Methylation-Specific Multiplex Ligation-Dependent Probe Amplification and Identification of Deletion Genetic Subtypes in Prader-Willi Syndrome

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Purpose: Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are complex neurodevelopmental disorders caused by loss of expression of imprinted genes from the 15q11-q13 region depending on the parent of origin. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) kits from MRC-Holland (Amsterdam, The Netherlands) were used to detect PWS and AS deletion subtypes. We report our experience with two versions of the MS-MLPA-PWS/AS kit (original A1 and newer B1) in determining methylation status and deletion subtypes in individuals with PWS. **Methods:** MS-MLPA analysis was performed on DNA isolated from a large cohort of PWS subjects with the MS-MLPA-PWS/AS-A1 and -B1 probe sets. **Results:** Both MS-MLPA kits will identify deletions in the 15q11-q13 region but the original MS-MLPA-A1 kit has a higher density of probes at the telomeric end of the 15q11-q13 region, which is more useful for identifying individuals with atypical deletions. The newer B1 kit contains more probes in the imprinting center (IC) and adjoining small noncoding RNAs useful in identifying small microdeletions. **Conclusion:** The A1 kit identified the typical deletions and smaller atypical deletions, whereas the B1 kit was more informative for identifying microdeletions including the IC and *SNORD116* regions. Both kits should be made available for accurate characterization of PWS/AS deletion subtypes as well as evaluating for IC and *SNORD116* microdeletions.

Introduction

PRADER-WILLI SYNDROME (PWS; OMIM# 176270) and Angelman syndrome (AS; OMIM# 105830) are complex neurodevelopmental disorders caused by loss of expression of imprinted genes in the 15q11-q13 region. Imprinted genes are differentially expressed depending on the parent of origin and controlled epigenetically by methylation, whereby methylated genes are not expressed and remain in the inactive state. PWS occurs when imprinted genes that are paternally expressed in the 15q11-q13 region are absent, either due to a paternal deletion or by inheriting two copies of the mother's chromosome 15, which are maternally silenced. This genetic state is known as maternal uniparental disomy (UPD) 15. AS is caused by a loss of the maternally expressed ubiquitin protein ligase *UBE3A*, either through deletion, paternal UPD, or mutation. Both PWS and AS can also result from non-

deletion methylation defects in the imprinting center (IC) within the 15q11-q13 region. PWS and AS are the most common genetic disorders caused by non-Mendelian inheritance (Nicholls *et al.*, 1989; Bittel and Butler, 2005; Butler, 2011).

Typical deletions in PWS and AS occur at either breakpoint 1 (BP1; located between 18.68 and 20.22 Mb) or BP2 (BP2; located between 20.81 and 21.36 Mb) at the centromeric end of the region with the resulting genetic subtypes designated as type I or type II deletions, respectively. Regardless of whether the deletion begins at BP1 or BP2, typical deletions generally end at BP3 (BP3, located between 25.94 and 27.28 Mb) at the telomeric end of the 15q11-q13 region (Fig. 1) (Butler *et al.*, 2008). *De novo* paternal deletions are responsible for about 70% of PWS and AS cases. Atypical 15q11-q13 deletions, microdeletions, and IC defects occur much less frequently and account for about 5% of cases. Maternal UPD 15 is seen in about 25% of PWS cases (Bittel and Butler, 2005).

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FIG. 1. Chromosome 15 ideogram, showing the locations of the genes within the 15q11-q13 region affected in AS and PWS and the areas deleted in Type I versus Type II deletions. Probe locations for the MS-MLPA PWS/AS A1 and B1 kits are illustrated in separate columns, with shared probes represented in the middle column. PWS; Prader-Willi syndrome; AS, Angelman syndrome; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification.

The 15q11-q13 region contains several genes, both coding and noncoding, with the region between BP1 and BP2 containing four genes: *TUBGCP5*, *CYFIP1*, *NIPA1*, and *NIPA2*. More than 15 genes are located between BPs BP2 and BP3, many of which are small noncoding RNAs (snoRNAs), believed to affect the expression of other genes. A specific gene of interest within this region is the paternally expressed small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene and its accompanying *SNRPN* upstream reading frame (*SNURF*) (Glenn *et al.*, 1996). The IC is within the same region of *SNURF-SNRPN* as well as noncoding RNAs including *SNORD116* (HBII-85) and *SNORD115* (HBII-52), all of which potentially play an important role in causation of PWS. Other imprinted genes between BP2 and BP3 include *MKRN3*, *MAGEL2*, and *NDN* (paternally expressed) and *UBE3A* and *ATP10A* (maternally expressed). Genes expressed from both alleles or of indeterminate origin include the *P* locus pig-

mentation gene *OCA2* and the *GABA* receptor subunit genes (Butler, 2011) (see Fig. 1).

