Molecular Diagnosis of Prader–Willi and Angelman Syndromes by Methylation-Specific Melting Analysis and Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

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Background: Approximately 99% of Prader–Willi syndrome (PWS) and 80% of Angelman syndrome (AS) cases have deletions at a common region in chromosome 15q11.2-q13, uniparental disomy for chromosome 15 (UPD15), or imprinting center defects affecting gene expression in this region. The resulting clinical phenotype (PWS or AS) in each class of genomic abnormalities depends on the parent of origin. Both disorders are characterized at the molecular level by abnormal methylation of imprinted regions at 15q11.2-q13. Other rare chromosome 15 rearrangements and a few smaller atypical deletions associated with abnormal methylation patterns also have symptoms overlapping with either PWS or AS.

Methods: We designed a methylation-specific melting analysis (MS-MA) method for a rapid screening of PWS/AS and evaluated methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for diagnosis of PWS/AS associated with deletions, UPD15, or rare duplications. Forty-nine previously genotyped samples were tested by MS-MA. We also tested 26 MS-MA genotyped samples and 1 additional sample with rare duplication of chromosome region 15q11-q12.

Results: PWS/AS genotyping results obtained by MS-MA and by MS-MLPA were fully concordant. In addition, MS-MLPA was superior in detecting deletions/rare duplications, possible UPD15, or imprinting center defects, which were usually determined by a laborious fluorescence in situ hybridization method or by chromosomal segregation analysis for the parental-origin using short-tandem repeat makers.

Conclusions: MS-MA appears to be an efficient primary method to diagnose PWS/AS, and use of the quantitative MS-MLPA method provides detailed information about deletions, rare duplications, and possibly UPD.

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DNA methylation is an epigenetic event that occurs frequently in the mammalian genome (1). It is implicated in multiple normal cellular processes, such as the regulation of tissue- and development-specific gene expression, imprinting, X-chromosome inactivation, DNA repair, and the suppression of parasitic DNA sequences (2, 3). Methylation usually takes place on the cytosine located within a cytosine-guanine dinucleotide, often called a CpG island, although the cytosine in CpA or CpT can also be methylated. Methylation within the CpG islands in the promoter region is often associated with transcriptional silencing (4). Somatic de novo methylation of CpG islands in tumor suppressor genes has been implicated in tumorigenesis, and aberrant methylation of imprinted genes is associated with several inherited human diseases (5).

Prader–Willi syndrome (PWS;[4] OMIM 176270) and Angelman syndrome (AS; OMIM 105830) are the most common genetic disorders involving non-Mendelian inheritance in the form of genomic imprinting (6). PWS occurs in 1 of every 15 000 live births and is characterized by severe hypotonia and feeding difficulties in early infancy, followed in later infancy or early childhood by

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[4] Nonstandard abbreviations: PWS, Prader–Willi syndrome; AS, Angelman syndrome; UPD, uniparental disomy; MS-MA, methylation-specific melting analysis; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; STR, short tandem repeat; and FISH, fluorescence in situ hybridization.

excessive eating and gradual development of morbid obesity. Approximately 70% of individuals with PWS have a 15q11.2-q13 deletion on the paternally inherited chromosome 15, whereas 25% have maternal uniparental disomy (UPD), <5% have an imprinting center sequence variant, and 1% have a structural chromosome rearrangement involving 15q11.2-q13 *(7–9)*. Regardless of the etiology, PWS patients obtain only the methylated allele(s) in the promoter region of the small nuclear ribonucleoprotein polypeptide N (*SNRPN*)[5] gene. AS is a neurogenic disorder caused by the loss of function of the imprinted ubiquitin protein ligase E3A (*UBE3A*) gene in 15q11.2-13, which is characterized by severe mental retardation with absence of speech, microcephaly, inappropriate laughter, seizures, and a stiff gait. Approximately 70% of individuals with AS have a 15q11.2-q13 deletion of the maternal-origin chromosome, 11% have a sequence variant in *UBE3A*, 7% of AS patients have paternal UPD, and 3% have an imprinting center sequence variant *(8–10)*.

