare the number of outliers in spontaneous abortions, stillbirths, and induced abortions. A *P* value of less than 5% was considered significant. Scatter plots were used to study a possible relationship between DNA methylation and gestational age. A linear regression (Pearson correlation, R^2) was calculated for each studied gene to infer the correlation between the dependent (methylation percentage) and the independent (age) variable.

Results

We performed box plot analyses of six functionally important DMRs in fetal muscles of 146 abortions and stillbirths (Figure 1). The analyzed regions represent both primary imprints (*H19*, *LIT1*, *PEG3*, and *SNRPN*), which are established in the germline and stably maintained after fertilization, as well as secondary imprints (*MEG3* and *NESP55*), which are established in the early embryo. In a

given sample, a gene-specific methylation imprint was designated as normal, if the average methylation percentage of all analyzed CpGs in the DMR fell in the T bars of the box plot for all analyzed fetal muscles. In the 55 spontaneous abortions, 15 of 330 (4.6%) analyzed DMR methylation values (for the six studied genes) represented outliers including four (1.2%) extreme outliers (Table 3). In the 57 stillbirths, the number of potentially abnormal methylation values was significantly higher (χ^2 test; $P < 0.01$): 35 of 342 (10.2%) methylation values were outliers and 8 (2.3%) extreme outliers. In contrast, the 34 induced abortions exhibited only 2 of 204 (1.0%) outliers and no extreme outlier, which is significantly ($P = 0.03$, respectively, $P < 0.001$) lower than in spontaneous abortions and stillbirths. We excluded that the higher rate of extreme methylation values in the stillbirth group, compared with abortions, was a consequence of a skewed age distribution. Linear regression analyses did not reveal significant effects of gestational age on DMR methylation (data not shown). It is interesting to note that 48 (92%) outliers represented hypermethylated and only four (8%) hypomethylated DMRs (Figure 1).

Table 2. Primers for QUASEP (*IGF2*) and Quantitative Real-Time RT-PCR (*MEG3*)

Gene	Primer	Sequence	cDNA amplicon length (bp)	cDNA localization (bp)
<i>IGF2</i>	Forward	5'-ACTAGAGTACAGGGGCCGAAGAGT-3'	63	ENST00000300632 466-529
	Reverse*	5'-GGGCTGGAATCCACCTCCT-3'		
	Sequencing	5'-GAGTCACCACCGAGC-3'		
<i>MEG3</i>	Forward	5'-ATCCGTCCACCTCCTTG-3'	277	ENST00000451743 115-392
	Reverse	5'-GAGCATAGCAAGGTCAGG-3'		

This table provides the primer sequences of an intron-spanning QUASEP assay for *IGF2*, which is under control of the analyzed *H19* DMR, and quantitative real-time RT-PCR analysis of *MEG3*, which is controlled by the analyzed *MEG3* DMR. The biotinylated QUASEP primer is indicated by an asterisk.

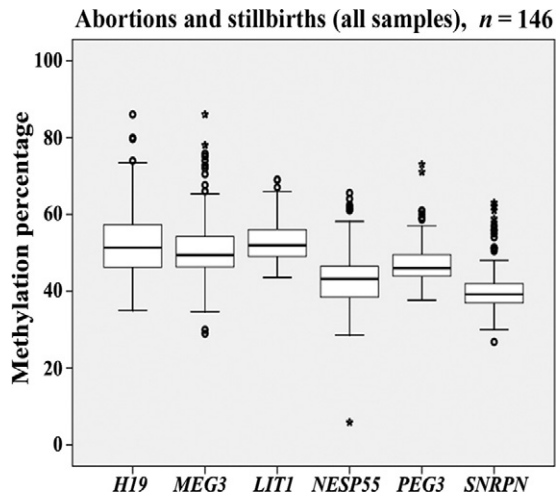


Figure 1. Methylation values of imprinted genes in 146 fetal muscle samples from abortions and stillbirths. The box plots show the distribution of DMR methylation values for *H19*, *MEG3*, *LIT1*, *NESP55*, *PEG3*, and *SNRPN*, respectively. The median is represented by horizontal lines. The **bottom** of the box indicates the 25th percentile, the **top** the 75th percentile. Outliers are shown as **open circles**, extreme outliers as **stars**.

