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# Comprehensive and quantitative multilocus methylation analysis reveals the susceptibility of specific imprinted differentially methylated regions to aberrant methylation in Beckwith–Wiedemann syndrome with epimutations

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**Purpose:** Expression of imprinted genes is regulated by DNA methylation of differentially methylated regions (DMRs). Beckwith–Wiedemann syndrome is an imprinting disorder caused by epimutations of DMRs at 11p15.5. To date, multiple methylation defects have been reported in Beckwith–Wiedemann syndrome patients with epimutations; however, limited numbers of DMRs have been analyzed. The susceptibility of DMRs to aberrant methylation, alteration of gene expression due to aberrant methylation, and causative factors for multiple methylation defects remain undetermined.

**Methods:** Comprehensive methylation analysis with two quantitative methods, matrix-assisted laser desorption/ionization mass spectrometry and bisulfite pyrosequencing, was conducted across 29 DMRs in 54 Beckwith–Wiedemann syndrome patients with epimutations. Allelic expressions of three genes with aberrant methylation were analyzed. All DMRs with aberrant methylation were sequenced.

**Results:** Thirty-four percent of *KvDMR1*–loss of methylation patients and 30% of *H19DMR*–gain of methylation patients showed multiple methylation defects. Maternally methylated DMRs were susceptible to aberrant hypomethylation in *KvDMR1*–loss of methylation patients. Biallelic expression of the genes was associated with aberrant methylation. *Cis*-acting pathological variations were not found in any aberrantly methylated DMR.

**Conclusion:** Maternally methylated DMRs may be vulnerable to DNA demethylation during the preimplantation stage, when hypomethylation of *KvDMR1* occurs, and aberrant methylation of DMRs affects imprinted gene expression. *Cis*-acting variations of the DMRs are not involved in the multiple methylation defects.

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**Key Words:** Beckwith–Wiedemann syndrome; DNA methylation; differentially methylated region; genomic imprinting; multiple methylation defects

## INTRODUCTION

Genomic imprinting is an epigenetic phenomenon that leads to parent-specific differential expression of a subset of mammalian genes. Most imprinted genes are clustered in regions called imprinting domains, and the expression of imprinted genes within these domains is regulated by imprinting control regions.<sup>1,2</sup> Differentially methylated regions (DMRs), which are defined as having DNA methylation on only one of the two parental alleles, play critical roles in the regulation of imprinting. There are two kinds of DMRs: maternally methylated DMRs (matDMRs) and paternally methylated DMRs (patDMRs). In

addition, there is another classification, gametic DMRs and somatic DMRs, based on the timing of the establishment of differential methylation. Gametic DMRs acquire DNA methylation during gametogenesis, and the methylation is maintained from zygote to somatic cells during all developmental stages. Most gametic DMRs are identical to imprinting control regions. On the other hand, somatic DMRs are established during early embryogenesis after fertilization under the control of nearby imprinting control regions.<sup>1,2</sup> Because imprinted genes play an important role in the growth and development of embryos, placental formation, and metabolism, aberrant expression of

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imprinted genes due to epigenetic or genetic abnormalities is implicated in the pathogenesis of some human disorders, such as congenital anomalies and tumors.<sup>1,2</sup>

Beckwith–Wiedemann syndrome (BWS; Online Mendelian Inheritance in Man (OMIM) #130650) is an imprinting disease that is characterized by prenatal and postnatal macrosomia, macroglossia, abdominal wall defects, and variable minor features. The relevant imprinted chromosomal region in BWS is 11p15.5, which consists of two imprinted domains, *IGF2/H19* and *CDKN1C/KCNQ1OT1*, *H19DMR* and *KvDMR1* being the respective imprinting control regions.<sup>3–5</sup> Among several causative alterations identified so far, loss of methylation (LOM) at *KvDMR1* and gain of methylation (GOM) at *H19DMR* are isolated epimutations. Hypomethylation at multiple imprinted DMRs has been reported in patients with transient neonatal diabetes mellitus type 1,<sup>6</sup> and the same phenomenon, referred to as multiple methylation defects (MMDs), has been reported in BWS patients with *KvDMR1*-LOM.<sup>7–13</sup> However, although the human genome contains more than 30 imprinting domains (<http://www.geneimprint.com>), a limited number of imprinted DMRs have been analyzed so far, with the exception of a report by Court et al.<sup>12</sup> In addition, methods used for methylation analysis have ranged from nonquantitative to quantitative approaches, and although some studies have used only one method for methylation analysis,<sup>8,9,11</sup> others have used two or more in conjunction.<sup>7,10–13</sup> Furthermore, the questions of whether susceptibility to aberrant methylation is different in each type of DMR, whether aberrant methylation indeed affects imprinted gene expression, and what causative factors are responsible for MMDs still remain unanswered. To clarify these issues, we have conducted a comprehensive methylation screening in BWS patients with *KvDMR1*-LOM or *H19DMR*-GOM with a quantitative method, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS), on 29 imprinted DMRs, which represents the largest number of DMRs analyzed to date, followed by confirmation with another quantitative method, bisulfite pyrosequencing. We also performed gene expression analysis and sequencing of aberrantly methylated DMRs. We found that matDMRs are susceptible to aberrant methylation. We also found alterations in imprinted gene expression due to the aberrant methylation and no *cis*-acting pathological variations in DMRs with MMDs.