In molecular diagnosis, nucleic acid sequencing is considered the gold standard for methylation detection, but this may not be suitable for routine clinical testing as it is laborious and expensive. The most commonly used nonsequencing assays for detection of methylated CpG include restriction fragment length polymorphism analysis using methylation-sensitive enzymes followed by Southern blotting *(11)*, and methylation-specific PCR using bisulfite-treated DNA samples and allele-specific PCR, followed by gel electrophoresis *(12, 13)*. Other techniques, including denaturing HPLC *(14)*, Pyrosequencing™ *(15)*, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry *(16)* have been used recently in methylation studies. Although many of them are used as diagnostic assays, the post-PCR open-tube steps, laborious protocols, and expensive instrumentation are concerns.

Methylation-specific melting analysis (MS-MA) has been reported previously *(17)*. In the present study, we designed a new primer set and assay conditions to determine the methylation status of PWS or AS patients in a rapid and closed-tube assay. To further expand our study, we used a recently described methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) method to cross-validate the in-house–developed MS-MA assay. MS-MLPA is a novel and robust method that can detect changes in CpG island methylation status as well as copy numbers of genes located at the PWS/AS critical region 15q11.2-q12 *(18)*.

Materials and Methods

FLUORESCENT MS-MA ON THE LightCycler

Samples, DNA extraction, and sodium bisulfite conversion. We studied 52 patient blood samples, 19 of which had been

tested by the DNA Diagnostic Laboratory at the University of Utah by bisulfite-treated methylation-specific PCR for the *SNRPN* promoter region and found to be positive for either PWS *(12)* or AS *(7)*. The other 33 DNA samples were from unaffected individuals. All samples were deidentified according to Institutional Review Board protocol. Genomic DNA was extracted from whole peripheral blood (3 mL) by use of PUREGENE® DNA Purification Kits (Gentra Systems, Inc.) according to the manufacturer's instructions. For MS-MA analysis, extracted genomic DNA was treated with sodium bisulfite and the EZ DNA Methylation Kit™ (Zymo Research) according to the manufacturer's protocol.

Primer design and PCR conditions. After bisulfite treatment, we PCR-amplified a 322-bp fragment of the *SNRPN* gene promoter region and partial exon 1 corresponding to nucleotides g.5 to g.326 (GenBank accession no. L32702.1) and containing 22 CpG islands. The primers, PW/AS-F (5'-GAG GTT TTT TTT TAT TGT AAT AGT GTT GTG GGG TTT TAG-3') and PW/AS-R (5'-CTC CCC AAA CTA TCT CTT AAA AAA AAA ACC AC-3') amplified unmethylated as well as methylated alleles. PCR was performed in a 20-μL reaction volume using 1× buffer reagent from the LightCycler FastStart Master SYBR Green I reagent set (Roche Diagnostics), primers at 0.5 μM each, 4 mM MgCl$_2$, and 2 μL of bisulfite-treated DNA. The initial denaturing/polymerase activation step was at 95 °C for 10 min. In this protocol, PCR products were amplified by 30 cycles with a denaturing temperature of 95 °C for 10 s, followed by annealing at 68 °C for 30 s and an increase to 72 °C at a fast transition rate of 20 °C/s.

MS-MA assay. After PCR amplification, the PCR products were completely denatured at 95 °C, cooled to 40 °C at a thermal transition rate of 20 °C/s, and then heated to 95 °C at a thermal transition rate of 0.05 °C/s with continuous fluorescence monitoring in the F1 channel. Data analysis was performed with the LightCycler software, Ver. 5.32 (Roche Diagnostics), with the derivative of fluorescence changes on the *y* axis and temperature (°C) on the *x* axis.

MS-MLPA

Samples and DNA extraction. To test the MS-MLPA and the analysis algorithm chosen, we analyzed 26 genotype-confirmed samples (by MS-MA) by MS-MLPA, including 10 classified as wild type, 9 classified as PWS, and 7 classified as AS. In addition, 1 rare sample with a duplication at chromosome 15q11-q12 purchased from Coriell Cell Repositories (GM12135; Coriell Institute for Medical Research) was also tested by MS-MLPA. Of the 10 wild-type samples, 8 were routine clinical specimens and 2 were extracted DNA from Coriell. Of the 9 PWS samples, 6 were routine clinical specimens, 2 had known UPD status confirmed by either short tandem repeat (STR) or fluorescence in situ hybridization (FISH) assays, and the

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[5] Human genes: *SNRPN*, small nuclear ribonucleoprotein polypeptide N; *UBE3A*, ubiquitin protein ligase E3A; and *NDN*, human necdin-encoding gene.

remaining sample was purchased from Coriell as extracted DNA (NA11391). All of the AS samples were from routine clinical specimens with unknown UPD status. All genomic DNA was extracted by use of the PUREGENE DNA Purification Kit (Gentra Systems) except for the nucleic acid samples purchased from Coriell.

