Clinical Epigenetics

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# Comprehensive molecular and clinical findings in 29 patients with multi-locus imprinting disturbance

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## Abstract

**Background** Multi-locus imprinting disturbance (MLID) with methylation defects in various differentially methylated regions (DMRs) has recently been identified in approximately 150 cases with imprinting disorders (IDs), and deleterious variants have been found in genes related to methylation maintenance of DMRs, such as those encoding proteins constructing the subcortical maternal complex (SCMC), in a small fraction of patients and/or their mothers. However, integrated methylation analysis for DMRs and sequence analysis for MLID-causative genes in MLID cases and their mothers have been performed only in a single study focusing on Beckwith-Wiedemann syndrome (BWS) and Silver-Russell syndrome (SRS) phenotypes.

**Results** Of 783 patients with various IDs we have identified to date, we examined a total of 386 patients with confirmed epimutation and 71 patients with epimutation or uniparental disomy. Consequently, we identified MLID in 29 patients with epimutation confirmed by methylation analysis for multiple ID-associated DMRs using pyrosequencing and/or methylation-specific multiple ligation-dependent probe amplification. MLID was detected in approximately 12% of patients with BWS phenotype and approximately 5% of patients with SRS phenotype, but not in patients with Kagami-Ogata syndrome, Prader-Willi syndrome, or Angelman syndrome phenotypes. We next conducted array-based methylation analysis for 78 DMRs and whole-exome sequencing in the 29 patients, revealing hypomethylation-dominant aberrant methylation patterns in various DMRs of all the patients, eight probably deleterious variants in genes for SCMC in the mothers of patients, and one homozygous deleterious variant in *ZNF445* in one patient. These variants did not show gene-specific methylation disturbance patterns. Clinically, neurodevelopmental delay and/or intellectual developmental disorder (ND/IDD) was observed in about half of the MLID patients, with no association with the identified methylation disturbance patterns and genetic variants. Notably, seven patients with BWS phenotype were conceived by assisted reproductive technology (ART).

**Conclusions** The frequency of MLID was 7.5% (29/386) in IDs caused by confirmed epimutation. Furthermore, we revealed diverse patterns of hypomethylation-dominant methylation defects, nine deleterious variants, ND/IDD complications in about half of the MLID patients, and a high frequency of MLID in ART-conceived patients.

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**Keywords** Multi-locus imprinting disturbance, Imprinting disorders, Subcortical maternal complex, MS-MLPA, Pyrosequencing, Array-based methylation analysis, Whole-exome sequencing

## Background

Imprinted genes are expressed in a parent-of-origin-specific manner according to the methylation patterns of differentially methylated regions (DMRs) functioning as imprinting control centers [1, 2]. Aberrant expression of imprinted genes leads to imprinting disorders (IDs). Most imprinted genes are strongly expressed in the placenta, fetus, and brain, and therefore, patients with IDs frequently have prenatal and postnatal growth abnormalities and intellectual disability. In addition, some clinical features overlap among different IDs, such as overgrowth between Beckwith-Wiedemann syndrome (BWS) and Kagami-Ogata syndrome (KOS), growth restriction among Silver-Russell syndrome (SRS), Temple syndrome (TS14), Prader-Willi syndrome (PWS), and transient neonatal diabetes mellitus (TNDM), obesity between PWS and pseudohypoparathyroidism type 1B (PHP1B), intellectual disability among KOS, Angelman syndrome (AS), and PWS, hypotonia among SRS, TS14, and PWS, and hormonal abnormalities among BWS, PHP1B, PWS, and TNDM. The etiologies of IDs consist of single nucleotide variants in the disease-causative imprinted genes, copy number variations (CNVs) involving imprinted genes and/or DMRs, uniparental disomy (UPD), and imprinting defects of the disease-responsible DMRs without structural abnormalities of the DMRs, namely epimutation [1]. BWS, SRS, KOS, TS14, AS, PWS, PHP1B, and TNDM include epimutation as one of the etiologies of each disorder. Although the pathogenetic mechanisms of epimutations are unknown, familial cases with single locus epimutation have not been reported, except for twin cases [3].

Recent advances in analytical techniques have revealed multi-locus imprinting disturbance (MLID) with aberrant methylation of multiple DMRs at low frequency in IDs caused by epimutations [4]. Recently, an interim joint statement was released for clinical and molecular diagnosis for MLID, such as a set of DMRs included in the definition, methylation analysis methods for detecting MLID, and criteria for methylation disturbances in DMRs [5]. The defects in genes encoding proteins functioning for maintenance of methylation of CpG sites in the DMRs lead to MLID. Sex-specific DNA methylation of the CpG sites in the DMRs is established in the gonads and protected by maternal and fetal factors from genome-wide demethylation following fertilization [6]. The subcortical maternal complex (SCMC), consisting of NLRP2, NLRP5, NLRP7, PADI6, KHDC3L, OOEP, and TLE6, is expressed in oocytes and preimplantation embryos, thus functioning as a maternal factor [6]. In humans, pathogenic variants in *NLRP2, NLRP5, NLRP7, PADI6, TLE6,* and *KHDC3L* lead to female infertility, biparental hydatidiform mole, and recurrent miscarriage. In mice, deletions of *Nlrp2, Nlrp5, Padi6, Tle6,* and *Khdc3* result in female infertility and early embryonic arrest [7, 8]. Moreover, maternal loss-of-function variants of the *NLRP2, NLRP5, NLRP7, PADI6,* and *KHDC3L* genes cause MLID in their children [9]. Fetal factors, such as ZFP57 and ZNF445, also play an essential role in maintaining the methylation of DMRs after fertilization. *ZFP57* pathogenic variants were detected in approximately 30% of MLID cases with TNDM phenotype (TNDM-MLID) under homozygous conditions, whereas the *ZNF445* pathogenic variant was reported only in one TS14-MLID case under homozygous conditions [10, 11]. Approximately 150 cases with MLID [12–27] and familial MLID cases have been reported [28–30]. These cases showed various clinical features; some cases had ID-specific clinical features, and others had non-specific clinical features as IDs. Several studies reported the frequencies of MLID in BWS, SRS, PHP1B, and TNDM. However, the frequencies of MLID in the remaining IDs, such as TS14, KOS, PWS, and AS, have not been reported. In addition, comprehensive methylation analysis and mutation screening for known MLID-causative genes in cases with MLID and their mothers have been reported only in a single study targeting cases with BWS and SRS phenotypes [12].

Here, we identified 29 patients with MLID by methylation analysis for multiple ID-associated DMRs in patients with epimutation screened from 783 patients with various IDs and conducted comprehensive array-based methylation analysis and whole-exome sequencing (WES). Furthermore, we evaluated the association between their methylation disturbance patterns and their clinical features.

## Results

### Subjects

We included two groups in this study. Group A consisted of 697 patients with IDs diagnosed by methylation analysis for multiple ID-associated DMRs, namely, the *PLAGL1*:TSS-DMR (*PLAGL1*-DMR) on chromosome 6, *PEG10*:TSS-DMR (*PEG10*-DMR) or *GRB10*:alt-TSS-DMR and *MEST*:alt-TSS-DMR (*MEST*-DMR) on chromosome 7, *H19/IGF2*:IG-DMR (*H19*-DMR) and

*KCNQ1OT1*:TSS-DMR (*KCNQ1OT1*-DMR) on chromosome 11, *MEG3/DLK1*:IG-DMR and *MEG3*:TSS-DMR (*MEG3*-DMR) on chromosome 14, *SNRPN*:TSS-DMR (*SNRPN*-DMR) on chromosome 15, and *GNAS A/B*:TSS-DMR (*A/B*-DMR) on chromosome 20, using pyrosequencing and/or methylation-specific multiple ligation-dependent probe amplification (MS-MLPA) analysis with the SALSA MS-MLPA Probe-mix ME034 (MRC-Holland, Amsterdam, Netherlands) (ME034). We also obtained their clinical information in detail. Of 697 patients with IDs, 300 patients had epimutations and 71 patients had no structural abnormalities and were classified as epimutations or UPD due to no parental sample. As shown in Fig. 1, the patients in group A had BWS, SRS, TS14, KOS, PWS, AS, PHP, TNDM, BWS+PHP, or PWS+TS14 phenotypes, or clinical features, such as small for gestational age (SGA), overgrowth, or hypotonia. Group B consisted of 86 BWS patients with hypomethylation of *KCNQ1OT1*-DMR without detailed clinical information.