## MATERIALS AND METHODS

### Patients

Fifty-four BWS patients (25 boys, 26 girls, 3 gender-unspecified patients; average age: 3.0 years (0–13.9 years)) and their parents were enrolled in this study. Among them, 46 patients met clinical criteria for BWS as described by Weksberg et al.<sup>3</sup> and 6 patients met clinical criteria as described by DeBaun et al.<sup>14</sup> (Supplementary Table S1 online). Because two patients were clinically diagnosed more than 20 years ago, their specific diagnostic criteria were unknown. The methylation statuses of *H19DMR* and *KvDMR1*, paternal uniparental disomy of chromosome 11 (upd(11)pat), and *CDKN1C* mutations were

screened as described previously.<sup>15–17</sup> Peripheral blood samples of most patients were subjected to standard G-banding chromosome analysis and/or high-resolution G-band patterning of human chromosome 11, but neither assay showed any abnormalities in any patient (data not shown). Among the 54 patients, 44 displayed *KvDMR1*-LOM but did not show other causative alterations, including *H19DMR*-GOM, upd(11)pat, and *CDKN1C* mutations (data not shown). The remaining 10 patients displayed *H19DMR*-GOM but did not show other causative alterations (data not shown). We sequenced the entire *H19DMR* in *H19DMR*-GOM patients and found no mutations.<sup>18</sup> We used the peripheral blood samples of 24 children (11 boys, 13 girls; average age: 3.8 years (range of 0–8 years)) who visited the Department of Pediatrics, Saga University Hospital, as normal controls having only mild illness such as common cold. This study was approved by the Ethics Committee for Human Genome and Gene Analyses of the Faculty of Medicine, Saga University. Written informed consent was obtained from the parents or the guardians of the patients and participants.

### DNA isolation and bisulfite conversion

Genomic DNA was extracted from the peripheral blood of patients using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A total of 1 µg of genomic DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA), and then the converted DNA was eluted in 100 µl of water. Unmethylated control DNA was created by whole-genome amplification using the REPLI-g Mini Kit (Qiagen). To prepare fully methylated control DNA, the unmethylated DNA created by whole-genome amplification was treated twice with *SssI* methylase.

### Methylation analysis by MALDI-TOF MS

The DNA methylation status of imprinted DMRs was analyzed by MALDI-TOF MS analysis with a MassARRAY system (Sequenom, San Diego, CA) as previously described.<sup>19,20</sup> Briefly, each DMR was amplified by bisulfite-mediated polymerase chain reaction (PCR) using a primer set containing a primer carrying the T7 promoter sequence at the 5' end. In vitro transcription of the PCR product was performed with T7 RNA polymerase, and the transcript was subjected to uracil-specific cleavage with RNase A. MALDI-TOF MS analysis of the cleaved fragments produced signal pattern pairs indicative of nonmethylated and methylated DNA. Epityper software (Sequenom) analysis of the signals yielded a methylation index (MI) ranging from 0 (no methylation) to 1 (full methylation) for each CpG unit, which contained one or more CpG sites. Aberrant methylation of a CpG unit was defined as the condition in which the difference of MIs between each patient and the average of normal controls exceeded 0.15. This definition was based on our finding in methylation-sensitive Southern blots, which revealed that the differences in MI for *KvDMR1*-LOM or *H19DMR*-GOM in BWS patients were  $\geq 0.15$  (data not shown). Because the analyzed DMRs included several CpG units, aberrant methylation of each DMR was defined as the situation in which more

than 60% of the total number of analyzed CpG units showed aberrant methylation (with the MI difference exceeding 0.15). In the case of *IGF2*-DMR0, the three CpG sites were analyzed based on previous reports.<sup>21,22</sup> All primers used in this study are shown in **Supplementary Table S2** online.

### Methylation analysis by bisulfite pyrosequencing

The aberrant methylation status of DMRs identified by MALDI-TOF MS was confirmed by bisulfite pyrosequencing using QIAGEN PyroMark Q24 according to the manufacturer's instructions (Qiagen). Primers for bisulfite-mediated PCR and pyrosequencing were designed using PyroMark Assay Design 2.0 (Qiagen). In analogy with MALDI-TOF MS analysis, aberrant methylation of a CpG site was defined as the situation in which the difference of MIs between each patient and the average of normal controls exceeded 0.15. Aberrant methylation of each DMR was defined as the condition in which more than 60% of the total number of analyzed CpG sites showed aberrant methylation (with the MI difference exceeding 0.15).

### Bisulfite sequencing

Bisulfite sequencing was performed to analyze allelic methylation of *ZDBF2*-DMR. After PCR amplification, the PCR products were cloned into a pT7Blue T-Vector (Novagen, Darmstadt, Germany), and individual clones were sequenced. Parental alleles were distinguished by a single-nucleotide polymorphism (SNP, *rs1861437*) within the DMR.

### Expression analysis of *ZDBF2*, *FAM50B*, and *GNAS1A*

Total RNA was extracted from the peripheral blood of patients using the QIAamp RNA Blood Mini Kit (Qiagen). The RNA was treated with RNase-free DNase I, and reverse transcription was performed with random primers. We used SNPs for allelic expression to distinguish between the two parental alleles: *rs10932150* in exon 5 of *ZDBF2*; *rs6597007* in exon 2 of *FAM50B*; and *rs143800311*, which is a 5-bp deletion/insertion variation in exon 1A of *GNAS1A*. Reverse transcription-PCR (RT-PCR) products encompassing the SNPs of *ZDBF2* and *FAM50B* were directly sequenced. The products encompassing the deletion/insertion variation of *GNAS1A* were separated by electrophoresis on an Applied Biosystems 3130 genetic analyzer (Applied Biosystems, Foster City, CA) and then analyzed with GeneMapper software (Applied Biosystems). Total expression levels of *ZDBF2* and *FAM50B* were quantitated by real-time PCR with TaqMan probes (Applied Biosystems). The expression level of each gene was normalized against that of the housekeeping genes encoding hydroxymethylbilane synthase (*HMBS*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All quantitative RT-PCRs were performed in triplicate.

### Sequencing of aberrantly methylated DMRs

Direct sequencing of all DMRs showing aberrant methylation in *KvDMR1*-LOM patients was performed to determine whether there was any pathological variation.