In abnormally methylated samples usually more than one of the six analyzed genes was affected. Two of 55 (4%) spontaneous abortions and 10 of 57 (18%) stillbirths displayed hypermethylated DMRs in two to five genes (Table 4). In contrast, none of 34 induced abortions displayed multiple outliers. Eight of 12 (66%) abortions/stillbirths with multiple methylation abnormalities showed an impairment of villus maturation in the placenta; one fetus suffered from a lymphangioma, whereas nothing abnormal was detected in the remaining 11 fetuses. It is interesting to note that 10 of the 12 (83%) samples with multiple methylation abnormalities were male, whereas of all analyzed fetal samples 54% were male and 46% female. To exclude the possibility that the significant (χ^2 test; $P = 0.05$) excess of male abortions/stillbirths with methylation abnormalities was attributable to sex-specific differences, we performed separate box plot analyses for the 78 male and the 66 female samples (data not shown). Although the median methylation values differed slightly between male and female fetuses (53% versus 51% for *H19*, 53% versus 50% for *MEG3*, 53% versus 52% for *LIT1*, 45% versus 41% for *NESP55*, 48% versus 47% for

PEG3, and 42% versus 40% for *SNRPN*), the results obtained with the male and the female data sets were similar to those obtained with all samples. In both the male and the female group, extreme methylation values were more frequent in stillbirths than in spontaneous abortions. Eleven of the 12 abortions/stillbirths with multiple outliers in the box plot analysis of all fetal muscles also displayed abnormal methylation values in the male group and the female group analysis, respectively.

Hypermethylation of the *H19* DMR may lead to ectopic expression of *IGF2* from the normally hypomethylated maternal allele.²⁷ We performed QUASEP of six informative abortions/stillbirths with *H19* hypermethylation. Samples no. 53 (with 74% *H19* methylation) and 30 (80%) displayed significant biallelic *IGF2* expression (70% C allele versus 30% T allele and 61% C versus 39% T, respectively; Table 5). Abnormal methylation of the normally hypomethylated maternal *MEG3* allele may lead to gene silencing.²⁸ We compared the *MEG3* mRNA expression levels of three normally methylated (48% to 50%) and two hypermethylated fetal muscle samples (no. 27 with 64% and no. 118 with 75% *MEG3* methylation). REST analysis of triplicate measurements of the C_t values (Table 5) revealed a significant ($P < 0.01$) down-regulation of *MEG3* expression in sample no. 27 (relative expression 4%) and a trend ($P = 0.07$) toward down-regulation in sample no. 118 (relative expression 30%), compared with the normally methylated samples.

Discussion

Many imprinted genes play essential roles in mammalian development, notably in the regulation of placental, fetal, and/or postnatal growth. Paternally expressed genes such as *LIT1* and *PEG3* tend to enhance growth, whereas maternally expressed genes such as *H19* restrict growth.^{7,8,12,13} It is generally assumed that a parent-specific expression mechanism has been established during mammalian evolution to settle the conflicting interests between the paternal and maternal genomes on placental/fetal growth.²⁹ Their monoallelic expression makes imprinted genes particularly susceptible to genetic or epigenetic dysregulation. The methylation abnormalities that were observed in 4% of spontaneous abor-

Table 3. Extreme Methylation Values in Fetal Muscle Samples

Gene	All fetal muscles, <i>n</i> = 146 Median methylation	Spontaneous abortions, <i>n</i> = 55		Stillbirths, <i>n</i> = 57		Induced abortions, <i>n</i> = 34	
		Number of outliers	Extreme outliers	Number of outliers	Extreme outliers	Number of outliers	Extreme outliers
<i>H19</i>	51%	0	0	4	0	0	0
<i>MEG3</i>	49%	4	1	7	3	1	0
<i>LIT1</i>	52%	1	0	2	0	0	0
<i>NESP55</i>	43%	2	1	5	0	1	0
<i>PEG3</i>	46%	3	1	5	1	0	0
<i>SNRPN</i>	39%	5	1	12	4	0	0
		15/330	4/330	35/342	8/342	2/204	0/204
		4.6%	1.2%	10.2%	2.3%	1.0%	0%

This table presents the median methylation values and the number of extreme methylation values for six imprinted genes in fetal muscle samples from spontaneous abortions, stillbirths, and induced abortions.