### Identification of MLID

When patients with epimutation had methylation defects in the multiple ID-associated DMRs, we used molecular diagnosis for the patients with MLID. We conducted multi-locus methylation analysis for IDs-responsible DMRs using pyrosequencing from 2013 to 2021. In 2022, we changed the method of multi-locus methylation analysis from pyrosequencing to MS-MLPA (ME034). We used both methods for multi-locus methylation analysis in several patients, but not all. We identified 22 MLID patients in group A (Table 1 and Fig. 1). Therefore, the frequency of MLID in epimutations in group A was calculated at 5.9% based on the number of patients with epimutation and patients classified as epimutation or UPD and 7.3% based on the number of patients with epimutation (Table 1). The frequencies of MLID in TNDM-MLID, SRS-MLID, BWS-MLID, TS14-MLID, and PHP1B-MLID were 25.0%-33.3%, 4.4%, 12.3%, 7.7%-9.5%, and 2.1%-2.7%, respectively. MLID was not detected in the patients with KOS, AS, or PWS. For patients in group B, we conducted MS-MLPA analysis (ME034) and identified seven patients with MLID (Fig. 1). Of these, Patients 9, 12–14, 16, 18–21, and 23–25 have been previously reported [11, 13, 14, 31, 32] (Additional file 1: Table S1).

### MS-MLPA and pyrosequencing analyses

We summarized the results of methylation analyses targeting multiple ID-related DMRs using pyrosequencing and/or MS-MLPA analysis in 29 patients with MLID (Fig. 2). All patients showed methylation disturbances in two or more ID-associated germline DMRs by MS-MLPA and/or pyrosequencing (Fig. 2). All patients with BWS phenotype (Patients 1–15) and all patients with SRS phenotype (Patients 19–24) had hypomethylation of the *KCNQ1OT1*-DMR and hypomethylation of the *H19*-DMR, respectively. Patient 16 with BWS and

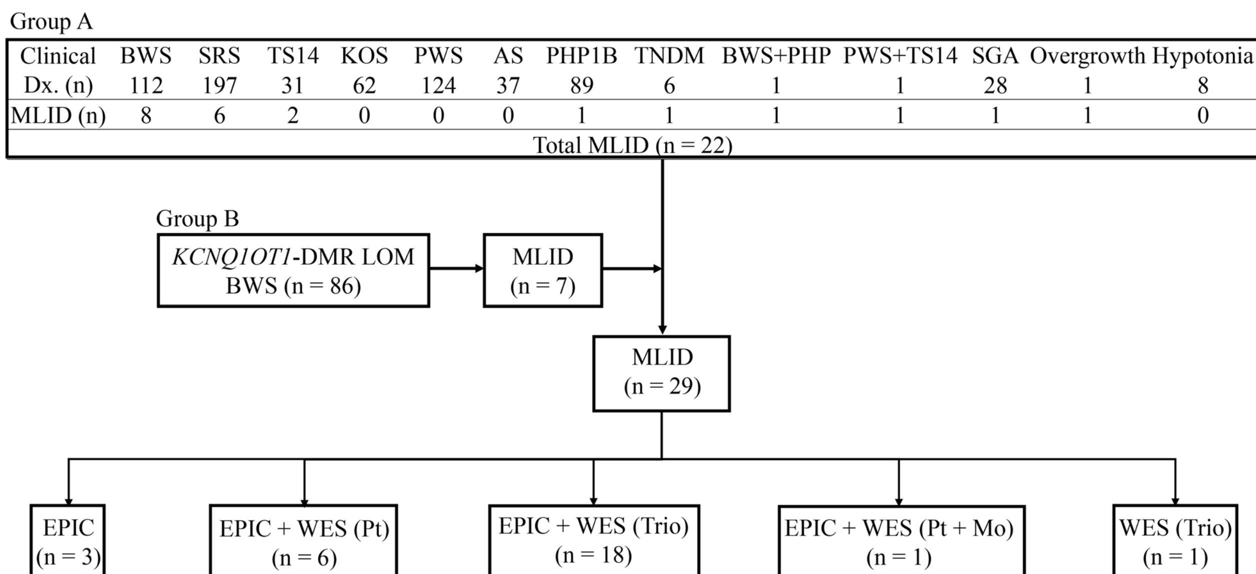


**Fig. 1** Study flowchart. Dx, diagnosis; BWS, Beckwith-Wiedemann syndrome; SRS, Silver-Russell syndrome; TS14, Temple syndrome; KOS, Kagami-Ogata syndrome; PWS, Prader-Willi syndrome; AS, Angelman syndrome; PHP1B, pseudohypoparathyroidism type 1B; TNDM, transient neonatal diabetes mellitus; SGA, small for gestational age; MLID, multi-locus imprinting disturbance; DMR, differentially methylated region; LOM, loss of methylation; EPIC, array-based methylation analysis using Infinium MethylationEPIC Kit (Illumina); WES, whole-exome sequencing; Pt, patient; Mo, mother

**Table 1** Frequency of imprinting disorders and multi-locus imprinting disturbance in group A

| Phenotypes | Genotypes (n) | | | | | | | Frequency of MLID | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Epimutation (MLID) | | UPD | | Epi or UPD | CNV | Total | max[a] | min[b] | Ochoa et al. (ref 39) |
| TNDM | *PLAGL1*:TSS-DMR LOM | 3 (1) | UPD(6)pat | 3 | 1 | 1 | 8 | 33.3% (1/3) | 25.0% (1/4) | 100.0% (2/2) |
| SRS | – | – | UPD(7)mat | 42 | – | – | 42 | – | – | – |
| SRS | *H19/IGF2*:IG-DMR LOM | 135 (6) | – | – | 6 | 141 | 4.4% (6/135) | 4.4% (6/135) | 11.8% (2/17) | – |
| BWS | *H19/IGF2*:IG-DMR GOM | 18 | UPD(11)pat | 36 | – | 7 | 108 | 12.3% (8/65) | 12.3% (8/65) | 39.5% (17/43) |
| BWS | *KCNQ1OT1*:TSS-DMR LOM | 47 (8) | | | | | | | | |
| TS14 | *MEG3*:TSS-DMR LOM | 21 (2) | UPD(14)mat | 26 | 5 | 4 | 56 | 9.5% (2/21) | 7.7% (2/26) | – |
| KOS | *MEG3*:TSS-DMR GOM | 18 (0) | UPD(14)pat | 26 | 4 | 14 | 62 | 0.0% (0/18) | 0.0% (0/22) | – |
| AS | *SNURF*:TSS-DMR LOM | 11 (0) | UPD(15)pat | 6 | 11 | 14 | 42 | 0.0% (0/11) | 0.0% (0/22) | – |
| PWS | *SNURF*:TSS-DMR GOM | 6 (0) | UPD(15)mat | 56 | 39 | 25 | 126 | 0.0% (0/6) | 0.0% (0/45) | – |
| PHP1B | *GNAS A/B*:TSS-DMR LOM | 37 (1) | UPD(20)pat | 5 | 11 | 37 | 90 | 2.7% (1/37) | 2.1% (1/48) | 0.0% (0/14) |
| SRS like | – | – | UPD(6)mat | 3 | – | – | 3 | – | – | – |
| SRS like | – | – | UPD(11)mat | 1 | – | – | 1 | – | – | – |
| SRS like | – | – | UPD(16)mat | 4 | – | – | 4 | – | – | – |
| SRS like | – | – | UPD(20)mat | 10 | – | – | 10 | – | – | – |
| PWS + TS14 | – | 1 (1) | | | | | 1 | – | – | – |
| BWS + PHP | – | 1 (1) | | | | | 1 | – | – | – |
| SGA | – | 1 (1) | | | | | 1 | – | – | – |
| Overgrowth | – | 1 (1) | | | | | 1 | – | – | – |
| All | – | 300 (22) | | 218 | 71 | 108 | 697 | 7.3% (22/300) | 5.9% (22/371) | 27.6% (21/76) |

[a] Frequency of MLID in epimutation. [b] Frequency of MLID in epimutation + epi or UPD. DMR, differentially methylated region; UPD, uniparental disomy; Epi, epimutation; CNV, copy number variant; MLID, multi-locus imprinting disturbance; TNDM, transient neonatal diabetes mellitus; SRS, Silver-Russell syndrome; BWS, Beckwith-Wiedemann syndrome; TS14, Temple syndrome; KOS, Kagami-Ogata syndrome; PWS, Prader-Willi syndrome; AS, Angelman syndrome; PHP, pseudohypoparathyroidism; SGA, small for gestational age; LOM, loss of methylation; GOM, gain of methylation; UPD(6)mat, maternal UPD of chromosome 6; UPD(6)pat, paternal UPD of chromosome 6; UPD(7)mat, maternal UPD of chromosome 7; UPD(7)pat, paternal UPD of chromosome 7; UPD(11)mat, maternal UPD of chromosome 11; UPD(11)pat, paternal UPD of chromosome 11; UPD(14)mat, maternal UPD of chromosome 14; UPD(14)pat, paternal UPD of chromosome 14; UPD(15)mat, maternal UPD of chromosome 15; UPD(15)pat, paternal UPD of chromosome 15; UPD(20)mat, maternal UPD of chromosome 20; UPD(20)pat, paternal UPD of chromosome 20