### Statistical analyses

Fisher's exact test was used for the comparison of aberrant methylated DMRs. Fisher's exact test or Mann-Whitney *U*-test was used for statistical analyses of clinical features between MMDs and monocus methylation defects in *KvDMR1*-LOM patients. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Validation of methylation analyses, MALDI-TOF MS, and bisulfite pyrosequencing

First, we selected 37 regions reported previously as imprinted DMRs in the human genome<sup>16,20,23</sup> (refer to <http://www.geneimprint.com/>). To validate the quantitative capability of MALDI-TOF MS methylation analysis, mixtures of the unmethylated control DNA and the fully methylated control DNA (0, 25, 50, 75, and 100% methylated DNA) were subjected to bisulfite conversion and analyzed. We found a significant correlation between the measured MIs and predicted MIs in all DMRs, except for *GRB10*, *PEG13*, and IG-DMR-CG4 (**Supplementary Figure S1** online). Furthermore, in normal leukocytes, two regions (*TCEB3C*, *USP29*) showed mostly full methylation and three regions (*TP73*, *SPTBN1*, *WT1-AS*) showed mostly no methylation, suggesting that these regions were not differentially methylated in leukocytes (data not shown). Therefore, we excluded these eight regions and decided to analyze the remaining 29 DMRs by MALDI-TOF MS. Second, we obtained MIs from 24 normal controls using MALDI-TOF MS and calculated the average and SD of each CpG unit. We excluded CpG units in which SDs were >0.1 from further analysis. Averages and SDs of all CpG units analyzed in this study are shown in **Supplementary Table S3** online. After the MALDI-TOF MS analysis, we used bisulfite pyrosequencing to confirm the aberrant methylation uncovered. We also obtained MIs from the 24 controls using bisulfite pyrosequencing and calculated the average and SD of each CpG site. We excluded one CpG site in *H19*DMR because its SD was >0.1 due to a known SNP (*rs10732516*). Averages and SDs of control CpG sites are shown in **Supplementary Table S3** online. Finally, we compared the MIs of MALDI-TOF MS and bisulfite pyrosequencing of each DMR and found a significant correlation (**Supplementary Figure S2** online).

### Multilocus methylation defects in BWS patients with epimutations

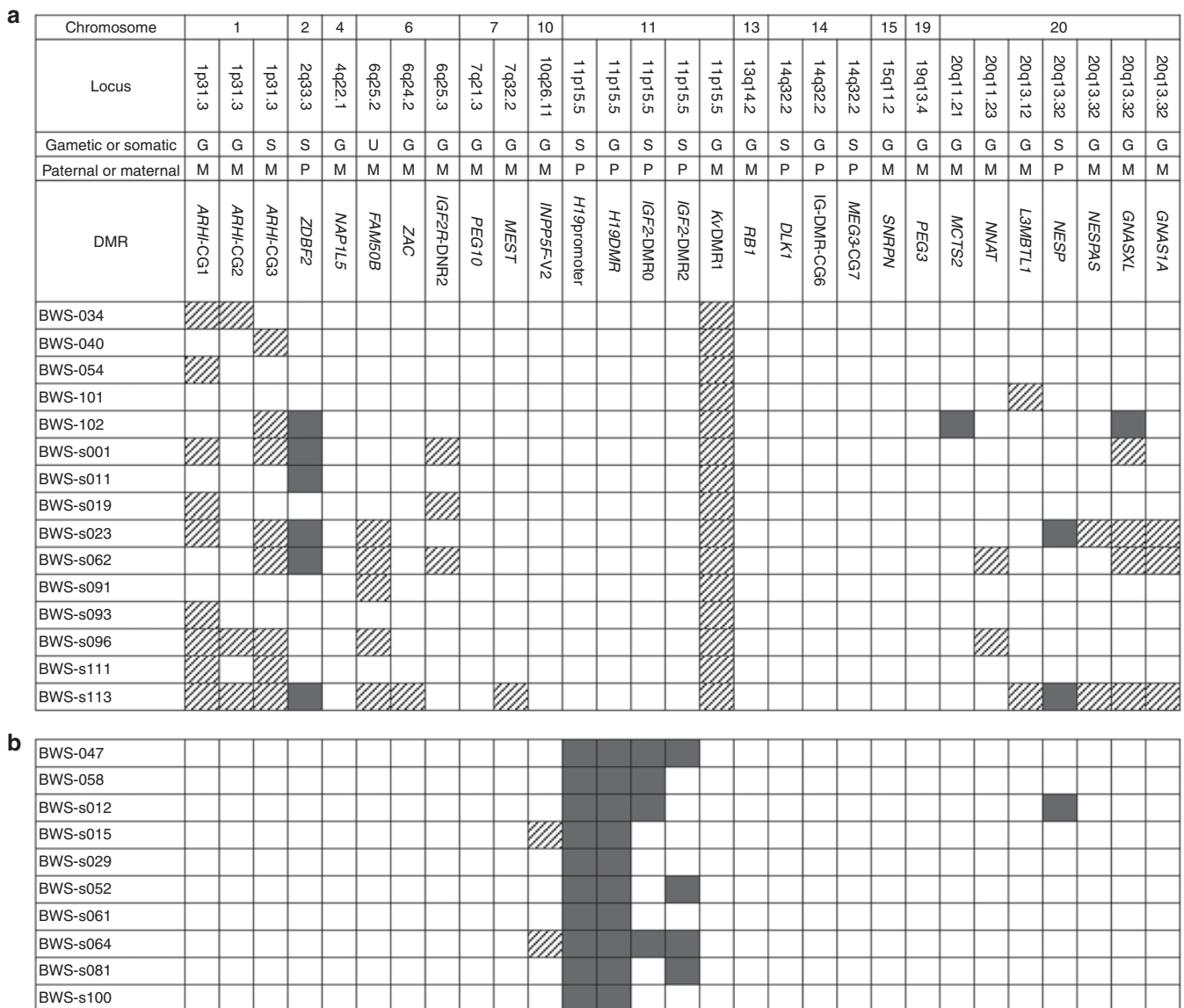
Among the 44 *KvDMR1*-LOM patients, 15 (34.1%) showed aberrantly methylated DMRs outside of *KvDMR1*: six showed aberrant methylation at only one DMR, and the other nine showed two or more methylated DMRs (**Figure 1a** and **Supplementary Figure S3** online). The greatest number of aberrantly methylated DMRs was found in patient BWS-s113, who exhibited 12 DMRs. Most of the aberrantly methylated DMRs demonstrated LOM, which was seen at *ARHI*-CG1, *ARHI*-CG2, *ARHI*-CG3, *FAM50B*, *ZAC*, *IGF2R*-DMR2, *MEST*, *NNAT*, *L3MBTL1*, *NESPAS*, *GNASXL*, and *GNAS1A*. Among them, the most frequently hypomethylated DMRs were

ARHI-CG1 and ARHI-CG3, found in nine (20.5%) and eight (18.2%) patients, respectively. By contrast, three DMRs, located at ZDBF2, NESP, and MCTS2, showed GOM, which was found in six (13.6%), two (4.5%), and one (2.3%) patients, respectively. GNASXL-DMR showed GOM in one patient (2.3%), whereas four patients (9.1%) showed LOM. The other 13 DMRs were not aberrantly methylated in any KvDMR1-LOM patient.