MS-MLPA setup and fragment analysis. For MS-MLPA analysis, genomic DNA samples tested were not treated with sodium bisulfite. This technique uses sequence-specific oligonucleotide probes tagged with common tails complementary to a universal primer set to hybridize with genomic DNA. After hybridization and ligation reactions, a methylation-sensitive restriction enzyme (*Hha*I) is added to digest only unmethylated genomic DNA, followed by PCR amplification using universal primers. If the CpG sites are methylated, then *Hha*I activity is blocked and PCR products are produced and detected by a capillary electrophoresis. However, no PCR products are detected when the CpG sites are unmethylated and therefore digested by *Hha*I. Genotyping using MS-MLPA was 100% concordant with the rapid, closed-tube MS-MA assay, and MS-MLPA has the capability of differentiating deletions from possible UPDs.

The MS-MLPA probe mixture (P028 PWS/AS) was purchased from MRC-Holland. This probe mixture contains 43 probes, 25 of which are specific to the genes in the PWS/AS critical region (15q11-q12). Among these specific probes, 15 probes (including 3 control probes) are methylation-sensitive and contain the *Hha*I restriction site. We investigated 5 of the probes in detail and ignored the other 7 methylation-sensitive probes because of the sample type used (DNA from blood), in which some of the targeted genes (such as *UBE3A*) always remain fully unmethylated and thus may be unsuitable for genotype differentiation (MRC-Holland instruction manual, Ver. 05) *(19)*. These 5 selected methylation-sensitive probes target the important imprinting region, with 4 probes located on the *SNRPN* promoter region with PCR fragment sizes of 142, 166, 190, and 247 bp, and 1 probe located on the human necdin-encoding gene (*NDN*) promoter region (exon 1) with a PCR fragment size of 418 bp.

To minimize sample-to-sample variation, we used a total of 80 ng of genomic DNA for each sample tested. We used 10 mmol/L Tris-EDTA (pH 8.0) for DNA dilution if necessary. After 16 h of hybridization at 60 °C, samples were split equally into 2 aliquots. The first aliquot underwent ligation only, whereas the second underwent ligation plus enzymatic digestion. The ligation, enzymatic digestion, and PCR amplification were performed according to manufacturer's instructions.

PCR products (2 μL) from each tube were mixed with 0.5 μL of internal size standard (ROX-500 Genescan; Applied Biosystems) and 8.5 μL of deionized formamide, and injected into an ABI-3100 genetic analyzer (Applied Biosystems) equipped with a 50-cm, 16-capillary array. All samples were injected for 7 s at 3 kV in a POP-6 polymer. Total run time was 3500 s at 15 kV. All MS-MLPA reactions were performed twice.

DETERMINATION OF COPY NUMBERS AND METHYLATION STATUS

Data normalization and analysis were performed with the built-in MLPA application in GeneMarker, Ver. 1.4 (Soft-Genetics). This MLPA analysis normalizes the peak height (fluorescence intensity) of each fragment by use of exponential fitting with either the internal control probes or the entire population (all fragments) so that the intensities of larger fragments can be normalized. Because of a potential for large deletions, we selected 15 control probes outside the chromosome 15 PWS/AS critical region to normalize our data. Resulting data were then fitted in a regression model and presented in an MLPA ratio analysis format, with the height ratio (normalized fluorescence intensity of each individual probe of a patient sample to a control DNA sample) on the *y* axis and fragment size on the *x* axis. Height ratios of ~1.5, 1.0, 0.5, or 0.0 represented tested regions with a duplication, the wild-type sequence, a deletion, or absence of the sequence, respectively. The upper and lower limits of the height ratio used to determine copy numbers were set at <0.65 for deletion and >1.35 for duplication. To determine the methylation status, we used the normalized height-ratio data of a ligation-treated sample and compared them with the height-ratio data of the same sample treated with both ligation and restriction digestion (by *Hha*I), using the 3 ligation control probes, 4 methylation-sensitive probes located on the *SNRPN* promoter region, and 1 methylation-sensitive probe located on the *NDN* promoter region.