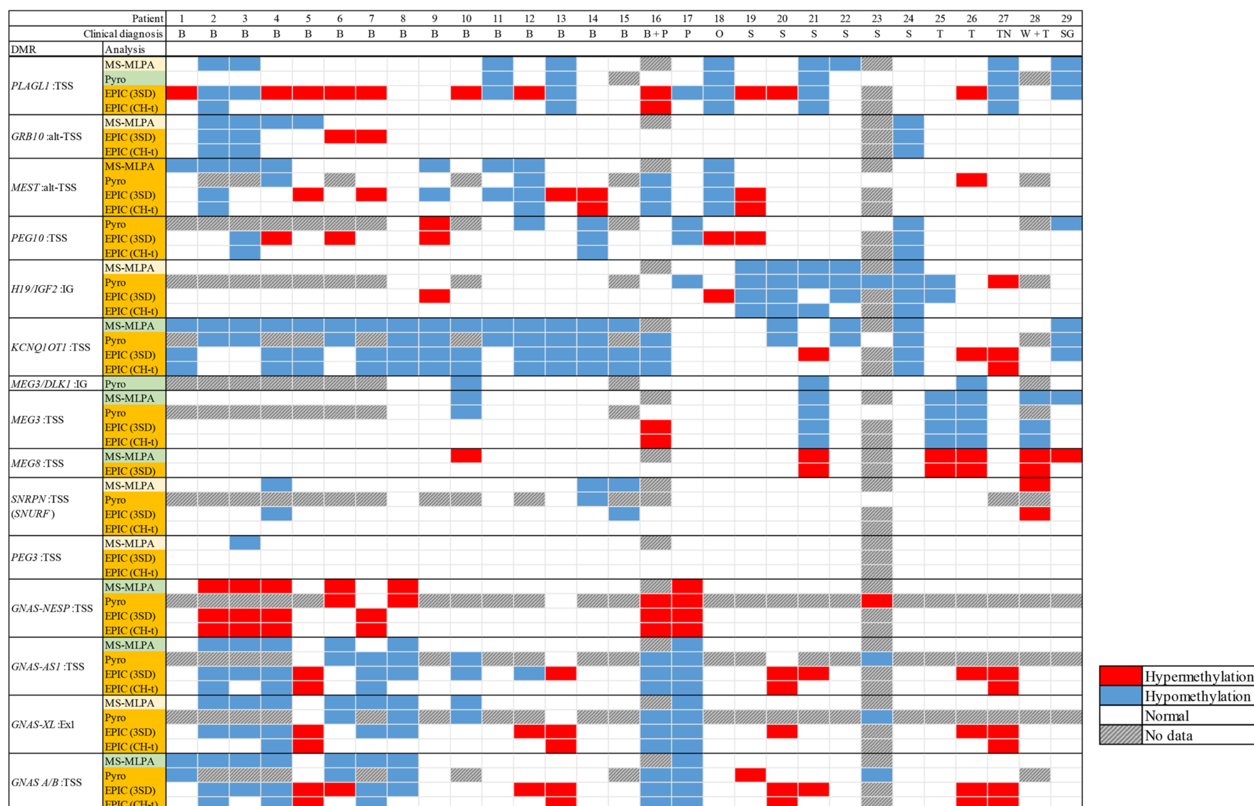


**Fig. 2** Results of methylation analyses using MS-MLPA, pyrosequencing, and array-based methylation analysis. The color-coded background in the Analysis row indicates the degree of coincidence of the locus of evaluated CpG sites in the DMR between MS-MLPA or pyrosequencing and array-based methylation analysis using Infinium MethylationEPIC Kit (Illumina): yellow for complete coincident, light yellow for partial coincident, and green for no coincident. DMR, differential methylated region; B, Beckwith-Wiedemann syndrome; P, pseudohypoparathyroidism type 1B; O, overgrowth; S, Silver-Russell syndrome; T, Temple syndrome; TN, transient neonatal diabetes mellitus; W, Prader-Willi syndrome; SG, small for gestational age; MS-MLPA, methylation-specific multiple ligation-dependent probe amplification; Pyro, pyrosequencing; EPIC (3SD), array-based methylation analysis using analysis method 1; EPIC (CH-t), array-based methylation analysis using analysis method 2

PHP1B phenotypes had hypomethylation of the *A/B*-DMR in addition to the *KCNQ1OT1*-DMR. Patient 17 with PHP1B phenotype, Patients 25 and 26 with TS14 phenotype, and Patient 27 with TNDM phenotype had aberrant methylation levels of four DMRs in the *GNAS* locus, hypomethylation of the *MEG3*-DMR, and hypomethylation of the *PLAGL1*-DMR, respectively. Patient 28 with some aspects of PWS and TS14 phenotypes had hypermethylation of the *SNRPN*-DMR and hypomethylation of the *MEG3*-DMR. Patient 18 with overgrowth had hypomethylation of the *PLAGL1*-DMR and *MEST*-DMR. Patient 29 with SGA had hypomethylation of the *PLAGL1*-DMR, *MEG3*-DMR, and *KCNQ1OT1*-DMR, and hypermethylation of the *MEG8*:TSS-DMR (*MEG8*-DMR).

### Array-based methylation analysis using EPIC

The methylation levels in all aberrant DMRs and raw data examined by array-based methylation analysis with Infinium MethylationEPIC Kit (EPIC) (Illumina) using analysis method 1 (see Methods section) are shown in Fig. 3 and Additional file 2: Table S2, respectively. We conducted array-based methylation analysis in all patients except Patient 23 together with normal controls. We extracted the data of methylation levels for 78 DMRs previously reported as DMRs by Monk [33] and Joshi [34] and compared them between patients and normal controls. Array-based methylation analysis identified the methylation disturbances in two or more clinically associated germline DMRs as with MS-MLPA and pyrosequencing analyses. Of the 78 DMRs, 56 DMRs had methylation disturbances in at least one patient. The median number of DMRs with aberrant methylation levels per patient was 17 (minimum–maximum: 3–27), the median number of abnormally hypomethylated DMRs was 12 (1–23), and the median number of abnormally hypermethylated DMRs was 4 (0–13). The most affected DMR was the *SNU13*:alt-TSS-DMR (*SNU13*-DMR), the most observed hypomethylated DMR was the *FANCC*:Int-DMR (*FANCC*-DMR),

and the most observed hypermethylated DMR was the *PLAGL1*-DMR. Although all patients with BWS phenotype had hypomethylation of the *KCNQ1OT1*-DMR by MS-MLPA and/or pyrosequencing analyses, the *KCNQ1OT1*-DMR in Patients 2, 3, 6, and 11 were classified as a normally methylated DMR by array-based methylation analysis despite some aberrantly hypomethylated CpG sites on this DMR. Similarly, all patients with SRS phenotypes had hypomethylation of the *H19*-DMR by MS-MLPA and/or pyrosequencing analyses. Although Patient 21 had some aberrantly hypermethylated CpG sites within *KCNQ1OT1*-DMR and several hypomethylated CpGs within *H19*-DMR by array-based methylation analysis using analysis method 1, microsatellite analysis for chromosome 11 using this patient's and parental samples showed biparental origin without mosaic (Additional file 3: Table S3). Patients 16 and 17 with resistance to parathyroid hormone (PTH) showed hypomethylation of the *A/B*-DMR, *GNAS-AS1*:TSS-DMR (*AS1*-DMR), and *GNAS-XL*:Ex1-DMR (*XL*-DMR) and hypermethylation of the *GNAS-NESP*:TSS-DMR (*NESP*-DMR). Patients 11, 12, and 15 with BWS-MLID had some SRS-like clinical features, such as feeding difficulties, hypotonia, and a protruding forehead without methylation disturbance of the *H19*-DMR responsible for SRS and the DMRs responsible for TS14 and PWS, which have overlapping clinical features with those of SRS. We obtained genomic DNA from the leukocytes of monozygotic twin siblings of Patients 9 and 12 and conducted array-based methylation analysis (Additional file 2: Table S2). Patient 9 and her twin sister had similar methylation status. Regarding Patient 12 and his twin brother, the DMRs with methylation defects were almost identical, although the numbers of CpGs with aberrant methylation in the DMRs and abnormal methylation levels in the CpG sites were more frequent and severe in Patient 12 than in his twin brother. No specific methylation disturbance pattern was observed in patients with neurodevelopmental delay and/or intellectual developmental disorder (ND/IDD) or in those who were conceived by assisted reproductive technology (ART).