Among the 10 H19DMR-GOM patients, all patients showed GOM at the H19 promoter DMR, which was usually observed with loss of imprinting of IGF2 (Figure 1b).<sup>24</sup> Four patients showed GOM at either IGF2-DMR0 or IGF2-DMR2; two patients showed GOM at both. Moreover, both LOM and GOM at other DMRs were found: LOM was found at INPP5Fv2-DMR

in patients BWS-s015 and BWS-s064, and GOM was found at NESP-DMR in patient BWS-s012.

In addition, to exclude aberrantly methylated DMRs resulting from chromosome abnormalities such as uniparental disomy and copy number abnormality, microsatellite analyses using patients' and their parents' DNA were performed on all DMRs showing aberrant methylation. For quantitative analyses, tetranucleotide repeat markers near the imprinted DMRs were used (Supplementary Materials and Methods online). We found that no DMRs, except for six DMRs in three patients, exhibited any chromosome abnormalities (summarized in Supplementary Figure S4 online). These results strongly suggest that the aberrant methylation of DMRs observed was



**Figure 1** Results of methylation analyses of 29 imprinted differentially methylated regions (DMRs) in Beckwith–Wiedemann syndrome patients with epimutations. (a) Results of patients with KvDMR1-LOM. Only the results of multiple methylation defects are shown. Aberrant methylation was confirmed by two quantitative methods: matrix-assisted laser desorption/ionization mass spectrometry and bisulfite pyrosequencing. The definition of aberrant methylation used here is described in the Materials and Methods section. Shaded rectangle: aberrant hypomethylation; dark gray rectangle: aberrant hypermethylation. (b) Results of all patients with H19DMR-GOM. GOM, gain of methylation; LOM, loss of methylation.

an isolated epimutation and was not due to chromosome abnormalities.

### Comparison of aberrantly methylated DMRs

We found that 34.1% (15 of 44) of *KvDMR1*-LOM patients and 30.0% (3 of 10) of *H19DMR*-GOM patients showed MMDs (Figure 1a). There was no statistical difference between them ( $P > 0.99$ , Fisher's exact test).

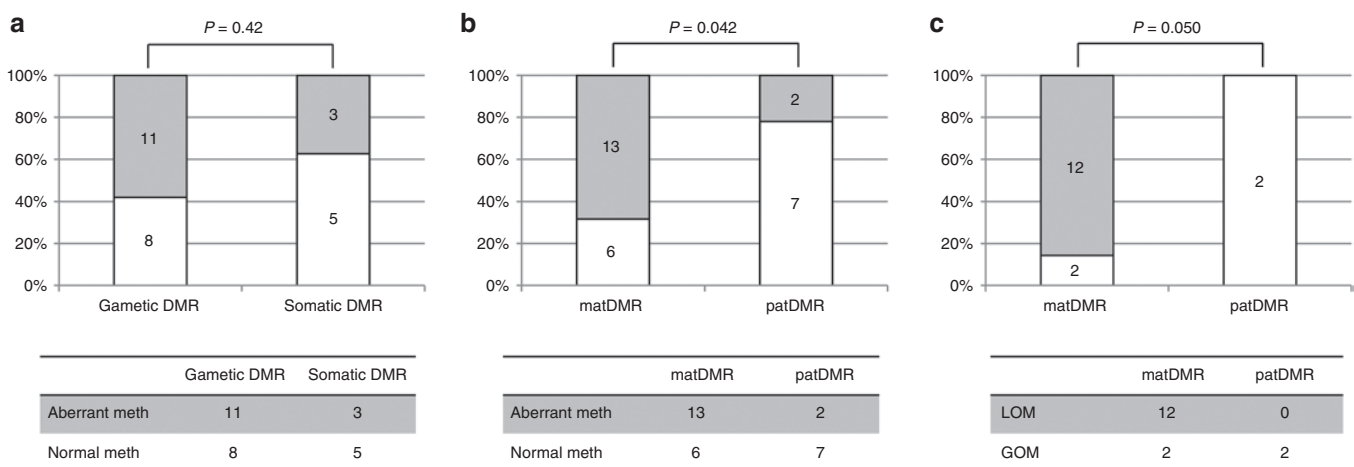
Among the 29 DMRs analyzed, there were 20 gametic DMRs and 8 somatic DMRs (Figure 1a). The timing of methylation establishment of one DMR (*FAM50B*-DMR) has not yet been determined. On the other hand, there were 20 matDMRs and 9 patDMRs. We investigated whether susceptibility to aberrant methylation differed for each type of DMR in *KvDMR1*-LOM patients. *KvDMR1* itself, a gametic and matDMR, was excluded from this analysis. Several DMRs were mapped to certain imprinted domains, e.g., three DMRs in the *ARHI* domain and four in the *GNAS* domain. However, these DMRs differed by type, and aberrant methylations of these DMRs were not always linked. We also had previously found that DMRs in the *GNAS* domain were independently aberrantly methylated in hepatoblastoma.<sup>20</sup> Therefore, we decided to perform statistical analyses assuming the independence of each DMR.