Table 4. Fetal Muscle Samples with Hypermethylation of Multiple Imprinted Genes

Sample number	Weeks gestation	Methylation values						Number of outliers	Phenotype		Karyotype/Genital sex
		<i>H19</i>	<i>MEG3</i>	<i>LIT1</i>	<i>NESP55</i>	<i>PEG3</i>	<i>SNRPN</i>		Placenta	Fetus	
Spontaneous abortions											
2	17	N	N	+	N	+	N	2	CHA	NAD	46,XY
118	20	N	++	N	N	++	+	3	RC	NAD	46,XY
Stillbirths											
1	35	+	++	N	+	N	++	4	VMI	NAD	Male
6	37	+	+	N	+	+	+	5	VMI	NAD	46,XY
7	32	N	N	N	+	N	+	2	VMI	NAD	46,XY
16	22	N	+	+	N	+	+	4	VMI	NAD	Female
30	36	+	++	N	+	++	++	5	VMI	NAD	46,XX
53	28	+	++	N	N	+	++	4	VMI	NAD	46,XY
93	22	N	+	N	N	N	+	2	VMI	NAD	46,XY
102	37	N	+	N	+	N	++	3	VMI	NAD	46,XY
104	26	N	+	N	+	+	++	4	CHA	NAD	46,XY
134	24	N	N	+	N	+	N	2	NAD	Lymphangioma	Male

This table presents abortions and stillbirths with extreme methylation values in at least two studied genes. Box plot analysis was performed for all 146 fetal muscle samples. "N" indicates normal methylation. Hypermethylated outliers are indicated by "+" and extreme outliers by "++." The observed placental phenotypes include chorionamnionitis (CHA), regressive changes (RC), and villus maturation impairment (VMI). NAD means nothing abnormal detected.

tions and 18% of stillbirths are consistent with somatic mosaicism that is the presence of hypermethylated and normally methylated cells in the same fetus. If an epimutation occurred in the germline, all cells of the individual should be affected, leading to clear DMR hypomethylation (<10%) or hypermethylation (>90%). In contrast, if an epimutation occurred during early embryogenesis, it affects only a subset of cells. Our results suggest a postzygotic gain of methylation at multiple imprinted genes, regardless of whether the hypomethylated allele is derived from the paternal or the maternal germline and regardless of whether it is a primary or secondary imprint. This may be attributable to relaxation of the so-far unknown mechanism that normally protects the hypomethylated allele from *de novo* methylation in the morula to blastocyst stage.¹¹ The preimplantation embryo is much less protected from environmental factors than the germline and, thus, may be particularly susceptible to epigenetic alterations.^{14,19}

Two stillbirths with *H19* hypermethylation showed biallelic *IGF2* expression. Loss of *IGF2* imprinting has been found in many different tumor types.³⁰ Importantly, *IGF2* was also dysregulated in normal mucosa and peripheral blood lymphocytes of colorectal cancer patients,³¹ indicating that relaxation of imprinting may predispose to tumorigenesis and, by extrapolation, to other multifactorial phenotypes. About 10% of blood samples from normal populations show biallelic *IGF2* expression.³² Similar to the situation in tumor cells,²³ in our study hypermethylation of the *MEG3* DMR was associated with transcriptional repression in fetal tissue samples. Although the methylation and expression patterns of imprinted genes may show considerable variation, one can envision a link between epigenetic alterations at multiple imprinted loci during critical stages of placental/fetal development and pregnancy loss. We propose a multifactorial threshold model for pregnancy loss. If extreme methylation values attributable to primary epimutations in imprinted and/or