**Comparison among methylation analyses**

First, we compared the methylation patterns between DMRs targeted by MS-MLPA (ME034) and/or pyrosequencing and array-based methylation analysis using analysis method 1 (see Methods section). Although CpG sites in the DMRs targeted by MS-MLPA Probe-mix ME034 (MRC-Holland), pyrosequencing, and array-based methylation analysis were not identical (Fig. 2 and Additional file 4: Table S4), over half of the DMRs with aberrant hypomethylation were consistent across these different methylation analysis methods. Array-based methylation analysis more frequently identified the DMRs showing hypermethylation, and aberrant hypermethylation was not consistent other than the *NESP*-DMR and *MEG8*-DMR. Next, to evaluate the DMRs with discrepancies in the results between MS-MLPA and/or pyrosequencing and array-based methylation analysis, we conducted both MS-MLPA and pyrosequencing analyses in these DMRs, although we could not conduct these analyses in Patients 16, 23, and 27 due to a shortage of DNA samples. In addition, we re-analyzed methylation-array data applying more stringent bioinformatic parameters (analysis method 2) (see Methods section). Additional analyses showed decreased numbers of DMRs with discrepancies in the results between MS-MLPA and/or pyrosequencing and array-based methylation analysis. In particular, the number of aberrantly hypermethylated DMRs in array-based methylation analysis with discrepancies in the results of MS-MLPA and/or pyrosequencing and array-based methylation analysis, such as the *PLAGL1*-DMR, *MEST*-DMR, *H19*-DMR, and *KCNQ1OT1*-DMR, decreased. Furthermore, we compared two analysis methods in array-based methylation analysis to examine the frequency-matching methylation patterns in the DMRs between methylation analysis using MS-MLPA or pyrosequencing and array-based methylation analysis. We evaluated a total of 351 DMRs, which had data of methylation analysis using MS-MLPA and/or pyrosequencing and array-based methylation analysis in 29 patients. Of 351 DMRs, methylation patterns detected by array-based methylation analysis using analysis methods 1 and 2 were the same as those by methylation

(See figure on next page.)

**Fig. 3** Heatmap of array-based methylation analysis using analysis method 1. The heatmap indicates 56 aberrant DMRs out of 78 examined DMRs. Each row represents a DMR; each column represents a patient. Germline DMRs are shown on a gray background, secondary DMRs on a light green background, and unclassifiable DMRs on a white background. Methylation disturbances of DMRs are classified into seven categories based on the degree. B, Beckwith-Wiedemann syndrome; P, pseudohypoparathyroidism type 1B; O, overgrowth; S, Silver-Russell syndrome; TS, Temple syndrome; TN, transient neonatal diabetes mellitus; W, Prader-Willi syndrome; SG, small for gestational age; N, neurodevelopmental delay and/or intellectual developmental disorder; A, assisted reproductive technology; T, monozygotic monochorionic diamniotic twins; V, variants of uncertain significance; L, likely pathogenic; DMR, differential methylated region; Chr, chromosome; MML, median methylation level of CpG sites in the DMR; SD, standard deviation; mean, mean MML of 16 healthy controls

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 24 | 25 | 26 | 27 | 28 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clinical diagnosis | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B | B+P | P | O | S | S | S | S | S | TS | TS | TN | W+TS | SG |
| Clinical feature | | | | N | | | | N | | N | N | N | | | | | | | | N | N | | N | N | N | | N | N |
| Other features | | A | A | A | | A | A | | T | A | | T | A | T | | | | | | | | | | | | | | |
| Variants | | V | V | | | | | | V | | V | | | | | | | | V | | | LP | LP | LP | | | | |

| DMR | Chr |
|---|---|
| *PPIEL* :Ex1 | 1 |
| *DIRAS3* :Ex2 | 1 |
| *DIRAS3* :TSS | 1 |
| *OBSCN* | 1 |
| *MIR1273E* | 1 |
| *LOC151121* | 1 |
| *PYHIN1* | 1 |
| *C1orf177* | 1 |
| *ZDBF2/GPR1* :IG | 2 |
| *NAPL1L5* :TSS | 4 |
| *JAKMIP1* | 4 |
| *FAM50B* :TSS | 6 |
| *PLAGL1* :alt-TSS | 6 |
| *IGF2R* :Int2 | 6 |
| *WDR27* :Int13 | 6 |
| *GRB10* :alt-TSS | 7 |
| *PEG10* :TSS | 7 |
| *MEST* :alt-TSS | 7 |
| *HTR5A* :TSS | 7 |
| *SVOPL* :alt-TSS | 7 |
| *RPS2P32* | 7 |
| *CHD7* | 8 |
| *LOC101927815_2* | 8 |
| *ERLIN2* :Int6 | 8 |
| *PEG13* :TSS | 8 |
| *FANCC* :Int1 | 9 |
| *INPP5F* :Int2 | 10 |
| *H19/IGF2* :IG | 11 |
| *IGF2* :Ex9 | 11 |
| *IGF2* :alt-TSS | 11 |
| *KCNQ1OT1* :TSS | 11 |
| *RB1* :Int2 | 13 |
| *DLK1* | 14 |
| *MEG3* :TSS | 14 |
| *MEG8* :Int2 | 14 |
| *PWRN4* | 15 |
| *SNRPN* -Int1 | 15 |
| *SNRPN*: alt-TSS | 15 |
| *SORD* | 15 |
| *SNURF* :TSS | 15 |
| *IGF1R* :Int2 | 15 |
| *MKRN3* :TSS | 15 |
| *ZNF597* :3' | 16 |
| *ZNF597* :TSS | 16 |
| *ZNF331* :alt-TSS1 | 19 |
| *ZNF331* :alt-TSS2 | 19 |
| *MCTS2P* :TSS | 20 |
| *NNAT* :TSS | 20 |
| *L3MBTL1* :alt | 20 |
| *GNAS-NES* P:TSS | 20 |
| *GNAS-AS1* :TSS | 20 |
| *GNAS-XL* :Ex1 | 20 |
| *GNAS A/B* :TSS | 20 |
| *WRB* :alt-TSS | 21 |
| *LIPI* | 21 |
| *SNU13* :alt-TSS | 22 |

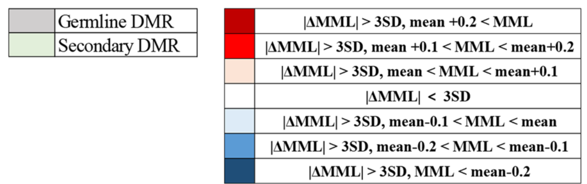


**Fig. 3** (See legend on previous page.)

analysis using MS-MLPA and/or pyrosequencing in 281 (80.1%) and 294 (83.8%) DMRs, respectively. In aberrantly hypomethylated DMRs, methylation patterns detected by array-based methylation analysis using analysis methods 1 and 2 were the same as those by methylation analysis using MS-MLPA and/or pyrosequencing in 67 (19.1%) and 47 (13.4%) DMRs, respectively. In aberrantly hypermethylated DMRs, methylation patterns detected by array-based methylation analysis using analysis methods 1 and 2 were the same as those by methylation analysis using MS-MLPA and/or pyrosequencing in 7 (2.0%) and 5 (1.4%) DMRs, respectively. In normally methylated DMRs, methylation patterns detected by array-based methylation analysis using analysis methods 1 and 2 were the same as those by methylation analysis using MS-MLPA and/or pyrosequencing in 207 (59.0%) and 242 (68.9%) DMRs, respectively.

#### Whole-exome sequencing (WES)

The results of the WES analysis are shown in Table 4. In mothers of MLID patients, we identified the following rare heterozygous variants in the genes encoding proteins constituting SCMC: one *NLRP2* frameshift variant, two *PADI6* frameshift variants, three *NLRP2* missense variants, one *NLRP5* missense variant, and one *PADI6* missense variant. Of five missense variants, the variants in *NLRP2* (rs1183506640, rs142785605) and *PADI6* (rs372065243) have been registered as dbSNPs (https://www.ncbi.nlm.nih.gov/snp/) with extremely rare allele frequencies, and the remaining two variants have not been registered. All missense variants were predicted to be pathogenic by at least one of the bioinformatic prediction tools. According to the American College of Medical Genetics and Genomics (ACMG) guidelines [35], these nine variants were classified as four likely pathogenic (LP) variants and five variants of uncertain significance (VUS). Patient 25 with homozygous pathogenic variants in *ZNF445* has been previously reported [11]. Patients 22, 23, 24, and 25 with LP variants had prenatal and postnatal growth failure with suspected SRS or TS14; their mothers conceived the children without ART (Fig. 3, Table 4, and Additional file 1: Table S1). The LP variants in *ZNF445*, *PADI6*, and *NLRP2* were observed only in one patient, two patients, and one patient, respectively. Therefore, we could not determine the association between methylation defect pattern and genotype.