We first compared gametic DMRs with somatic DMRs and found no significant difference in susceptibility ( $P = 0.42$ , Fisher's exact test; Figure 2a). *FAM50B*-DMR was excluded from this comparison. By contrast, matDMRs were aberrantly methylated more frequently than patDMRs ( $P = 0.042$ , Fisher's exact test; Figure 2b). In addition, among the aberrantly methylated DMRs, 12 showed LOM and 4 showed GOM. When we compared LOM with GOM, LOM preferentially occurred on matDMRs ( $P = 0.050$ , Fisher's exact test; Figure 2c). In this subanalysis, *GNASXL*-DMR was counted as having both GOM and LOM (Figure 1a). Furthermore, among the 12 DMRs with

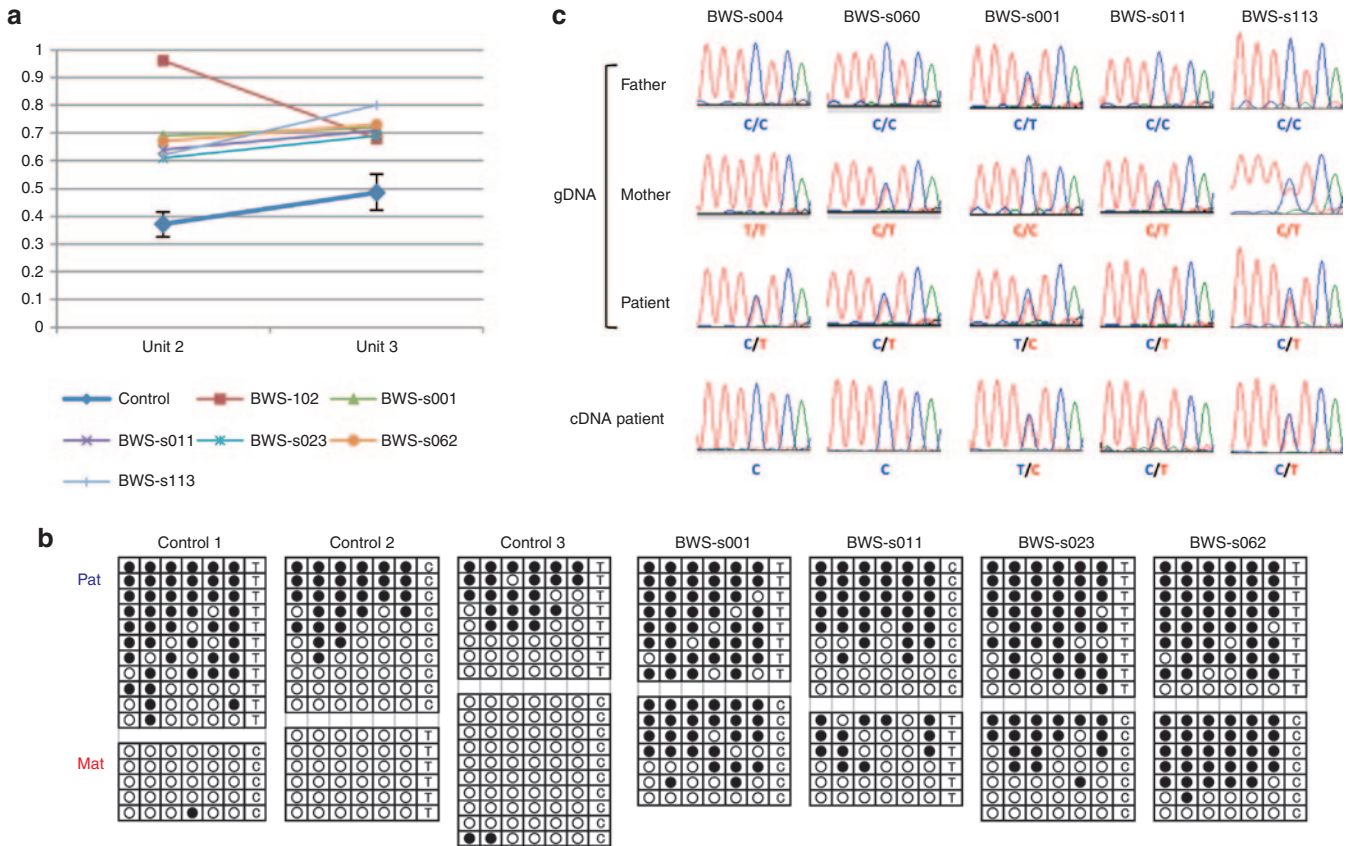
LOM, most of them (10) were gametic DMRs. These results suggest that matDMRs are susceptible to aberrant methylation and that gametic maternally methylated DMRs tend to be susceptible to LOM in *KvDMR1*-LOM patients.

### Biallelic expression of imprinted genes induced by aberrant methylation at their corresponding DMRs

We continued our investigation by determining whether allelic expression was associated with the methylation status of the corresponding DMR. We selected three genes (*ZDBF2*, *FAM50B*, and *GNAS1A*) expressed in lymphocytes.<sup>25–27</sup> In the case of *ZDBF2*, bisulfite sequencing of *ZDBF2*-DMR showed paternal monoallelic methylation in normal controls heterozygous for a specific SNP (*rs1861437*), whereas four BWS patients with GOM showed biallelic methylation: these findings were consistent with the results of MALDI-TOF MS and bisulfite pyrosequencing (Figure 3a,b and Supplementary Figure S5 online). Because paternal expression of the *ZDBF2* gene is coupled with methylation of *ZDBF2*-DMR on the paternal allele,<sup>25</sup> biallelic expression due to biallelic methylation was expected. Indeed, three BWS patients heterozygous for a coding SNP (*rs10932150*) with hypermethylated DMRs clearly showed biallelic expression, in contrast with the paternal monoallelic expression in patients with normally methylated DMRs (Figure 3c). *FAM50B* and *GNAS1A* were paternally expressed and were coupled with maternal methylation of corresponding DMRs. RT-PCR using coding SNPs (*rs6597007* for *FAM50B* and *rs143800311* for *GNAS1A*) revealed that both genes were expressed biallelically with LOM of each corresponding DMR, which was in contrast with monoallelic expression in the patients with normally methylated DMRs (Figure 4 and Supplementary Figure S5 online). It is intriguing that *FAM50B* in patient BWS-s096 and *GNAS1A* in patient BWS-s062 were expressed from the maternal allele despite low-grade LOM,



**Figure 2** Statistical analyses of aberrantly methylated differentially methylated region (DMRs). (a) Comparison of the number of aberrantly methylated DMRs between gametic DMRs and somatic DMRs in *KvDMR1*-LOM patients. There was no statistical difference between the two DMRs ( $P = 0.42$ , Fisher's exact test). (b) Comparison of the number of aberrantly methylated DMRs between matDMRs and patDMRs in *KvDMR1*-LOM patients. matDMRs were aberrantly methylated more frequently than patDMRs ( $P = 0.042$ , Fisher's exact test). (c) Comparison of the number of LOMs and GOMs between matDMRs and patDMRs among the aberrantly methylated DMRs in *KvDMR1*-LOM patients. LOM preferentially occurred on matDMRs ( $P = 0.050$ , Fisher's exact test). GOM, gain of methylation; LOM, loss of methylation; matDMR, maternally methylated DMR; patDMR, paternally methylated DMR.