Table 5. Expression Analysis of Hypermethylated Fetal Muscle Samples

Sample number	<i>H19</i> DMR methylation	QUASEP analysis of <i>IGF2</i>	
		C allele	T allele
1	86%	100 ± 0%	0 ± 0%
6	80%	100 ± 0%	0 ± 0%
7	71%	2.2 ± 0.3%	97.8 ± 0.3%
19	73%	1.7 ± 0.1%	98.3 ± 0.1%
30	80%	61.1 ± 1.2%	38.9 ± 1.2%
53	74%	69.8 ± 2.8%	30.2 ± 2.8%
Sample number	<i>MEG3</i> DMR methylation	Real-time RT-PCR of <i>MEG3</i> C ₁ value	
70 (control)	48%	16.1 ± 0.1	
77 (control)	50%	18.5 ± 0.2	
29 (control)	50%	18.2 ± 0.1	
27	64%	27.1 ± 0.3	
118	75%	26.2 ± 0.2	

The upper part of this table presents the QUASEP results (mean and standard deviation of duplicate tests) of fetal muscles with *H19* hypermethylation. Two samples (nos. 30 and 53) express both *IGF2* alleles, indicative of loss of imprinting. The bottom part presents quantitative measurements of *MEG3* expression (mean and standard deviation of triplicate tests) in two samples (nos. 27 and 118) with *MEG3* hypermethylation versus three controls.

other developmentally important genes exceed a critical threshold, the phenotype may become manifest. Similar to other multifactorial diseases, additional genetic and environmental factors also play a role.

In this study, we focused on methylation abnormalities of imprinted genes, which may serve as an indicator for more profound epigenetic defects. It is plausible to assume that the observed epimutations are not restricted to imprinted genes but also affect other developmentally important (non-imprinted) loci. A substantial fraction (>20%) of the spontaneous abortions and stillbirths had malformations that are difficult to explain by imprinting disorders. Interestingly, none of the 12 fetuses with multiple methylation abnormalities displayed detectable malformations; one fetus suffered from a mediastinal lymphangioma. Epimutations are known to play a role in tumorigenesis.^{22,30} On the other hand, most placentae of pregnancies with multiple methylation abnormalities displayed villus maturation impairment or other pathological features. Overall, epigenetic defects were predominantly associated with unexplained pregnancy loss, in particular in the second half of pregnancy.

The majority (>80%) of abortions/stillbirths with multiple methylation abnormalities were male. This is consistent with the observed tendency toward 1% to 4% higher methylation levels at selected loci in male fetuses, compared with females. There are more male than female embryos at conception and a male excess of fetal deaths, which is little understood.³³ In Japan, the male-to-female ratio of fetal deaths increased since the 1970s, reaching more than two in the late 1990s.³⁴ Maternal exposure to severe life events before and/or during the time of conception has been associated with a differential loss of male pregnancies.³⁵ Epigenetic mechanisms are thought to mediate environmental influences on gene regulation and to modulate disease risk.^{20,22-24} In this context it is interesting to speculate that an increased vulnerability of the male embryo to methylation reprogramming defects contributes to the greater male to female risk of fetal mortality.

Our study is limited by the fact that we could analyze only one fetal tissue and that it is difficult to collect adequate controls. Fetal muscle biopsies from healthy pregnancies are ethically out of discussion. Chorionic villus samples (ie, excess materials from prenatal diagnosis) could be easily obtained, but are not suitable for studying imprinted gene regulation in the fetus. In contrast to somatic tissues, imprinting in the placenta appears to be primarily controlled by histone modifications independent of DNA methylation.³⁶ Possibly because of their more direct contact with the environment, trophoblast and placenta may also be much more susceptible to changes in DNA methylation patterns than embryonic or fetal tissues.³⁷ At present, we cannot exclude the formal possibility that the observed methylation patterns represent normal variation during fetal development. Moreover, it is possible that the observed extreme methylation values in aborted fetuses are not the cause but a consequence of aberrant development. In any case, this is the first study presenting baseline data on imprinted gene methylation

in fetal tissues and a first step in detailing what kind of variation to expect.

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