#### Clinical characteristics

We show the clinical characteristics of 29 patients with MLID in Additional file 1: Table S1 and summarize their clinical characteristics in each clinical diagnostic category (Table 2). The clinically suspected diagnoses at the time referred for genetic analysis were BWS in 15 patients, SRS in six patients, PHP1B in a single patient, BWS and PHP1B in a single patient, TS14 in two patients, TNDM in a single patient, overgrowth in a single patient, PWS and TS14 in a single patient, and SGA infant in a single patient. Eleven patients were male, and the remaining 18 patients were female. The median gestational age was 37 weeks, and the median paternal and maternal ages were 35 years and 33 years, respectively. Seven out of 28 patients (25.0%) were conceived by ART and all seven of these patients had BWS-MLID. Namely, seven out of 14 BWS-MLID patients were born following ART (50.0%). Over 70% of patients were first births. Of the mothers with accessible pregnancy history, one mother experienced a miscarriage. Neurodevelopmental delay and/or intellectual developmental disorder was observed in about half of the patients with MLID. There were no patients with tumor complications. The median BWS spectrum (BWSp) score [36] in BWS-MLID was 7 and the median Netchine-Harbison clinical scoring system (NH-CSS) [37] in SRS-MLID was 5 (Table 3). Correlation between the BWSp score and the number of aberrantly methylated DMRs in patients with BWS-MLID was not observed ($r = 0.48$, $P = 0.059$). Correlation between the NH-CSS score and the number of aberrantly methylated DMRs in patients with SRS-MLID and TS14-MLID was also not identified ($r = -0.08$, $P = 0.867$). In the comparison of the frequencies of clinical features, body asymmetry, polyhydramnios/placentomegaly, and ND/IDD were significantly higher in BWS-MLID than in BWS with hypomethylation of the *KCNQ1OT1*-DMR alone (*KCNQ1OT1*-BWS) caused by epimutation. The median BWSp scores were significantly higher in BWS-MLID than in *KCNQ1OT1*-BWS. Similarly, protruding forehead and ND/IDD were significantly more common in SRS-MLID than in SRS with hypomethylation of the *H19*-DMR alone (*H19*-SRS) caused by epimutation. Clinical features of each patient with MLID are shown in Additional file 1: Table S1. Patients 11, 12, and 15 in the BWS-MLID group exhibited mild hypotonia, feeding difficulties, and a protruding forehead, respectively, which are frequently observed in SRS. Patient 28 showed both PWS and TS14 phenotypes, including almond-shaped palpebral fissures, small hands, and severe hypotonia. Patient 29 presented with a mixed phenotype of some IDs, including prenatal growth restriction, microcephaly, macroglossia, and intellectual disability. Scoliosis was present in two patients (Patients 1 and 12) and café-au-lait spots in two patients (Patients 18 and 20). Round face and brachydactyly included Albright hereditary osteodystrophy (AHO) features and hypocalcemia, which are commonly observed in PHP [38], were detected only

**Table 2** Summary of clinical findings in patients with multi-locus imprinting disturbance

| Diagnosis | BWS | BWS + PHP1B | PHP1B | Overgrowth | SRS | TS14 | TNDM | PWS + TS14 | SGA | All |
|---|---|---|---|---|---|---|---|---|---|---|
| Number of patients | 15 | 1 | 1 | 1 | 6 | 2 | 1 | 1 | 1 | 29 |
| | Pts. 1–15 | Pt. 16 | Pt. 17 | Pt. 18 | Pts. 19–24 | Pts. 25–26 | Pt. 27 | Pt. 28 | Pt. 29 | |
| BL (SDS) | 1.6 [– 0.5 to 4.0] | 2.1 | 2.0 | 2.7 | – 3.5 [– 4.1 to – 1.1] | – 2.4 [– 2.9 to – 1.9] | 1.0 | – 2.5 | – 1 | 0.8 [– 4.1 to 4.0] |
| BW (SDS) | 2.3 [0.4 to 5.2] | 2.1 | 3.8 | 1.2 | – 3.7 [– 4.1 to – 1.9] | – 3.5 [– 3.8 to – 3.2] | – 0.4 | – 4.1 | – 3.6 | 1.6 [– 4.1 to 5.2] |
| BOFC (SDS) | 1.5 [– 1.8 to 2.3] | ND | ND | – 0.8 | – 1.1 [– 1.4 to 0.6] | – 0.7 [– 0.9 to – 0.4] | 0.4 | – 1.1 | 0.9 | 0.4 [– 1.8 to 2.3] |
| GA (weeks:days) | 36:5 [23:2 to 41:1] | 35:0 | 37:0 | 42:0 | 37:2 [30:2 to 37:6] | 37:6 [34:6 to 41:0] | 38:0 | 31:0 | 35:0 | 37:0 [23:2 to 42:0] |
| Paternal age (y) | 36 [28 to 48] | 35 | 50 | 26 | 33.5 [25 to 45] | 33 [31 to 35] | 32 | 36 | 41 | 35 [25 to 50] |
| Maternal age (y) | 33 [24 to 41] | 34 | 47 | 26 | 32.5 [23 to 38] | 32.5 [27 to 38] | 22 | 38 | 29 | 33 [22 to 47] |
| First childbirth | 11/15 | 1/1 | 0/1 | 1/1 | 4/5 | 1/2 | 1/1 | 0/1 | 1/1 | 20/28 |
| Miscarriage | 0/9 | 0/1 | ND | 0/1 | 1/2 | ND | 0/1 | 0/1 | 0/1 | 1/16 |
| ART | 7/14 | 0/1 | 0/1 | 0/1 | 0/6 | 0/2 | 0/1 | 0/1 | 0/1 | 7/28 |
| BWSp scores | 7 [3–11] | 9 | 1 | 0 | 2 [1–3] | 2 [2] | 0 | 1 | 3 | 3 [0–11] |
| Macroglossia | 15/15 | 1/1 | 0/1 | 0/1 | 0/5 | 0/2 | ND | 0/1 | 1/1 | 17/27 |
| Exomphalos | 5/12 | 1/1 | 0/1 | 0/1 | 0/4 | 0/2 | 0/1 | 0/1 | 0/1 | 6/24 |
| Hypoglycemia | 6/14 | 1/1 | ND | 0/1 | 1/3 | ND | 0/1 | 0/1 | ND | 8/21 |
| Overgrowth at birth | 11/15 | 1/1 | 1/1 | 0/1 | 0/6 | 0/2 | 0/1 | 0/1 | 0/1 | 13/29 |
| Facial naevus simplex | 5/15 | 0/1 | 0/1 | 0/1 | 1/4 | 0/2 | ND | 0/1 | 0/1 | 6/26 |
| Ear creases/pits | 8/15 | 0/1 | ND | 0/1 | 0/6 | 0/2 | ND | 0/1 | 0/1 | 8/27 |
| Umbilical hernia | 10/14 | 0/1 | 0/1 | 0/1 | 0/3 | 0/2 | ND | 0/1 | 1/1 | 11/24 |
| Polyhydramnios/ Placentomegaly | 7/11 | 1/1 | ND | 0/1 | 0/3 | 0/2 | ND | 1/1 | 0/1 | 9/20 |
| Organomegaly | 1/15 | 0/1 | 0/1 | 0/1 | 0/3 | 0/2 | 0/1 | 0/1 | 0/1 | 1/26 |
| NH-CSS | 0 [0–1] | 1 | 0 | 0 | 5 [4, 5] | 5.5 [5, 6] | 0 | 4 | 2 | 0 [0–6] |
| SGA | 0/15 | 0/1 | 0/1 | 0/1 | 5/6 | 2/2 | 0/1 | 1/1 | 1/1 | 9/29 |
| Feeding difficulty | 1/6 | 0/1 | 0/1 | 0/1 | 2/6 | 1/2 | ND | 1/1 | ND | 5/18 |
| Protruding fore-head | 1/8 | 0/1 | 0/1 | 0/1 | 6/6 | 2/2 | ND | 0/1 | 0/1 | 9/21 |
| Postnatal growth failure | 0/10 | 0/1 | 0/1 | 0/1 | 6/6 | 2/2 | ND | 1/1 | 0/1 | 9/23 |
| Body asymmetry | 7/15 | 1/1 | 0/1 | 0/1 | 4/6 | 2/2 | ND | 0/1 | 0/1 | 14/28 |
| Relative macro-cephaly | 0/9 | 0/1 | 0/1 | 0/1 | 6/6 | 2/2 | 0/1 | 1/1 | 1/1 | 10/23 |

**Table 2** (continued)

| Diagnosis | BWS | BWS + PHP1B | PHP1B | Overgrowth | SRS | TS14 | TNDM | PWS + TS14 | SGA | All |
|---|---|---|---|---|---|---|---|---|---|---|
| **Number of patients** | **15** | **1** | **1** | **1** | **6** | **2** | **1** | **1** | **1** | **29** |
| | **Pts. 1–15** | **Pt. 16** | **Pt. 17** | **Pt. 18** | **Pts. 19–24** | **Pts. 25–26** | **Pt. 27** | **Pt. 28** | **Pt. 29** | |
| Hypotonia | 1/6 | 0/1 | 0/1 | 0/1 | 3/6 | 2/2 | 0/1 | 1/1 | ND | 7/19 |
| ND/IDD | 5/12 | 0/1 | 0/1 | 0/1 | 3/5 | 2/2 | 0/1 | 1/1 | 1/1 | 12/25 |
| Tumor | 0/15 | 0/1 | 0/1 | 0/1 | 0/4 | 0/2 | 0/1 | 0/1 | 0/1 | 0/27 |
| Other features (n) | Scoliosis (2) | Round face, PTH resistance | Brachydactyly, PTH resistance | Cafe-au-lait spots | Cafe-au-lait spots (1) , Low-set ears (1) | S–H (2), J-H (2), High palate (1) | – | S–H, A-S, Round face | Microcephaly | |
| Variant (n)[a] | - | - | - | - | *PADI6* (2), *NLPR2* (1) | *ZNF445* (1) | - | - | - | |