Results

CLOSED-TUBE MS-MA

In this study, sequences of the promoter region of the *SNRPN* gene were used to develop a rapid screening method for PWS/AS by differentiation of their aberrant methylation patterns. After conversion of unmethylated cytosine to uracil by sodium bisulfite and subsequent PCR-mediated conversion of uracil to thymine, methylated and unmethylated alleles are predicted to differ in thermal stability because of different GC contents. We expected that a PWS amplicon would melt at a higher temperature (~87 °C) because of higher GC contents derived from methylated maternal alleles, that the same length AS amplicon would melt at a lower temperature (~83 °C) because of the paternally derived unmethylated allele, and that the same length wild-type amplicon should possess both peaks in the melting spectrum (Fig. 1). When we used specifically designed primers situated outside the CpG sites, there was no discrimination of amplification (between methylated and unmethylated alleles).

Among 33 whole-blood samples from patients known to be wild-type for either PWS or AS, 1 sample failed to amplify because of low DNA quality. The remaining 32
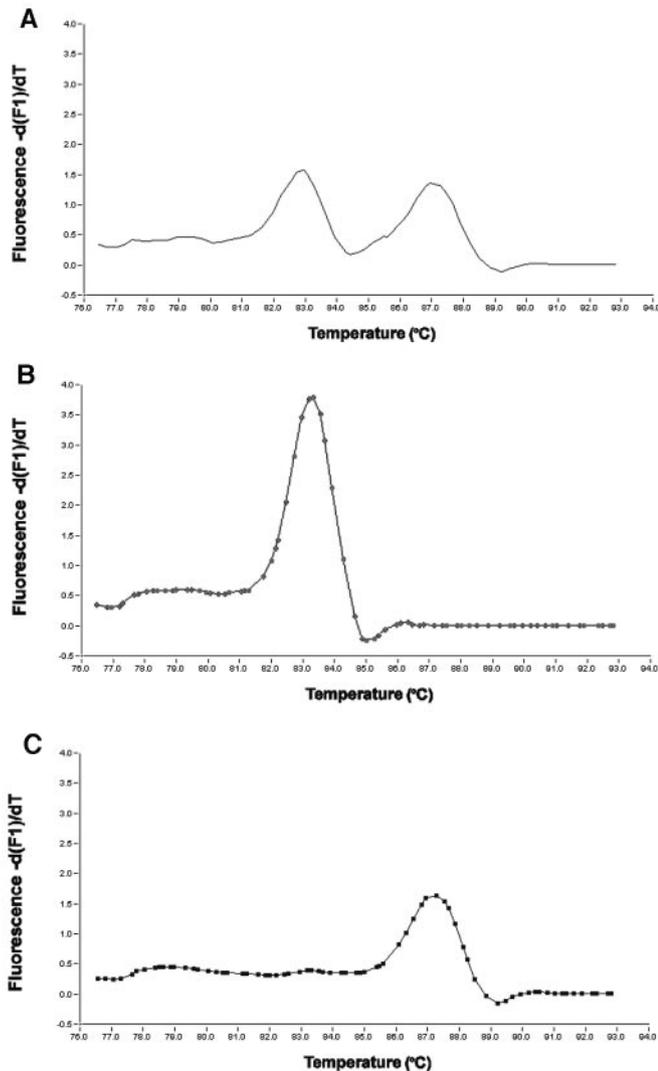
Fig. 1. MS-MA assay for the determination of PWS/AS.