Although the same chromosomal region is affected in both PWS and AS, their clinical features are very different. Common findings in PWS include hypotonia, a poor suck and failure to thrive, hypogonadism/hypogonitalism, hyperphagia and associated obesity, growth retardation, small hands and feet, behavioral problems, and mild intellectual disabilities (Cassidy *et al.*, 1997; Butler *et al.*, 2006; Butler, 2011). Several clinical features are influenced by the genetic subtype. For example, subjects with PWS and the 15q11-q13 deletion exhibit more characteristic facial features, a higher incidence of behavioral problems such as obsessive-compulsions and skin-picking, lower verbal intelligence quotient, and hypopigmentation (Cassidy *et al.*, 1997; Butler *et al.*, 2004). Within the subset of PWS subjects with typical deletions, additional differences depend on whether the individual has a larger

type I or a smaller type II typical deletion (Dimitropoulos *et al.*, 2000; Hartley *et al.*, 2005). PWS subjects with UPD exhibit a higher incidence of psychoses and social impairment and lower skill levels at tasks requiring spatial abilities (Dykens, 2002; Zarcone *et al.*, 2007). Conversely, AS is characterized by severe developmental and speech impairment, seizures, an unusually happy demeanor, behavioral problems, and ataxia (Jiang *et al.*, 1999; Williams *et al.*, 2010).

Identification of DNA methylation anomalies, copy number variation, and BPs in the 15q11-q13 region can be achieved using small amounts of DNA with relatively little time and expense with the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) kits available from MRC-Holland. MS-MLPA is a method wherein both the methylation status and the copy number of genes are identified (Procter *et al.*, 2006; Bittel *et al.*, 2007).

The original kit (A1) contained 25 probes located throughout the 15q11-q13 region encompassing *TUBGCP5* through *APBA2*, including two probes in the *OCA2* gene and six located in the IC/snoRNA region. The newer kit (B1) contains 32 probes encompassing *TUBGCP5* through *APBA2*, with 15 probes in the IC/snoRNA region but none within *OCA2*. The older A1 kit contains probes that extend further into the telomeric end of the deletion (near BP3), whereas the newer B1 kit has a higher concentration of probes within the IC/snoRNA region. It is important to know the precise genetic subtype including the location of deletion BPs, parent of origin, and/or presence of imprinting defects for medical management and genetic counseling. Herein, we report our experience using the two MS-MLPA kits for comparison purposes in a large cohort of PWS individuals with 15q11-q13 deletions.

Materials and Methods

Study subjects

Twenty-four subjects with confirmed PWS and 15q11-q13 deletions previously found by chromosome and fluorescence *in situ* hybridization (FISH) analyses were recruited from the PWS/AS/Rett rare disease consortium at the University of Kansas Medical Center and University of California at Irvine. Data from subjects at two other PWS/AS/Rett research sites, University of Florida ($N=88$) and Vanderbilt University ($N=10$), and data from 42 additional subjects previously studied with the A1 kit (Bittel *et al.*, 2007) were incorporated into the final analysis. All subjects were consented for study.

MS-MLPA assay

DNA samples for use in the MS-MLPA assay were first isolated using either buccal cells or peripheral blood from each subject with Qiagen (Valencia, CA) DNA isolation kits. The SALSA MS-MLPA PWS/AS-A1 and -B1 kits were obtained from MRC-Holland (Amsterdam, The Netherlands). The MS-MLPA assay and statistical analyses were performed according to manufacturer's protocol and as previously described (Bittel *et al.*, 2007). Briefly, probes were simultaneously hybridized to regions throughout the 15q11-q13 region and then exposed to methylation-specific digestion, followed by polymerase chain reaction (PCR) amplification of the hybridized regions in a manner dependent on copy number and methylation status of the target sequence.