Data are presented as median [min–max]. [a] Pathogenic and Likely pathogenic (ACMG guidelines). BWS, Beckwith-Wiedemann syndrome; PHP1B, pseudohypoparathyroidism type 1B; SRS, Silver-Russell syndrome; TS14, Temple syndrome; TNDM, transient neonatal diabetes mellitus; PWS, Prader-Willi syndrome; SGA, small for gestational age; Pt., patient; BL, birth length; BW, birth weight; BOFC, birth occipitofrontal circumference; SDS, standard deviation score; GA, gestational age; w, week; y, year; ART, assisted reproductive technology; BWSp, BWS spectrum; NH-CSS, Netchine-Harbison clinical scoring system, ND/IDD, neurodevelopmental delay and/or intellectual developmental disorder; PTH, parathyroid hormone; S–H, small hands; J-H, joint hypermobility; A-S, almond-shaped eyes; ND, no data

**Table 3** Comparison of clinical features between MLID and non-MLID

| Clinical features | Group A | | | | *P* value in group A | | Previous report [ref 12] | |
|---|---|---|---|---|---|---|---|---|
| | BWS-MLID | *KCNQ1OT1*-BWS[a*] | SRS-MLID | *H19*-SRS[b*] | BWS-MLID vs BWS | SRS-MLID vs SRS | BWS-MLID | SRS-MLID |
| GA (weeks:days) median [min–max] | 36:5 [23–41] | 36:4 [24:0–40:5] | 37:2 [30:2–37:6] | 37:1 [29:0–41:6] | 0.882 | 0.630 | 36.1 (mean) | 35.5 (mean) |
| BL (SDS) | 1.6 ± 1.3 | 1.0 ± 1.2 | −3.2 ± 1.1 | −3.3 ± 1.0 | 0.180 | 0.689 | 0.51 | −2.8 |
| BW (SDS) | 2.5 ± 1.1 | 2.0 ± 1.1 | −3.4 ± 0.8 | −3.4 ± 1.0 | 0.191 | 0.980 | 0.5 | −2.6 |
| Macroglossia | 100% (15/15) | 100% (29/29) | | | 0.999 | | 87.5% (14/16) | |
| Exomphalos | 41.7% (5/12) | 42.9% (12/28) | | | 0.999 | | 42.9% (6/14) | |
| Body asymmetry | 46.7% (7/15) | 8.3% (2/24) | | | **0.015** | | 61.5% (8/13) | |
| Overgrowth at birth | 73.3% (11/15) | 55.6% (15/27) | | | 0.330 | | 15.4% (2/13) | |
| Facial naevus simplex | 33.3% (5/15) | 37.9% (11/29) | | | 0.999 | | 83.3% (10/12) | |
| Polyhydramnios/Placentomegaly | 63.6% (7/11) | 23.1% (6/26) | | | **0.028** | | 60.0% (9/15) | |
| Ear creases/pits | 53.3% (8/15) | 78.6% (22/28) | | | 0.162 | | 60.0% (9/15) | |
| Hypoglycemia | 42.9% (6/14) | 37.0% (10/27) | | | 0.747 | | 43.8% (7/16) | |
| Nephromegaly/Hepatomegaly | 6.7% (1/15) | 20.0% (5/25) | | | 0.381 | | 7.1% (1/14) | |
| Umbilical hernia/Diastasis recti | 71.4% (10/14) | 57.1% (16/28) | | | 0.505 | | 40.0% (6/15) | |
| BWSp scores median [min–max] | 7 [3–11] | 6 [3–8] | | | **0.041** | | 7 [2–13] | |
| SGA | | | 83.3% (5/6) | 98.1% (54/55) | | 0.189 | | 87.5% (7/8) |
| Postnatal growth failure | | | 100.0% (6/6) | 94.1% (48/51) | | 0.999 | | 75.0% (6/8) |
| Relative Macro-cephaly | | | 100.0% (6/6) | 87.5% (42/48) | | 0.999 | | 71.4% (5/7) |
| Protruding fore-head | | | 100.0% (6/6) | 51.0% (26/51) | | **0.030** | | 85.7% (6/7) |
| Body asymmetry | | | 66.7% (4/6) | 44.2% (23/52) | | 0.402 | | 75.0% (6/8) |
| Feeding difficulty | | | 33.3% (2/6) | 43.8% (21/48) | | 0.999 | | 42.9% (3/7) |
| NH-CSS median [min–max] | | | 5 [4–5] | 4 [2–6] | | 0.063 | | 4 [2–5] |
| ART | 50.0% (7/14) | 23.1% (6/26) | 0.0% (0/6) | 18.6% (8/43) | 0.155 | 0.571 | 37.5% (6/16) | 12.5% (1/8) |
| ND/IDD | 41.7% (5/12) | 0.0% (0/29) | 60.0% (3/5) | 14.3% (6/42) | **0.001** | **0.042** | 35.7% (5/14) | 50.0% (3/6) |

[*] We collected clinical features in *KCNQ1OT1*-BWS and *H19*-SRS from the attending physicians of the patients who received genetic diagnoses in our laboratory (unpublished data). [a] BWS with loss of methylation at the *KCNQ1OT1*:TSS-differentially methylated region alone. [b] SRS with loss of methylation at the *H19/IGF2*:IG-differentially methylated region alone. BWS, Beckwith-Wiedemann syndrome; MLID, multi-locus imprinting disturbance; SRS, Silver-Russell syndrome; BWSp, Beckwith-Wiedemann spectrum; SGA, small for gestational age; NH-CSS, Netchine-Harbison Clinical Scoring System; ART, assisted reproductive technology; ND/IDD, neurodevelopmental delay and/or intellectual developmental disorder; GA, gestational age; BL, birth length; BW, birth weight; SDS, standard deviation score. Bold means $P < 0.05$

in Patients 16 and 17. Two patients with SRS-MLID, two patients with TS14-MLID, and one patient with PWS + TS14-MLID underwent growth hormone treatment. Patients 8, 9, and 12 with BWS-MLID were born as monozygotic monochorionic diamniotic twins. In all three patients, their twin siblings had no BWS phenotype (BWSp score: 0), although the twin brother of Patient 12 had mild intellectual disability.

## Discussion

We conducted comprehensive molecular and clinical analyses in 29 MLID patients with various ID-associated phenotypes detected by pyrosequencing and/or MS-MLPA and revealed the following findings. First, our study consisting of patients with eight IDs caused by epimutation showed that the frequency of MLID ranged from 5.9% to 7.3%. Ochoa et al. reported that

**Table 4** Results of whole-exome sequencing

| Individuals with rare variants | Genes | Variant | Protein | dbSNP | GnomAD allele frequency | Prediction | | | | ClinVar | ACMG guidelines | Zygosity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | v4.0.0 | CADD PHRED | SIFT | Poly-phen2 | Mutation taster | | | |
| Pt. 2 and Mo | *NLRP2* | c.1564G > A | p.(Glu522Lys) | rs1183506640 | 0.000001253 | 0.8 | T | PD | Benign | – | VUS (PM2) | hetero |
| Pt. 9 and Mo | *NLRP2* | c.592C > T | p.(Pro198Ser) | - | - | 19.5 | T | PD | Benign | – | VUS (PM2) | hetero |
| Mo of Pt. 11 | *NLRP2* | c.1521G > T | p.(Gln507His) | rs142785605 | 0.00004213 | 23.4 | D | D | Benign | – | VUS (PM2 + PP3) | hetero |
| Pt. 24 and Mo | *NLRP2* | c.492_493insGA | p.(Ala164fs) | – | – | – | – | – | Deleterious | – | Likely Pathogenic (PVS1 + PM2) | hetero |
| Mo of Pt. 3 | *NLRP5* | c.437 T > C | p.(Met146Thr) | – | – | 19.0 | T | PD | Benign | – | VUS (PM2) | hetero |
| Pt. 19 and Mo | *PADI6* | c.1247 T > C | p.(Ile416Thr) | rs372065243 | 0.00006011 | 23.4 | D | PD | – | LP[a] | VUS (PM1 + PM2 + PP3) | hetero |
| Mo of Pt. 22 | *PADI6* | c.526dupA | p.(Lys175fs) | – | – | – | – | – | – | – | Likely Pathogenic (PVS1 + PM2) | hetero |
| Pt. 23 and Mo | *PADI6* | c.609_612del | p.(Leu203fs) | – | – | – | – | – | – | – | Likely Pathogenic (PVS1 + PM2) | hetero |
| Pt. 25 | *ZNF445* | c.2803C > T | p.(Gln935*) | – | – | 35.0 | – | – | Deleterious | – | Likely Pathogenic (PVS1 + PM2) | homo |

[a] Biallelic variants in early embryonic arrest. Pt., patient; Mo, mother; CADD, combined annotation-dependent depletion; SIFT, Sorting Intolerant from Tolerant; T, tolerated; D, damaging; PD, possibly damaging; LP, likely pathogenic; VUS, variant of unknown significance; hetero, heterozygote; homo, homozygote

21 of 76 (27.6%) cases only with BWS, SRS, PHP1B, and TNDM caused by epimutation were detected with MLID by ImprintSeq using a custom targeted methylation sequencing panel (Table 1) [39]. These findings suggest that differences in target IDs, analysis methods, and definitions of MLID result in different frequencies of MLID. Recently, an interim joint statement for clinical and molecular diagnosis of MLID has been published [5]. Based on this agreement, further accumulation of cases with MLID and progress in research on MLID are expected. Focusing on the results of each ID, MLID was detected in approximately 12% of patients with BWS phenotype and approximately 5% of patients with SRS phenotype, but not patients with KOS, PWS, or AS phenotypes. Consistent with this, MLID has been most frequently reported in cases with BWS and SRS [12, 13, 15, 17–22, 24–30]. On the other hand, MLID has been reported in only one case with PWS and AS and none with KOS [2, 40]. The frequency of epimutation differs in each ID. Epimutation has a higher frequency in etiologies of BWS and SRS, but a lower frequency in KOS, AS, and PWS. Furthermore, patients with MLID had more aberrantly hypomethylated DMRs than hypermethylated DMRs. A higher frequency of MLID in BWS and SRS may be associated with these matters.