(*A*), 2 amplicon melting peaks detected from a wild-type sample, with T_ms at ~82.37 and 86.74 °C; (*B*), single-amplicon melting peak with a T_m at 83 °C, indicating either AS deletion or paternal UPD15; (*C*), single-amplicon melting peak with a T_m at 86.68 °C, indicating either PWS deletion or maternal UPD15.

samples showed wild-type results with 2 melting peaks, one at a mean (SD) of 82.37 (0.74) °C, and the second at 86.74 (0.98) °C. Among DNA samples from PWS and AS patients previously genotyped by methylation-specific PCR, MS-MA analysis revealed, in 10 of 12 PWS samples, melting peaks of 86.68 (1.20) °C, consistent with a PWS genotype result. The remaining 2 samples could not be amplified, presumably because of insufficient DNA. The 7 AS samples yielded melting peaks at 83 (0.31) °C, consistent with an AS genotype.

DETERMINATION OF PWS BY MS-MLPA

In general, by comparing the normalized height-ratio data obtained with the selected 5 methylation-sensitive probes (4 *SNRPN* and 1 *NDN*) for a sample treated with ligation and ligation/digestion reactions, the PWS and AS dele-

tions can be easily distinguished based on different methylation status and gene copy numbers. Additionally, we found that distinct height-ratio patterns ruled out PWS/AS attributable to deletions, leaving the remaining possibilities of PWS/AS attributable to either UPD or imprinting center defects. STR analysis of both patient and parental samples is needed for UPD status confirmation (Table 1).

Because a normal (wild-type) individual has 1 copy of unmethylated paternal allele and 1 copy of methylated maternal allele from the parents, the height ratios of all probes should remain in a normal copy-number region (~1.0) after probe ligation and PCR amplification (Fig. 2A). Because of the digestion of unmethylated paternal allele by a methylation-specific enzyme, *Hha*I, the height ratios of the 5 methylation-sensitive probes (4 *SNRPN* and 1 *NDN*) should all decrease by one half (~0.5) after sequential processes of ligation, restriction digestion, and PCR amplification (Fig. 2B). To ensure that enzymatic digestion is complete, 3 methylation-sensitive control probes outside chromosome 15 (fragment sizes of 148, 346, and 463 bp) must be evaluated, and the height ratios of all 3 probes should be zero, confirming complete digestion and no PCR products produced (Fig. 2B, blue squares). These probe-height-ratio patterns of 1 (ligation) and 0.5 (ligation/digestion) were consistent among all wild-type samples tested in this study (Table 1).

Approximately 70% of PWS cases result from a 2×10^6-to-3×10^6-bp (Mb) deletion of the paternal allele in chromosome 15, region q11.2-q13 *(20, 21)*. Therefore, MS-MLPA height-ratio patterns of all chromosome 15 probes (except internal control probes) should be ~0.5 after probe ligation and PCR amplification. A height ratio of 0.5 should be also obtained for all 5 selected methylation-specific probes after ligation, restriction digestion, and PCR amplification because of the presence of a methylation-protected maternal allele (Table 1). Representative graphs to illustrate this PWS probe pattern, obtained with a genotype-confirmed clinical specimen, are shown in Fig. 2, C and D. This PWS probe pattern was differentiated from the wild-type control and was consistent for all genotype-confirmed samples tested with the MS-MLPA method.

Table 1. Determination of PWS/AS genotypes based on probe-height ratio between a ligation reaction and a ligation/digestion reaction using methylation-sensitive probes (4 *SNRPN* probes and 1 *NDN* probe).

