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Methylation-Specific Multiplex Ligation-Dependent Probe Amplification Analysis of Subjects with Chromosome 15 Abnormalities

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ABSTRACT

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are neurodevelopmental disorders caused by loss of expression of imprinted genes from the 15q11-q13 region. They arise from similar defects in the region but differ in parent of origin. There are two recognized typical 15q11-q13 deletions depending on size and several diagnostic assays are available but each has limitations. We evaluated the usefulness of a methylation-specific multiplex ligation-dependent probe amplification (MLPA) kit consisting of 43 probes to detect copy number changes and methylation status in the region. We used the MLPA kit to genotype 82 subjects with chromosome 15 abnormalities (62 PWS, 10 AS and 10 individuals with other chromosome 15 abnormalities) and 13 with normal cytogenetic findings. We developed an algorithm for MLPA probe analysis which correctly identified methylation abnormalities associated with PWS and AS and accurately determined copy number in previously assigned genetic subtypes including microdeletions of the imprinting center. Furthermore, MLPA analysis identified copy number changes in those with distal 15q deletions and ring 15s. MLPA is a relatively simple, cost-effective technique found to be useful and accurate for methylation status, copy number and analysis of genetic subtype in PWS and AS, as well as other chromosome 15 abnormalities.

INTRODUCTION

Prader-Willi syndrome (PWS; OMIM 176270) and Angelman syndrome (AS; OMIM 105830) are the most common genetic disorders involving non-Mendelian inheritance with defects in imprinted genes on chromosome 15 (Bittel and Butler 2005). Prader–Willi syndrome is a neurodevelopmental disorder due to the functional loss of paternally expressed genes from the 15q11-q13 region. About 70% of PWS subjects result from a de novo paternal deletion of 15q11-q13; 25% have maternal uniparental disomy 15 (mUPD) and the remaining cases have defects in the imprinting center (microdeletions or epimutations) or chromosomal translocations (Bittel and Butler 2005). Angelman syndrome results from functional defects in the maternally expressed *UBE3A* gene, located at 15q11.2, caused by a variety of genetic abnormalities of the chromosome 15q11-q13 region. These include about 70% with a de novo maternal deletion of 15q11- q13; about 5% with paternal uniparental disomy 15 (pUPD); imprinting center defects in 3–10%; point mutations or small deletions within the *UBE3A* gene in 5–10% and the remaining AS subjects (10–14%) have no identifiable molecular abnormality (Jiang et al. 1999). *UBE3A* shows tissue-specific imprinting, being expressed exclusively from the maternal allele in brain tissue. Different genetic subtypes in both PWS and AS have been reported to correlate with specific clinical findings of varying severity (Saitoh et al. 1997; Lossie et al. 2001; Butler et al. 2004). Thus, PWS and AS are sister imprinting disorders arising from similar defects in the 15q11-q13 region but originating on the paternal or maternal chromosome, respectively.

DNA methylation involved in the imprinting process results from epigenetic events implicated in many cellular processes. These include tissue- and development-specific gene expression, parent of origin gene expression (imprinting) and X-chromosome inactivation. Methylation usually takes place on cytosine nucleotides located within a CpG island in a promoter sequence of the gene resulting in transcriptional silencing.

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PWS individuals obtain only methylated allele(s) (maternal copy) in the promoter region of the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene while AS subjects obtain only an unmethylated allele(s) (paternal copy) of the *SNRPN* gene.

The typical deletion responsible for PWS or AS encompasses most of the 15q11-q13 region. However, studies have shown that the proximal deletion breakpoint may occur at one of two sites within either of two large duplicons centromeric to locus ZNF127 (Nicholls and Knepper 2001). The precise location of the breakpoints within the duplicons may vary but the breakpoints appear to be confined to a relatively small region of chromosome 15 which allows for the identification of two classes of typical deletion subjects. Breakpoint 1 (BP1) is proximal to D15S541/S1035 loci and BP2 lies between loci D15S541/S1035 and D15S543 (Ungaro et al. 2001). The type I (TI) typical deletion involves BP1 which is close to the centromere while the smaller type II (TII) typical deletion involves breakpoint BP2 and located approximately 500 kb distal to BP1. Therefore, the TI deletion results in the loss of approximately 500 kb of genetic material in addition to what is missing in the TII deletion.

A number of studies examining genotype/phenotype relationships between the genetic subtypes in PWS and AS have been reported (Bittel and Butler 2005). Most have focused on the differences between subjects with typical deletions and uniparental disomy. More recently, reports have examined the relationship between the two different classes of deletion size and their effect on phenotypic outcomes in PWS (Butler et al. 2004; Milner et al. 2005; Varela et al. 2005) and AS (Lossie et al. 2001). We previously presented the first assessment of clinical differences in individuals with PWS who were categorized as having TI or TII deletions using a large existing clinical and behavioral data set (Butler et al. 2004). Significant differences were found between the two typical deletion groups and those with UPD. For example, adaptive behavior scores were generally worse in individuals with PWS and the TI deletion, as were specific obsessive compulsive behaviors compared with subjects with PWS resulting from UPD (Butler et al. 2004). Individuals with PWS with TI deletions had poorer reading and math skills and visual motor integration. In general, individuals with TI deletions had more behavioral and psychological problems than individuals with the TII deletion or UPD.