Second, our study revealed a high frequency of ART-conceived patients. All ART-conceived patients had BWS-MLID and seven of 14 (50.0%) patients with BWS-MLID were ART-conceived patients. The frequency of ART-conceived livebirths in the general population of Japan in 2021 was 8.6% [http://www.mhlw.go.jp/toukei/list/81-1.html, https://www.jsog.or.jp/]. Previously, our group reported that the frequency of ART-conceived cases was 25.8% of cases with BWS caused by epimutation [32]. In this report, four of 31 cases with BWS had MLID, and three cases with BWS-MLID were natural pregnancies. In brief, seven of the 27 cases with single locus epimutation (25.9%) were ART-conceived cases. These findings suggest that ART increases the risk of development of BWS-MLID, and *KCNQ1OT1*-DMR is a DMR with susceptibility to the development of methylation defects by ART, as previously reported [12]. Regarding the history of miscarriage, only a single mother (6.3%) experienced miscarriage (Table 2), but she had no pathogenic variant in MLID-causative genes. In the previous report, a history of miscarriage was detected in 20.8% of the mothers of MLID cases, and 40% of them had candidate variants in MLID-causative genes [12]. In our study, the mothers with rare variants in the genes encoding proteins that are maternal factors had no history of miscarriage or use of ART. We assumed that the variants detected in the mothers did not cause infertility. To date, approximately 60 candidate variants in the MLID-causative genes have been identified in the mothers of the cases with MLID [9, 28, 41], and only a single mother had a pathogenic variant and needed ART for conception [28]. When women with pathogenic variants in MLID-causative genes require ART for pregnancy, more severe phenotypes, such as early embryonic arrest, but not MLID, may occur.

Third, we characterized several clinical presentations in patients with MLID and identified a higher frequency of ND/IDD complications. Even though ND/IDD is not a primary feature of IDs other than PWS, AS, or KOS [2], we identified ND/IDD in 48.0% (12/25) of MLID cases, as in the previous study detecting ND in 35% of BWS-MLID and 50% of SRS-MLID [12]. Of our MLID patients with ND/IDD, Patients 4 and 28 showed aberrant methylation of the DMRs in the PWS/AS imprinted region, which can lead to ND/IDD. However, the remaining patients showed normal methylation levels of these DMRs. Aberrant methylation of DMRs regulating the imprinted genes with unknown functions may be relevant to ND/IDD. When the cases with IDs having no ND/IDD, such as BWS and SRS, show ND/IDD, we need to consider the possibility of MLID. The median BWSp score in BWS-MLID was significantly higher than that in *KCNQ1OT1*-BWS, and body asymmetry and polyhydramnios/placentomegaly were significantly higher in BWS-MLID than in *KCNQ1OT1*-BWS, as in the previous study (Table 3) [12]. Aberrant methylation of DMRs other than the *KCNQ1OT1*-DMR may contribute to body asymmetry and polyhydramnios/placentomegaly, although candidate DMRs are unclear. Three patients in the BWS-MLID group exhibited clinical features frequently observed in SRS, but they had no abnormal methylation of the DMRs associated with SRS phenotype, including hypomethylation of the *H19*-DMR, hypomethylation of the *MEG3*-DMR, and hypermethylation of the *SNRPN*-DMR. On the other hand, Patient 29, having atypical clinical features of various IDs, including SGA, postnatal normal growth, macroglossia, microcephaly, and ND/IDD, showed hypomethylation of the *PLAGL1*-DMR leading to growth restriction and hypomethylation of the *KCNQ1OT1*-DMR leading to a BWS phenotype. The association between methylation disturbance of DMRs and clinical features remains to be completely elucidated. Hypomethylation of the *A/B*-DMR causes PHP1B, leading to resistance to PTH in almost all cases and AHO features in some cases. In our study, only two of six patients with hypomethylation of the *A/B*-DMR detected by array-based methylation analysis had resistance to PTH and some AHO features. Methylation levels of the *A/B*-DMR in these two patients were much lower than in the remaining patients without PTH resistance (Additional file 2: Table S2) as well as in previous

studies [12, 27]. Hypomethylation of the *A/B*-DMR below a certain threshold may result in PTH resistance in MLID cases. No tumor complications have been reported in MLID cases, as in our study. Although BWS has the risk of tumor complications, *KCNQ1OT1*-BWS has a relatively low risk [36]. BWS-MLID with hypomethylation of the *KCNQ1OT1*-DMR may have a low risk of tumor complications.

Fourth, we identified the characteristics of methylation disturbances in 29 patients with MLID. Aberrant hypomethylated DMRs were more common than aberrant hypermethylated DMRs in MLID cases. In methylation analysis using pyrosequencing and/or MS-MLPA and array-based methylation analysis, the aberrant hypomethylated pattern was more consistent than the aberrant hypermethylated pattern (Fig. 2, Fig. 3, and Additional file 2: Table S2). Comparison of two analysis methods in array-based methylation analysis for the frequency-matching methylation patterns in the DMRs between methylation analysis using MS-MLPA or pyrosequencing and array-based methylation analysis showed that method 1 had high sensitivity, and analysis method 2 using the more stringent bioinformatic parameters had high specificity. In fact, the number of aberrantly hypermethylated DMRs in array-based methylation analysis with discrepancies in the results of MS-MLPA and/or pyrosequencing and array-based methylation analysis, such as the *PLAGL1*-DMR, *MEST*-DMR, *H19*-DMR, and *KCNQ1OT1*-DMR, decreased in analysis method 2. These differences in the methylation pattern of DMRs among the different methylation analysis methods may depend on the differences in the targeted CpGs within the DMR and definitions of aberrant methylation among methylation analyses (Additional file 4: Table S4). To determine MLID, we need to pay attention to the differences in the methylation analysis methods. In array-based methylation analysis, the *SNU13*-DMR and *FANCC*-DMR most frequently had methylation disturbance similar to other studies [12, 39]. Patients with different clinical phenotypes showed aberrant hypermethylation or hypomethylation in the *SNU13*-DMR and *FANCC*-DMR. These findings suggest that these DMRs are susceptible to methylation defects, and methylation defects in these DMRs are not associated with their clinical features. Patients 8, 9, and 12 in our study were monochorionic diamniotic twin cases and had a BWS phenotype. Their twin siblings had a normal phenotype, although the methylation disturbance patterns in leukocytes were similar between cases and twin siblings (Additional file 2: Table S2). Previously reported monochorionic diamniotic twin cases with BWS-MLID had similar methylation disturbance patterns of the DMRs [12]. All three twin siblings had a normal phenotype. Two offered their genomic DNA samples from leukocytes and had aberrant methylated DMRs similar to the twin patients with MLID. These findings suggest that hematopoietic stem cells with aberrantly methylated DMRs derive from the common yolk sac, and other tissues obtain methylation patterns in each twin after twining. Unfortunately, we could not obtain tissues other than leukocytes in twin patients and siblings (Table 4).

Lastly, in this study, nine MLID families had MLID candidate variants. A single mother of the patient and two mothers had frameshift variants in *NLRP2* and *PADI6*, respectively. One of these families had no history of miscarriage, although the remaining two families had no information about pregnancy history. These three patients showed hypomethylation of the *H19*-DMR; however, other previously reported families with truncating frameshift variants (three cases with *NLRP2* variants and three with *PADI6* variants) showed no hypomethylation at the *H19*-DMR [9, 28, 42]. These findings suggest no specific methylation disturbance patterns of DMRs based on variants of MLID-causative genes, and methylation disturbance occurs stochastically. The *PADI6* variant (p.Ile416Thr) detected in Case 19 has been reported as a homozygous pathogenic variant in a case of early embryonic arrest [43]. The mother with this variant in the heterozygous condition delivered healthy children with a different father, so we classified the *PADI6* variant as VUS. We assessed pathogenicity according to ACMG guidelines. Because pathogenic variants of genes coding proteins constituting the SCMC are detected in mothers but not necessarily in offspring, pathogenicity may be underestimated according to ACMG guidelines [27]. It is difficult to assess the pathogenicity of variants in MLID-causative genes without a family history of ID and miscarriage or in cases with an unknown mother's reproductive history, as well as in our study. Further accumulation of MLID cases is required.