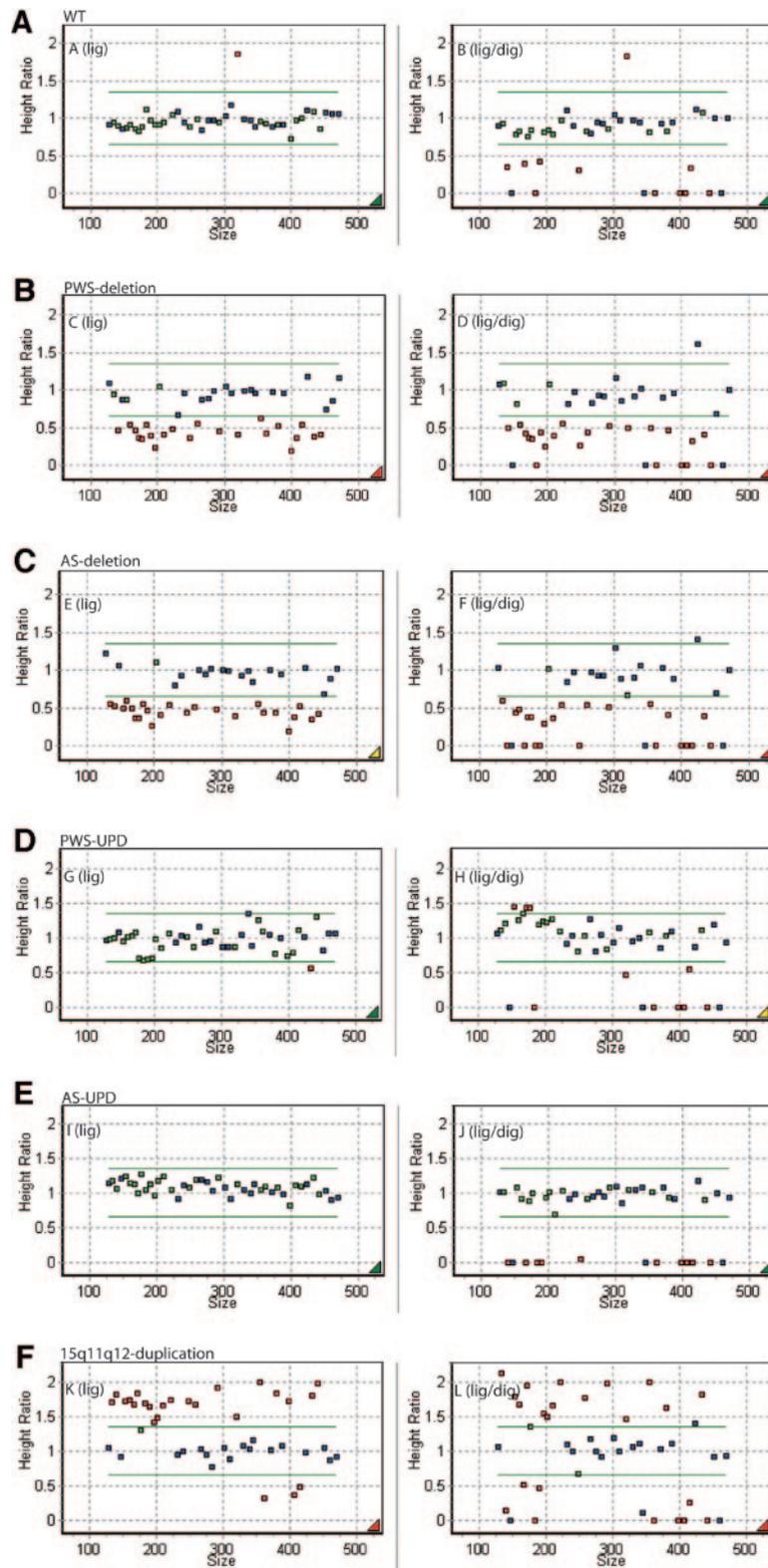| Genotype	No. of samples analyzed by MS-MLPA (total of 27 samples)	Probe-height ratio, ligation only/ ligation + digestion
Wild-type	10	1/0.5
PWS-deletion	6	0.5/0.5
PWS-UPD (potential)	3	1/1
AS-deletion	5	0.5/0
AS-UPD (potential)	2	1/0
Duplication	1	1.5/0.5

Fig. 2. MS-MLPA analysis for the determination of PWS/AS.

x axes represent fragment size in bp, and *y axes* represent probe-height ratios. *Blue*, internal control probes; *green*, targeted probes; *red*, either deleted probes (height ratio <0.65) or duplicated probes (height ratio >1.35). *WT*, wild-type; *lig*, samples treated with ligation reaction only; *lig/dig*, samples treated with both ligation reaction and enzymatic digestion. (*A* and *B*), probe-height-ratio pattern of a wild-type individual. (*C* and *D*), probe-height-ratio pattern of a genotyped PWS deletion sample. (*E* and *F*), probe-height-ratio pattern of a genotyped AS deletion sample. (*G* and *H*), probe-height-ratio pattern of a genotyped PWS sample with UPD confirmed by STR analysis. (*I* and *J*), probe-height-ratio pattern of a genotyped AS sample associated with a potential UPD. (*K* and *L*), probe-height-ratio pattern of a Coriell cell line (GM12135) with confirmed rare duplications in chromosome 15.