Many genetic techniques have been used to diagnose PWS and AS. Sequencing the promoter for methylation status is probably the most accurate means for molecular diagnosis but is not suitable for routine clinical testing as it is laborious and expensive. The most commonly used nonsequencing assays for detection of methylated CpG nucleotides include restriction fragment length polymorphism analysis using methylation-sensitive enzymes followed by Southern blotting or methylation-specific PCR using bisulfite-treated DNA samples and allele-specific PCR, followed by gel electrophoresis. However, other techniques, including denaturing HPLC and pyrosequencing have been used recently in methylation studies (Tost et al. 2003; Betz et al. 2004). Although many are used as diagnostic assays each has limitations primarily due to cost, time or reliability which make them less than ideal. Methylation-specific multiplex ligation-dependent probe amplification (MLPA), a relatively simple and cost-effective technique has been introduced only recently for genetic analysis of copy number variation and methylation status (Nygren et al. 2005; Procter et al. 2006; Dikow et al. 2007). There is a paucity of data in the application of this technique in the laboratory diagnosis of those with PWS, AS or other chromosome 15 abnormalities.

Herein, we applied MLPA testing to the analysis of subjects with PWS and AS with known genetic subtypes, and subjects with other chromosome 15 aberrations including ring 15s, pseudo-isodicentric (marker) chromosome 15s, proximal 15q duplications, distal 15q deletions and chromosome 15 translocations for determination of genetic subtype classification. In our hands, MLPA appeared to be a robust method for analyzing the methylation status and copy number in the PWS/AS 15q11-q13 critical region using small amounts of DNA from subjects with PWS and AS as well as those presenting with other chromosome 15 aberrations.

MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board and each subject was consented before entry into the study. Ninety-five individuals with abnormalities of chromosome 15 and controls were included in the study. These included 62 with PWS (34 females, 28 males; average age = 23.1y; age range 1 to 44y): 10 with AS (1 female, 9 males; average age = 9.3y; age range 3 to 20y) and 10 with other chromosome 15 abnormalities including ring 15s, marker 15s, proximal 15q duplications, distal 15q deletions and chromosome 15 translocations (5 females, 5 males; age range 4 to 25y) and 13 control subjects (9 females, 4 males; average age = 22.1y; age range 12–39y).

Methods

MLPA reagents were obtained from MRC-Holland (Amsterdam, The Netherlands; SALSA MLPA kit ME028). Approximately 50 ng of genomic DNA in 5 μl of TE buffer [10 mM Tris–HCl (pH 8.5) and 1 mM EDTA] was denatured for 10 min at 98°C. SALSA MLPA buffer (1.5 μl) and methylation specific (MS)-MLPA probes (1 fmol each in 1.5 μl vol) were then added. After incubation for 1 min at 95°C, the probes were allowed to hybridize to their respective targets for ~16 h at 60°C. After hybridization, the mixture was diluted at room temperature with water and 3 μl ligase buffer A to a final volume of 20 μl and then equally divided in two tubes. While at 49°C, a mixture of 0.25 μl ligase-65 (MRC-Holland), 5 U *HhaI* (Promega; Madison, WI) and 1.5 μl ligase buffer B in a total volume of 10 μl was added to one tube. The second tube was treated identically except that the *HhaI* enzyme was replaced with water. Simultaneous ligation and digestion were then performed by incubating for 30 min at 49°C, followed by 5 min heat inactivation of the enzymes at 98°C. The ligation products were PCR amplified by the addition of 5 μl of ligation mixture to 20 μl PCR mixture containing PCR buffer, dNTPs, SALSA polymerase and PCR primers (one unlabeled and one D4-labeled) at 60°C.

PCR products (1 μl) from each tube were mixed with 0.5 μl of an internal size standard (ROX-400 Genescan; Applied

Biosystems, Foster City, CA) and 9 μl of deionized formamide, and injected into an ABI-3100 Avant genetic analyzer (Applied Biosystems) equipped with a 50-cm, 4-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 MLPA reactions were performed twice to determine copy number and methylation status for each subject.

Data analysis was performed by exporting the peak heights and areas into the Excel program (Microsoft, Seattle, WA). For copy number quantification, the height and area of each peak from chromosome 15 was divided by the closest smaller and larger peaks from the printout and outside of chromosome 15. The copy number was obtained by comparing this ratio with the same peak ratio obtained from a control (biallelic) individual. By taking the mean normalized ratio and rounding to 0.5, 1, 1.5 or 2 and then multiplying by 2 (normal or biallelic copy number), the copy number can be determined. Thus, our subjects with 15q11-q13 deletions having normalized ratios of approximately 0.5 have a copy number of 1 indicating they have a single allele.