## Conclusion

Our study detected MLID in approximately 7% of patients with various IDs caused by epimutation. Clinical analysis in 29 patients with MLID revealed a high frequency of ART-conceived patients and ND/IDD complications. Nine rare variants in MLID-causative genes did not show gene-specific methylation disturbance patterns and phenotypes. This study should contribute to future MLID research and enhance the diagnosis and management of MLID cases.

## Methods

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development.

### Patients

Out of patients referred to our laboratory for genetic testing of IDs, 697 patients had methylation disturbances of DMRs related to IDs detected by combined bisulfite restriction analysis, methylation-specific PCR, pyrosequencing, or MS-MLPA analysis using the SALSA MS-MLPA Probe-mix ME028, ME030, ME032, ME031, or ME033 (MRC-Holland) (group A). The methods of combined bisulfite restriction analysis and pyrosequencing were previously reported [44, 45], and MS-MLPA analysis was conducted according to the manufacturer's protocol. For all patients with IDs, we conducted multi-locus methylation analysis for ID-related DMRs by MS-MLPA analysis and/or pyrosequencing (see below) and identified 22 patients with MLID. In addition, we identified seven patients with MLID identified by MS-MLPA analysis using the SALSA MS-MLPA Probe-mix ME034 in group B consisting of 86 BWS patients with hypomethylation of the *KCNQ1OT1*-DMR. Finally, we examined these 29 patients with MLID in the study (Fig. 1). We collected detailed clinical findings of all patients from their attending physicians using a comprehensive questionnaire.

### Identification of MLID

To detect MLID, we conducted MS-MLPA using ME034 Probe-mix and/or pyrosequencing for multi-locus IDs-related DMRs, as previously reported [46]. For DMRs without consistent methylation pattern between MS-MLPA and/or pyrosequencing and array-based methylation analysis, we conducted methylation analysis using both MS-MLPA and pyrosequencing, although we could not conduct additional analyses in Patients 16, 23, and 27 due to a shortage of genomic DNA samples.

### Array-based methylation analysis using EPIC

We conducted genome-wide methylation analysis using EPIC and obtained β values indicating the methylation levels for 842 CpGs on 78 imprinted DMRs as previously reported [11]. We defined aberrantly methylated DMR based on previous reports [12, 39] (analysis method 1). In brief, the median β value for each CpG within a DMR was determined as the MML (median methylation level) of the DMR. An aberrantly methylated DMR was defined as $|MML| > 3$ SD obtained from the mean of MML in 16 healthy child controls. The aberrantly methylated DMRs were further classified as mild ($\Delta MML < 0.1$), moderate ($0.2 \geq \Delta MML \geq 0.1$), and extreme ($\Delta MML > 0.2$) according to the difference between the MML of each patient and the mean of MML in the controls. In addition, we re-analyzed methylation-array data applying more stringent bioinformatic parameters using the Crawford-Howell *t*-test [47] and defined the aberrantly methylated DMRs. In brief, we considered a probe as differentially methylated, with an absolute value of Δβ ($|\Delta\beta| > 0.1$) and a false discovery rate < 0.05. When we detected two or more consecutive probes differentially methylated levels within a DMR (including at least four probes), we defined the DMR as aberrantly methylated (analysis method 2).

### WES

We conducted trio WES in Patients 1–3, 9, 11–13, 15, 16, 18, 19, 21–26, 28, and 29. Because we could not obtain the parental samples, we carried out WES only in Patients 4, 10, 14, 17, 20, and 27 and only in Patient 8 and the mother. We used SureSelect Human All Exon V6 (Agilent Technologies) for WES. Captured libraries were sequenced by NextSeq 500 (Illumina) with 150-bp paired-end reads. Processing of exome data, variant calling, and variant annotation were conducted following previously established procedures [48]. We searched for a variant(s) of reported MLID-related genes (*NLRP2, NLRP5, NLRP7, PADI6, KHDC3L, ZFP57*, and *ZNF445*) and other candidate genes (*OOEP, ZAR1, TLE6, ARID4A, UHRF1, NLRP14, DPPA3, DNMT3A, DNMT3B, DNMT3L, DNMT1, SETDB2, TRIM28*, and *WHSC1*). We extracted rare variants with minor allele frequencies of ≤ 0.01 in public databases and in-house database as previously reported [11]. We also searched for other causative genes for genetic diseases other than IDs. We evaluated pathogenicity of identified rare variants using the following in silico analyses: (1) CADD (http://cadd.gs.washington.edu/), (2) PP2_HVAR (http://genetics.bwh.harvard.edu/pph2/), (3) SIFT (http://sift.jcvi.org/), and (4) MutationTaster (http://www.mutationtaster.org/).

### Statistical analysis

The statistical significance of the median, mean, and frequency of data obtained from patients with MLID and patients with epimutation only in ID-associated DMR(s) was examined using the Mann–Whitney *U* test, Student's *t*-test, and Fisher's exact probability test. $P < 0.05$ was considered significant. To evaluate the correlation between the BWSp score and the number of aberrantly methylated DMRs in patients with BWS-MLID and between the NH-CSS score and the number of aberrantly methylated DMRs in patients with SRS-MLID and TS14-MLID, we used Pearson's correlation coefficients. The R environment was used for these analyses.

## Consent for publication

We obtained written informed consent from the patients or the patients' parents to publish patients' clinical and molecular information.

## Competing interests

The authors declare no competing interests.

### Abbreviations

| | |
|---|---|
| A/B | GNAS A/B:TSS |
| ACMG | American College of Medical Genetics and Genomics |
| AHO | Albright hereditary osteodystrophy |
| ART | Assisted reproductive technology |
| AS | Angelman syndrome |
| AS1 | GNAS-AS1:TSS-DMR |
| BWS | Beckwith-Wiedemann syndrome |
| BWSp | BWS spectrum |
| CNV | Copy number variation |
| DMR | Differentially methylated region |
| EPIC | Array-based methylation analysis using Infinium MethylationEPIC Kit (Illumina) |
| FANCC | FANCC:Int |
| H19 | H19/IGF2:IG |
| H19-SRS | SRS with hypomethylation of the *H19*-DMR alone |
| ID | Imprinting disorder |
| KCNQ1OT1 | KCNQ1OT1:TSS |
| KCNQ1OT1-BWS | BWS with hypomethylation of the *KCNQ1OT1*-DMR alone |
| KOS | Kagami-Ogata syndrome |
| LP | Likely pathogenic |
| MEG3 | MEG3TSS |
| MEG8 | MEG8:TSS |
| MEST | MEST:alt-TSS |
| ME034 | SALSA MS-MLPA Probe-mix ME034 (MRC-Holland, Amsterdam, Netherlands) |
| MLID | Multi-locus imprinting disturbance |
| MML | Median methylation level |
| MS-MLPA | Methylation-specific multiple ligation-dependent probe amplification |
| NESP | GNAS-NESP:TSS-DMR |
| ND/IDD | Neurodevelopmental delay and/or intellectual developmental disorder |
| NH-CSS | Netchine-Harbison clinical scoring system |
| PEG10 | PEG10:TSS |
| PHP1B | Pseudohypoparathyroidism type 1B |
| PLAGL1 | PLAGL1:TSS |
| PTH | Parathyroid hormone |
| PWS | Prader-Willi syndrome |
| SCMC | Subcortical maternal complex |
| SGA | Small for gestational age |
| SNRPN | SNRPN:TSS |
| SRS | Silver-Russell syndrome |
| SNU13 | SNU13:alt-TSS |
| UPD | Uniparental disomy |
| TNDM | Transient neonatal diabetes mellitus |
| TS14 | Temple syndrome |
| VUS | Variant of uncertain significance |
| WES | Whole-exome sequencing |
| XL | GNAS-XL:Ex1-DMR |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01744-5.

**Additional file 1**. Table S1. Clinical findings of all patients.

**Additional file 2**. Table S2. The raw data of EPIC analysis.

**Additional file 3**. Table S3. Result of microsatellite analysis in Patient 21.

**Additional file 4**. Table S4. Comparison of assessment sites between methylation analyses.

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### Author contributions

TU performed the molecular and data analyses and wrote the paper. HS, KY, K H-I, AN, SK, HN, KY, and KM performed the molecular and data analysis. HS, RK, YN, KY, TH, YM, and TI obtained clinical information of patients. HS, MF, SS, and TO reviewed the paper and supervised the project. MK designed the project, performed the molecular analysis, obtained clinical information of patients, wrote the paper, and gave the final approval of the version to be published. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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