In contrast, when the deletion occurs on the maternal allele, the result is AS, and a different probe-height ratio should be expected after both ligation and ligation/ digestion processing because a methylation-protected maternal allele is absent *(8)*. When we tested a genotype-confirmed AS clinical sample, as we predicted, the height ratios of all chromosome 15 probes (except internal control probes) remained near 0.5 after ligation and PCR amplification, which was similar to the PWS ligation data pattern (Fig. 2, C and E). However, a probe-height ratio of 0 (representing zero copies) was observed for all 5 selected methylation-sensitive probes after ligation, digestion, and PCR amplification, which was distinct from both of the wild-type and PWS ligation/digestion data patterns (Fig. 2, B, D, and F; Table 1).

DETERMINATION OF UPD STATUS FOR PWS AND AS
Approximately 25% of PWS cases result from UPD, in which both allele copies are inherited from the mother *(22, 23)*. In this study, we tested a clinically confirmed PWS-UPD sample (by STR analysis; data not shown) by MS-MLPA and predicted that a different height-ratio pattern should be observed. All chromosome 15 probes (including control probes) had a probe-height ratio of 1.0 after ligation and PCR amplification (Fig. 2G), and the ratio was close to 1.0 for all 5 selected methylation-sensitive probes after ligation, digestion, and PCR amplification (Fig. 2H; Table 1). This distinct probe-height-ratio pattern (ligation to ligation/digestion = 1.0/1.0) was consistent when tested on 2 additional genotyped PWS samples with confirmed UPD status. This particular height-ratio pattern for all 5 methylation-sensitive probes can be explained by the presence of 2 methylated maternal copies, ignoring possibilities of imprinting center defects.

In contrast, if a sample has lost the maternal allele and inherited both copies from the father, a less frequent AS-UPD is called after STR analysis *(24)*. Because of a lack of positive controls for AS-UPD, we speculated that 2 of the genotyped AS samples (by MS-MA) should be AS-UPD, with a probe-height ratio of 1.0 for all probes after ligation and PCR amplification (Fig. 2I) but a ratio of 0.0 for all 5 selected methylation-sensitive probes after the sequential processes of ligation, digestion, and PCR amplification (Fig. 2J; Table 1). This pattern (1.0/0.0) is also predicted under the assumption that both paternally inherited alleles are unmethylated and will be digested by *Hha*I and that no PCR products will therefore be produced and detected. These 2 potential AS-UPD samples will undergo STR analysis for further confirmation.

DETECTION OF RARE DUPLICATED CHROMOSOME 15 BY MS-MLPA
In addition to deletion and UPD, duplications of chromosome 15 q11-q12 have been described previously in patients with PWS-like or AS-like symptoms *(25)*. To evaluate the possibility of using MS-MLPA to diagnose this

rare duplication, we analyzed a cell line obtained from Coriell Repository (GM12135). Results showed all chromosome 15 probes except 3 (362, 406, and 416 bp) had a probe-height ratio of ~1.5 after ligation and PCR amplification (Fig. 2K). Interestingly, after ligation, digestion, and PCR amplification, we obtained a probe-height ratio of ~0.5 for all 5 selected methylation-sensitive probes and a probe-height ratio of ~1.5 for the remaining chromosome 15 probes (non–methylation-specific; Fig. 2L). This distinct MS-MLPA ratio pattern (1.5/0.5, Table 1) suggested duplication in the PWS/AS critical region of a paternally inherited allele.