**Figure 3 Methylation analysis of *ZDBF2*-DMR and expression analysis of the *ZDBF2* gene.** (a) Results of matrix-assisted laser desorption/ionization mass spectrometry analysis. Averages with SD of 24 normal controls are shown in blue. Methylation indexes of the patients showing GOM are indicated in different colors. Units 1 and 2 included two and one CpG sites, respectively. (b) Results of bisulfite sequencing. Normal controls show monoallelic differential methylation, whereas four Beckwith–Wiedemann syndrome (BWS) patients (BWS-s001, BWS-s011, BWS-s023, and BWS-s060) show biallelic methylation. Two parental alleles were distinguished by a SNP (*rs1861437*). Mat, maternal allele; Pat, paternal allele. (c) Results of expression analysis of the *ZDBF2* gene. Three BWS patients (BWS-s001, BWS-s011, and BWS-s113) heterozygous for a coding SNP (*rs10932150*) with GOM clearly showed biallelic expression; by contrast, two patients with normally methylated differentially methylated region (DMRs) exhibited paternal monoallelic expression (patients BWS-s004 and BWS-s060). gDNA, genomic DNA; GOM, gain of methylation; SNP, single-nucleotide polymorphism.

which suggests that our definition of aberrant methylation is appropriate. In addition, we investigated the expression levels of *ZDBF2* and *FAM50B* by quantitative RT-PCR. The expression levels in patients with aberrantly methylated DMRs were higher than those in patients with normally methylated DMRs (Supplementary Figure S6 online). These results indicate that allelic expression and expression levels were indeed associated with the methylation status of the corresponding DMR in patients with MMDs.

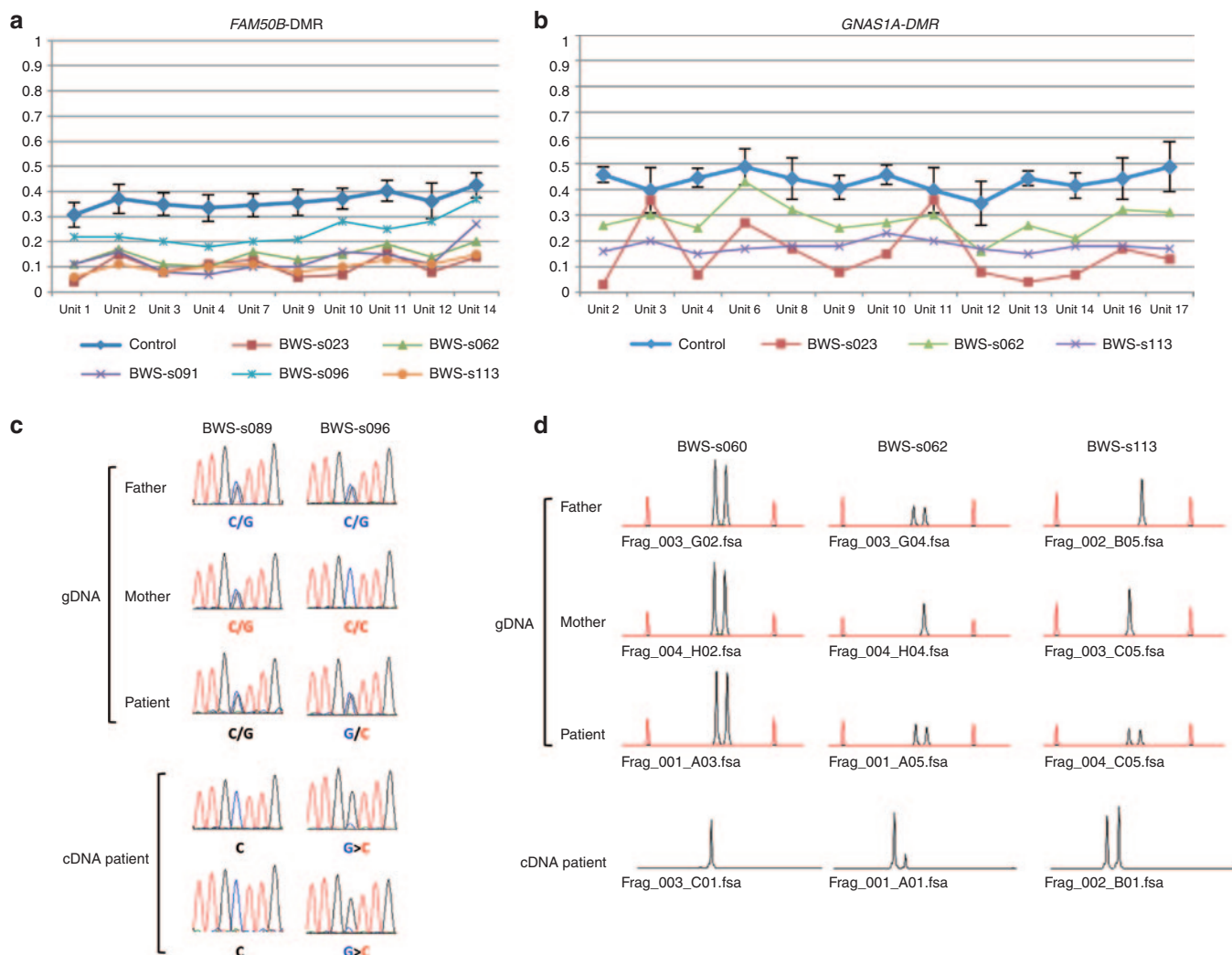
**Lack of pathological variation in all aberrantly methylated DMRs in *KvDMR1*-LOM patients**

Because the genetic aberrations of *H19DMR* explained only ~20% of BWS patients with *H19DMR*-GOM,<sup>28</sup> we hypothesized the existence of *cis*-acting variations within aberrantly methylated DMRs. Therefore, we sequenced all aberrantly methylated DMRs, including *KvDMR1*, in *KvDMR1*-LOM patients. However, no variations were found in any aberrantly hypomethylated DMRs, except for four known SNPs (summarized in Supplementary Figure S7 online), suggesting that

*cis*-acting pathological variations are not involved in aberrant methylation of these DMRs.