Results

DNA methylation status

All subjects with confirmed genetic diagnosis of PWS displayed the expected pattern of gene methylation using both kits. Imprinted genes are only methylated on one allele in a parent of origin-specific manner, and therefore, fragments from these genes are digested by about 50% in unaffected individuals. Each MS-MLPA-PWS/AS kit contains five probes that are about 50% digested in control samples (indicated by arrows in Fig. 2), including one probe from the *NDN* gene and four from the *SNRPN* region. The fragments from these genes are 100% methylated in PWS subjects regardless of the genetic subtype.

Copy number determination

Both MS-MLPA kits are effective at determining whether PWS subjects have a typical type I, type II, or nondeletion genetic status. Control subjects will have two copies of all genes, whereas PWS subjects with typical deletions will have a copy number of 1 for the genes within the 15q11-q13 region. Individuals with type I deletions (Fig. 3, panels A and B) have only one copy of all genes in the region from BP1 to BP3 and those with type II deletions (Fig. 3, panels C and D) have a normal copy number of 2 for the genes between the BP1 and BP2 region. Between BP1 and BP2, the A1 kit contains probes for *TUBGCP5* and *CYFIP1*, roughly 122 kb apart, whereas the B1 kit contains probes for *TUBGCP5* and *NIPA1*, roughly 214 kb apart. The A1 kit also contains one additional probe in both the *MKRN3* and *MAGEL2* genes, thereby increasing the probe density just distal to BP2. The *APBA2* gene is also on chromosome 15, but is located roughly 1 Mb distal to *OCA2* and about 2.5 Mb distal to the *GABRB3* gene and not within the typical PWS deletion region. PWS subjects with UPD (data not shown) have a normal copy number of 2 for all gene probes and a typical PWS methylation pattern.

Combining the MS-MLPA data from new PWS deletion subjects from multiple PWS study sites ($N=122$) and from PWS subjects previously reported ($N=42$) (Bittel *et al.*, 2007), a total of 164 PWS subjects with deletions were studied. Subjects with the UPD genetic subtype were not included as MS-MLPA testing is primarily designed to distinguish between deletion subtypes and for methylation status. Most PWS subjects are previously studied with chromosome and molecular cytogenetic techniques along with methylation analysis to confirm the diagnosis and the deletion vs. nondeletion status prior to MS-MLPA testing. Those subjects with a PWS methylation pattern and deletion assignment are candidates for deletion subtype studies with the MS-MLPA kits. In addition, those PWS subjects with abnormal methylation and biparental inheritance of chromosome 15 by genotyping become candidates for use of the B1 kit for identification of IC defects.

Of the 164 PWS subjects with recognized deletions included in this study, 41.5% had type I deletions, 49.4% had type II deletions, and 9.1% had atypical deletions or unbalanced chromosomal 15q translocations. Christian *et al.* (1995) previously reported PWS subjects with typical deletions analyzed with microsatellite analysis and found that 42% had type I deletions and 58% with type II deletions. Notably, there were no subjects with atypical deletions identified in their

FIG. 2. MS-MLPA gene methylation status of a representative control subject (**A, B**) and a representative PWS subject (**C, D**) with the MS-MLPA PWS/AS A1 (**A, C**) or MS-MLPA PWS/AS B1 (**B, D**) kits. Each kit contains five probes that are methylated at 40%–60% in control subjects (indicated by arrows in each panel). These probes are 80%–100% methylated in subjects with PWS.

report possibly because of less-sensitive genetic testing methods. Of the subjects in the present study with only typical deletions, 45.6% had type I deletions and 54.4% had type II deletions (Table 1).

Atypical deletions

The differences between the two kits are highlighted in individuals with atypical deletions. Of the PWS deletion subjects reported in this study, 15 individuals had atypical deletions (Table 1). Four of these individuals had deletions that were detected only by the A1 kit, because their atypical deletions ended proximally to BP3 and were missed because of the lack of *OCA2* gene probes with the B1 kit. To demonstrate the different strengths of the two MS-MLPA kits, detailed descriptions of five representative subjects with atypical deletions are provided.