Discussion

Detection of complex epigenetic defects is a growing field in molecular diagnosis. PWS and AS are caused by epigenetic defects, such as large deletions, UPD, or imprinting defects on chromosome 15q11.2-q13, the PWS/AS critical region *(8–10)*. The *SNRPN* gene is located at the PWS/AS critical region, in which the promoter and partial exon 1 regions are fully methylated on the maternal chromosome and completely devoid of methylation on the paternal chromosome. This *SNRPN* region is often used for PCR-gel–based methylation analysis, with >99% detection rate for PWS patients and 80% for the AS patients *(12, 13)*. The remaining 1% of PWS patients with alterations (e.g., single-base variants or small deletions) in the PWS/AS critical region and 20% of AS patients with either a sequence variant in the *UBE3A* gene or unknown causes are not detected by this assay *(8)*. In addition, PCR-gel–based methylation analyses are usually time-consuming and may pose a risk of cross-contamination. To substitute this PCR-gel–based analysis with a rapid, closed-tube system, we describe an MS-MA assay that can detect PWS and AS by monitoring amplicon melting *(26, 27)*. MS-MA can determine aberrant DNA methylation patterns of the promoter region and partial exon 1 of the *SNRPN* gene. Once the bisulfite conversion of sample DNA has been performed, screening of samples can be completed within 45 min on a LightCycler in a closed-tube assay *(27, 28)*. The MS-MA assay genotyped 49 PWS/AS samples with 100% accuracy. Therefore, this rapid MS-MA is a good primary screening method that can be implemented in a diagnostic laboratory to determine the methylation patterns of patients with suspected PWS or AS. However, the use of MS-MA for PWS/AS determination has some limitations, including insufficient data to differentiate the PWS or AS deletions from UPD, and rare imprinting patterns attributable to chromosome duplication of the PWS/AS critical regions. MS-MA should be followed by segregation analysis for the parental origin of the chromosomes, using highly polymorphic STR markers on chromosome 15 *(29)*. In addition, UPD confirmation by STR analysis requires samples from the proband and both parents, making it impractical in some cases *(30)*. Finally, an analytical limitation is that the MS-MA assay requires a large

amount of DNA for the bisulfite treatment. If there is insufficient DNA for amplification the assay will fail.

With the recently introduced MS-MLPA, the limitations of MS-MA are compensated for, and information on both methylation status and copy number changes of PWS/AS patients can be obtained by use of a restriction enzyme specific to methylation sites (18, 31). This new molecular approach has several advantages over traditional PCR- and cytogenetics-based analyses. For example, the molecular approach requires no samples from parents and less patient DNA (20–200 ng), compared with the most popularly used method, Southern blotting. In our study, we standardized all of our initial DNA input to a total of 80 ng to reduce sample-to-sample variations. In addition, this technique compares both ligation and ligation/digestion reactions within the same sample; therefore, quantitative information on copy number changes, methylation status, and parental origin of the alleles can be obtained with less internal variation. With these advantages, the main concern of MS-MLPA is the presence of single-base variations within the probe-binding regions or alterations in the recognition site of the restriction enzyme, which could potentially lead to false-positive or -negative interpretations. In addition, one limitation of MS-MLPA is its inability to differentiate between UPD and imprinting defects. Although the frequency of imprinting defects in either PWS or AS is low, these defects cannot be ignored, and STR analysis is required to confirm UPD status. In this study, we used MS-MLPA to test our genotyped PWS/AS samples and generated specific probe-height-ratio patterns to differentiate PWS/AS associated with deletions or PWS/AS associated with potential UPD.

In addition, DNA from a cell line (GM12135; Coriell Repository), which has been described as originating from a 19-year-old female with short stature, small hands and feet, brown hair and irides, cleft palate, and development delay was also analyzed by MS-MLPA (32). High-resolution karyotype analysis for this individual indicated a duplication of chromosome 15 region q11-q12, which was confirmed by FISH analysis. Our MS-MLPA results were not only concordant with the cytogenetic results, but also provided additional evidence to determine the inherited paternal duplication, confirming the featured Prader–Willi-like phenotype.

In conclusion, our in-house MS-MA assay is a rapid and accurate screening strategy to diagnose PWS/AS. This methylation-specific method is a good candidate for confirmation of clinically suspected PWS or AS, and the closed-tube format substantially reduces the chance of carryover contamination in a molecular laboratory. Although STR analysis is still required to confirm UPD status, by combining MS-MA and MS-MLPA, this molecular approach may well substitute for traditional cytogenetics tests such as FISH, providing detailed information of the etiologic mechanisms of these diseases. Additional study with more samples and different types of pathogenesis for both PWS and AS may be necessary to determine the sensitivity and accuracy of both the MS-MA and MS-MLPA assays. In particular, samples with discordant results between Southern blotting with the PW71 probe and methylation-specific PCR for the SNRPN region would be valuable.

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