**No difference in clinical features between MMDs and monolocus methylation defects**

In *KvDMR1*-LOM patients, there was no significant difference in clinical features between MMDs and monolocus methylation defects, which demonstrated LOM only at *KvDMR1* (Table 1). Among 27 patients with *KvDMR1*-LOM for whom information on conception was available, one patient was conceived using intracytoplasmic sperm injection, two were from artificial insemination by the husband, and two were from ovulation stimulation. We searched for a link between assisted reproductive technology and MMD but could find no relationship (Table 1). The average age of neither the mother nor the father differed between patients with MMDs versus those with monolocus methylation defects (Table 1). The fact that monozygotic twins discordant for BWS were found predominantly for females suggests an insufficient amount of DNA methyltransferase 1 (DNMT1) to maintain *KvDMR1* methylation during the overlap in timing



**Figure 4** Methylation analysis of *FAM50B*- and *GNAS1A*-DMRs and expression analysis of the *FAM50B* and *GNAS1A* genes. (a,b) Results of matrix-assisted laser desorption/ionization mass spectrometry analysis. Averages with SD of 24 normal controls are shown in blue. Methylation indexes of patients showing LOM are indicated in different colors. Ten CpG units analyzed for *FAM50B*-DMR covered 13 CpG sites, and 13 CpG units analyzed for *GNAS1A*-DMR covered 18 CpG sites. (c) Results of expression analysis of the *FAM50B* gene. Beckwith–Wiedemann syndrome (BWS) patient BWS-s096 was heterozygous for a coding SNP (*rs6597007*) with LOM and showed biallelic expression with a low peak of maternal expression, whereas monoallelic expression was seen in a patient with normally methylated differentially methylated regions (DMRs) (patient BWS-s089). In patient BWS-s096, maternal expression was noted in two independent analyses despite low-grade LOM. gDNA, genomic DNA. (d) Results of expression analysis of the *GNAS1A* gene. Patients BWS-s062 and BWS-s113, heterozygous for a deletion/insertion variation (*rs143800311*) with LOM, showed biallelic expression, whereas patient BWS-s060 possessed normally methylated DMRs and exhibited monoallelic expression. Maternal expression was noted despite low-grade LOM in patient BWS-s062. Red peaks are molecular markers. GOM, gain of methylation; LOM, loss of methylation.

with X-chromosome inactivation and twinning.<sup>29</sup> This hypothesis suggests that females might tend to suffer from MMDs. We compared the frequency of female patients with MMDs with the frequency of those with monolocus methylation defects, but no significant difference could be found (Table 1).

## DISCUSSION

Currently, most reports have studied 3–10 imprinted DMRs in BWS patients,<sup>7–10,13</sup> with the exception of two reports in which 16 and 27 DMRs were analyzed.<sup>11,12</sup> In addition, the quantitative capability of methods used for multiple methylation analyses has been variable, and few studies have conducted multiple

checks to confirm the methylation statuses of all DMRs showing aberrant methylation.<sup>7–13</sup> To resolve these matters, we analyzed 29 DMRs and confirmed all aberrantly methylated DMRs using MALDI-TOF MS and bisulfite pyrosequencing, which are the most reliable quantitative methods of methylation analysis available at present.<sup>19,30,31</sup> We found that 34.1% of *KvDMR1*-LOM patients exhibited MMDs. The frequency was higher than that in previous reports, which can be summarized as reporting an overall frequency of 20.6% (102 of 495 patients).<sup>7–13</sup> However, within these reports, the frequency in studies that analyzed 10 or fewer DMRs is 19.0% (82 of 431),<sup>7–10,13</sup> and the frequency in studies that analyzed more than 10 DMRs is 31.3% (20 of

**Table 1** Clinical features of *Kv*DMR-LOM patients with monolocus methylation defect and those with multilocus methylation defects

	Methylation defect		P value
	Monolocus	Multilocus	
Sex			0.22
Male	15	5	
Female	13	9	
Average age of patients	3.3	2.4	0.098 <sup>a</sup>
Average age of parents			
Father	31.8	33.8	0.93 <sup>a</sup>
Mother	31.8	30.3	0.37 <sup>a</sup>
Assisted reproduction technology	3/19 (20%) (AIH 2, OS 1)	2/8 (29%) (ICSI 1, OS 1)	0.47
Standard deviation of average birth weight	+1.9	+2.0	0.58 <sup>a</sup>
Overgrowth	21/28 (75%)	9/13 (69%)	0.78
Abdominal wall defect	22/29 (76%)	12/13 (92%)	0.21
Macroglossia	29/29 (100%)	12/12 (100%)	0.60
Hypoglycemia	14/27 (52%)	5/12 (42%)	0.41
Ear pits and creases	19/27 (70.4%)	8/12 (67%)	0.73
Nevus flammeus	9/26 (35%)	4/10 (40%)	0.53
Hemihypertrophy	6/27 (22%)	6/13 (46%)	0.12
Renal anomaly	2/26 (8%)	0/11 (0%)	0.49
Renal enlargement	6/28 (21%)	1/13 (8%)	0.27
Adrenal enlargement	1/27 (4%)	0/11 (0%)	0.71
Hepatomegaly	5/29 (17%)	2/12 (17%)	0.67
Splenomegaly	6/29 (21%)	2/12 (17%)	0.57
Abnormal external genitalia	2/28 (7%)	0/12 (0%)	0.49
Increased bone age	2/15 (13%)	0/3 (0%)	0.69
Cardiac anomaly	2/23 (9%)	0/11 (0%)	0.82
Developmental retardation	6/22 (27%)	0/9 (0%)	0.10
Childhood tumor	5/26 (19%)	0/11 (0%)	0.15

AIH, artificial insemination by husband; ICSI, intracytoplasmic sperm injection; LOM, loss of methylation; OS, ovulation stimulation.