PWS Subject 1 (Fig. 4) has an atypical proximal BP and a typical distal BP at BP3. Both MS-MLPA kits indicate that the proximal deletion BP was located between the *NDN* and *SNRPN* genes (between 21.48 and 22.62 Mb). PWS Subject 2

(Fig. 5) has a smaller atypical deletion with both atypical proximal and distal BP sites. The proximal BP for Subject 2 was located between the *NDN* and *SNRPN* genes (between 21.48 and 22.62 Mb), and the distal BP was located between the *ATP10A* and *GABRB3* genes at 23.66 and 24.34 Mb (Fig. 5, panels A and B). The deleted region in Subject 2 was determined to be the same with both the A1 and B1 kits. The atypical deletion observed in PWS Subject 2 does not include the *NDN* gene; therefore, the methylation pattern for the *NDN* probe was consistent with that of biallelic inheritance, whereas the probes within the *SNRPN* region had a typical PWS methylation pattern (Fig. 5, panels C and D).

PWS Subject 3 (data not shown) has a smaller atypical deletion. This subject with an unusual deletion was previously reported with a submicroscopic deletion in the 15q11-q13 region using FISH, Southern hybridization, and PCR analyses of DNA markers within the region (Butler *et al.*, 1996). MS-MLPA testing on this subject with the A1 kit indicated a deletion between the *SNRPN* u1B and exon u3 probes (between 22.32 and 22.72 Mb), whereas the B1 kit indicated a deletion between the *SNRPN* u2 and u5 probes (between 22.70

FIG. 3. MS-MLPA gene copy number compiled data from PWS type I deletion subjects for the MS-MLPA-A1 (A, *n* = 9) and B1 kits (B, *n* = 3) and from PWS type II deletion subjects for the MS-MLPA-A1 (C, *n* = 9) and B1 kits (D, *n* = 4).

and 22.72 Mb), a more precise BP assignment. The distal BP shown by the A1 kit was located between the *SNRPN* exon 3 and *UBE3A* exon 15 probes (22.76 and 23.14 Mb). The B1 kit indicated a BP between the *SNORD116* snoRNA cluster and *UBE3A* exon 15 probes (22.89 and 23.14 Mb), again a more specific region for the distal BP. The B1 kit indicates a deletion range of 170–440 kb versus a wider range (40–820 kb) for the A1 kit. Taken together, the data for Subject 3 indicate that the B1 kit, with its higher density of probes in the region, proves

more accurate in determining smaller deletions within the *SNRPN* noncoding region.

PWS Subject 4 (Fig. 6) has a proximal BP located in the typical BP2 region. However, the B1 kit indicates that this subject has a typical distal BP at BP3, whereas the A1 kit demonstrates that the actual distal BP was between the *GABRB3* and *OCA2* genes (24.57–25.77 Mb). This indicated a deletion that was at least 200 kb smaller than the typical type II deletion. Because the B1 kit does not contain probes

TABLE 1. METHYLATION-SPECIFIC MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION RESULTS FROM INDIVIDUALS WITH PRADER-WILLI SYNDROME GROUPED BY DELETION GENETIC SUBTYPE

PWS genetic subtype	PWS study subjects published in 2007 ^a (n = 42)	PWS study subjects from four sites (n = 122)	Total PWS study subjects (n = 164)
Type I deletion	52.4% (22)	37.7% (46)	41.5% (68)
Type II deletion	40.5% (17)	52.5% (64)	49.4% (81)
Atypical deletion or unbalanced chromosomal 15q translocation	7.1% (3)	9.8% (12)	9.1% (15)

^aBittel *et al.* (2007).
PWS, Prader-Willi syndrome.

FIG. 6. MS-MLPA copy number determination for PWS Subject 4 with an atypical deletion. Deleted region is indicated between the vertical dashed lines for MS-MLPA PWS/AS A1 (**A**) and B1 (**B**) kits.

between the *GABRB3* and *APBA2* probes, this atypical deletion was not detected.

PWS Subject 5 (Fig. 7) is an individual with an unbalanced translocation between chromosomes 2 (2q37.3) and 15 (15q11.2-q13.3), resulting in a larger than the typical deletion. The unbalanced translocation affects an 11.86-Mb fragment of chromosome 15 and a 77-kb fragment of chromosome 2 by microarray analysis. As a result, the critical PWS 15q11-q13 region is missing plus additional chromosome 15 material including the *APBA2* gene (at 27.2 Mb), as indicated by both MS-MLPA kits. For subjects with PWS, including Subject 5 with significantly larger deletions or translocations, it would be advantageous for the MS-MLPA-PWS/AS kits to include probes for chromosome 15 regions distal to BP3.