<sup>a</sup>Mann–Whitney *U*-test. Fisher's exact test was used for other analyses.

64).<sup>11,12</sup> In addition, we found that 30.0% of *H19*DMR-GOM patients showed MMDs, which is surprising considering that no MMDs were found in two previous reports in which 10 and 16 DMRs were analyzed.<sup>8,11</sup> These data suggest that the greater the number of DMRs analyzed, the higher the frequency of MMDs observed. In future, all DMRs in the genome should be analyzed to understand the precise frequency of MMDs, which DMRs become preferentially aberrantly methylated, and the mechanism by which MMDs occur.

In both *Kv*DMR1-LOM patients and *H19*DMR-GOM patients, we found MMDs in which not only LOM but also GOM were seen. We also found that both matDMRs and patDMRs were aberrantly methylated in both patient groups. It is noteworthy that matDMRs, probably gametic maternally methylated DMRs, were more susceptible to aberrant methylation than patDMRs in *Kv*DMR1-LOM patients, although no particular parent-based pattern of aberrant methylation has

been reported previously.<sup>12</sup> This suggests that gametic maternally methylated DMRs are vulnerable to DNA demethylation during the preimplantation stage of early embryogenesis when *Kv*DMR1-LOM occurs.

Although it has not been reported that aberrant methylation of the corresponding DMR affects imprinted gene expression in MMD patients, we found biallelic expression of three imprinted genes (*ZDBF2*, *FAM50B*, and *GNAS1A*) to be associated with the aberrant methylation of their respective DMRs. Because biallelic expression increased the total expression levels of *ZDBF2* and *FAM50B*, we expect that had we measured the expression levels of *GNAS1A*, we would have observed an increase. Therefore, alteration of gene expression levels due to MMDs might affect the phenotype; however, clinical features between MMDs and monolocus methylation defects were not different in our study. This lack of difference has been previously reported,<sup>7,9,10,13</sup> although a few groups have reported a



difference in clinical features.<sup>8,11,12</sup> Two reasons for this similarity in terms of clinical features could be suggested. First, the mosaic ratio might be different in each organ. Because aberrant methylation was generally partial, it would occur after fertilization, and the patients would be mosaic. A high mosaic ratio would be a critical factor in the emergence of a distinct phenotype in BWS patients with monolocus methylation defects. Second, the imprinted locus at 11p15 might be epidominant over other imprinted loci because all MMD patients were clinically diagnosed as BWS.

Regarding the causative factor(s) for MMD, we could not find any pathological variation in any aberrantly methylated DMR, including *KvDMR1*, suggesting that *cis*-acting variations of each specific DMR itself were not involved in the genesis of MMDs. On the other hand, the involvement of *trans*-acting factors has been advocated in other reports because mutations of *ZFP57* (which are required for the postfertilization maintenance of maternal and paternal methylation imprinting at multiple loci) have been found in transient neonatal diabetes mellitus type 1 patients with multilocus hypomethylation.<sup>32</sup> Mutations of *NLRP2* were also identified in a BWS patient with *KvDMR1*-LOM and *MEST*-LOM in a family with complex consanguinity and in a Silver–Russell syndrome patient with multilocus hypomethylation.<sup>12,33</sup> In addition, *TRIM28*, *NLRP7*, *KHDC3L*, and *DNMT3L* have been considered to be candidate *trans*-acting factors. However, no mutations in any of these candidates or other genes, such as *DNMT1*, *DNMT3A*, and *DNMT3B*, were found in our BWS patients with MMDs, as determined by exome sequencing (K. Sasaki and K. Hata, personal communication). Recently, Lorthongpanich et al.<sup>34</sup> reported that the absence of maternal *Trim28* until zygotic gene activation at the two-cell late stage caused mosaicism of MMDs randomly, suggesting that insufficient expression of the candidate gene(s) at very early embryogenesis is an important event in the generation of MMDs in human imprinted diseases. Whole-genome sequencing and whole-genome bisulfite sequencing, including the regulatory regions of the candidate genes, and transcriptome analysis in early embryogenesis would be useful to identify the cause(s) of MMDs.

In our *H19DMR*-GOM patients, we also found GOM of *IGF2*-DMR0 and *IGF2*-DMR2 to be associated with GOM of *H19DMR* and *H19promoter* DMR, in agreement with previous reports.<sup>22,35,36</sup> Two patients showed simultaneous GOM at both *IGF2*-DMRs. Because *Igf2*-DMRs were established at the post-implantation stage under the control of *H19DMR* in mice,<sup>37</sup> GOM of *IGF2*-DMRs in BWS is likely to occur at the same stage. Although the function of *IGF2*-DMR0 is still unknown, methylated *Igf2*-DMR2 plays a role in transcription initiation of *Igf2* in mice.<sup>38</sup> GOM of the DMRs might change the high-order chromatin structure of the maternal allele and increase the expression of *IGF2* in cooperation with *H19DMR*-GOM in BWS patients.

In conclusion, our comprehensive and quantitative methylation analysis of multiple imprinted DMRs revealed several new findings: (i) matDMRs, probably gametic maternally methylated DMRs, are more susceptible to aberrant methylation

during the preimplantation stage, when *KvDMR1*-LOM occurs; (ii) aberrant methylation indeed alters imprinted gene expression; and (iii) *cis*-acting pathological variations of each DMR are not involved in the MMDs analyzed. Moreover, our study confirmed the simultaneous aberrant hypermethylation of *IGF2*-DMR0 and/or -DMR2 with isolated *H19DMR*-GOM. These findings may help us to understand the molecular mechanisms and pathophysiological features of MMDs.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked in the online version of the paper at <http://www.nature.com/gim>.

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## DISCLOSURE

The authors declare no conflict of interest.

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