Discussion

This study was undertaken to evaluate the efficacy of the new version MS-MLPA-PWS/AS-B1 kit for determining PWS

genetic subtypes and to compare with the older version A1 kit previously used, reported, and validated (Procter *et al.*, 2006; Bittel *et al.*, 2007; Butler *et al.*, 2008). The A1 kit is no longer commercially available. The MS-MLPA assay effectively demonstrates methylation patterns indicative of PWS/AS and whether subjects have deletions or normal copy number. Both the older and newer MS-MLPA-PWS/AS kits yield data regarding typical deletions, but in subjects with atypical deletions, differences in the kits are accentuated.

The B1 kit has a higher density of probes in the IC and the noncoding RNA region. The number of probes in the *SNRPN* region was doubled from 6 to 12, and three probes in the *SNORD116* region were included, a region that was not covered in the A1 kit. The most notable loss from the A1 kit is two probes from the *OCA2* gene. As demonstrated by four PWS subjects with atypical deletions (e.g., Fig. 6 and unpublished data), the *OCA2* probes are critical for identifying atypical deletions at the distal end of the PWS/AS 15q11-q13 region. The A1 kit also contains a higher density of probes at

FIG. 7. MS-MLPA copy number determination for PWS Subject 5 with an atypical deletion. Deleted region is indicated between the vertical dashed lines for MS-MLPA PWS/AS A1 (**A**) and B1 (**B**) kits.

the proximal end of the typical PWS/AS 15q11-q13 deletion region. As the deletion breakpoints in PWS and AS deletion subjects do not always fall at BP1, BP2, or BP3, a higher density of probes around these BP locations is crucial.

Although the A1 kit more effectively identifies atypical deletions around the proximal and distal BP regions, the B1 kit has a higher density of probes near the IC and is better for characterizing smaller deletions in this critical region, as demonstrated by PWS Subject 3 in our study. The probes that detect the snoRNA *SNORD116* region were an important addition to the B1 kit as subjects with typical PWS features and small deletions of this snoRNA region only have been reported (Sahoo *et al.*, 2008; de Smith *et al.*, 2009). Noncoding RNAs present within the PWS imprinted region include *SNORD116* (HBII-85), *SNORD115* (HBII-52), and others. These snoRNAs function to modify other RNA molecules and apparently regulate protein isoform production (Rogelj, 2006; Kishore *et al.*, 2010). MS-MLPA analysis was performed on the subject with a small deletion reported by de Smith *et al.* (2009) using the A1 kit, and one probe, the most distal *SNRPN* (exon 3) probe (at 22.76 Mb), was shown to be deleted. However, had this subject been analyzed using the B1 kit, five probes would have shown a deletion spanning from the *SNRPN* exon 3 probe through the most distal *SNORD116* probe (a range from 22.76 to 22.89 Mb). Although the absolute size of the deletion is most thoroughly characterized via microarray technology, the B1 kit would have given a relatively accurate range for the deletion in this subject at a lower cost.

Another potentially important snoRNA in the PWS imprinting region is *SNORD115*, although there is currently no probe for *SNORD115* in either MS-MLPA kit. Deletion of the *SNORD115* snoRNA alone does not appear to cause PWS; however, its known role in isoform production of a serotonin receptor (Kishore and Stamm, 2006) may influence the PWS phenotype (Runte *et al.*, 2005) and justifies the addition of a *SNORD115* probe to the MS-MLPA-PWS/AS kits.

Overall, we conclude that analysis of subjects with 15q11-q13 deletions can be carried out successfully using the A1 kit as it provides better characterization of large, more typically sized PWS and AS deletions. The newer B1 kit has a higher density of probes in the IC and noncoding RNA region, which is helpful for characterizing smaller deletions in and around the imprinting center. The availability and use of both kits is important for the initial screening of subjects when carrying out genetic research or genetic subtype classification of individuals with PWS and AS. Because the majority (~70%) of PWS and AS subjects have typical or larger atypical deletions, our study supports the availability of both the older A1 kit, which is no longer commercially available, and the newer B1 kit for study and identification of a wide range of chromosome 15q11-q13 rearrangements. We encourage the manufacturer to return to market the availability of the MS-MLPA-PWS/AS-A1 kit or to redistribute the probe coverage balancing the IC and BP3 regions in a single kit.

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Disclosure Statement

No competing financial interests exist.

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