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 duplicate sample digested with methyl sensitive *HhaI*. The kit contained primers for four methylated sequences located in the maternal *SNRPN* promoter region, and one methylated sequence located in the maternal *NDN* gene promoter region (see Table 1). Amplification of these is not possible after digestion with *HhaI* except for methylated sequences since methylation prevents restriction. In control individuals, the amplification will be from the maternal copy only and peak height or area will be one-half of that in reactions using unrestricted (undigested) DNA as template; a ratio of 1 to 0.5. Samples amplified using DNA from PWS subjects with a typical deletion will have peak heights approximately one-half of the control undigested peak and the ratio between digested and undigested PWS deletion samples will be 0.5 to 0.5 because the template is unaffected by restriction since the unmethylated paternal sequences are absent. Samples from PWS subjects with mUPD will also have a 1 to 1 ratio as both copies of the template are maternal (methylated) and therefore undigested. Conversely, these amplicons will not amplify in reactions using *HhaI* digested DNA from Angelman syndrome subjects since no maternal methylated DNA is present and restriction digest will remove all available template. Thus, in reactions using DNA from Angelman syndrome (AS) subjects the ratio of undigested to digested peak height or area will be 0.5 to 0 in AS deletion subjects and 1 to 0 in AS pUPD subjects.

RESULTS

We analyzed 95 individuals, (62 with PWS, 10 with AS, 10 with other chromosome 15 aberrations and 13 cytogenetically normal) (Table 1). All PWS and AS subjects were previously diagnosed by methylation analysis, DNA microsatellite studies and/or cytogenetic/FISH analysis. Genetic subtypes (e.g., deletion type I or type II) in the PWS and AS subjects were established by microsatellite analysis as previously described (Butler et al. 2004). Chromosomal translocations and other aberrations were established by high resolution cytogenetic G-banding and/or FISH analysis.

The MLPA kit included two pairs of primers for sequences between breakpoints BP1 and BP2. Seven pairs of primers are

Table 1. Number, Gender, and Mean Age (Range) of Subjects Analyzed Using Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

Category	n	*Gender M:F*	*Mean age in years (range)*
Prader-Willi syndrome	62	28:34	23.1 (0.2–44)
Type I deletion	22	12:10	25.2 (10–41)
Type II deletion	17	6:11	18.8 (1–31)
mUPD	14	6:8	26.0 (13–44)
Imprinting center (IC-PWS)	6	4:2	11.7 (2–20)
Atypical 15q11-q13 deletion (150 kb del)	1	0:1	5
Deletion 15q11-q14	1	0:1	0.2
PWS type II deletion and proximal 15q11 duplication	1	0:1	17
Angelman syndrome	10	9:1	9.3 (3–20)
Type I deletion	1	0:1	8
Type II deletion	5	4:1	5.0 (3–7)
pUPD	4	4:0	14.9 (6–20)
Other chromosome 15 abnormalities	10	5:5	10.0 (1–25)
Proximal 15q11-q13 duplication	2	0:2	5 and 15
Pseudoisodicentric (marker) 15	2	1:1	10 and 25
Translocation	3	1:2	9.7 (1–25)
Ring 15	2	2:0	4 and 11
Deletion 15q26.1	1	0:1	1
Control	13	4:9	22.1 (12–39)

specific for the SNRPN promoter, four of which are specific for a maternally methylated sequence containing a *HhaI* restriction site. In addition, another pair of primers is specific for a maternally methylated sequence in *NDN*, giving five sites for analysis of methylation status (only 4 shown in Figure 1A, peaks labeled "a",). If the site is not methylated (paternal copies of chromosome 15), *HhaI* will cut the genomic DNA. Maternal methylated sequences will be protected from *HhaI* digestion and available for PCR resulting in a reaction with peak height (or area) of one-half that of undigested DNA. Enzymatic digestion of genomic DNA can be monitored by digestion of three unmethylated sequences outside of the PWS/AS critical region (only 2 shown in Figure 1A, peaks labeled "b"). These target sequences will not produce a PCR fragment in *HhaI* digested DNA if digestion is complete. In addition, two pairs of primers are included which amplify target sequences methylated on both maternal and paternal chromosomes. These will have a similar peak (or area) size in digested and undigested templates (Figure 1A, peaks labeled "c").

PRADER-WILLI SYNDROME AND ANGELMAN SYNDROME

Methylation analysis

The MLPA analysis identified altered methylation PWS patterns in all subjects with PWS while controls had a normal methylation pattern. Individuals with normal chromosome 15s had an approximately 50% reduction in peak height (or area) after digestion with *HhaI* for peaks derived from paternally unmethylated, maternally methylated sequences compared to peak height (or area) of undigested template DNA (Figure 1A, peaks labeled "a"). In subjects with PWS with typical deletions the unmethylated paternal sequences were absent; thus, there was no change in peak height (or area) when comparing *HhaI* digested vs. undigested template DNA (Figure 1B, peaks labeled "a").

PWS subjects with mUPD have two maternal copies of chromosome 15, both of which are methylated. *HhaI* digestion does not affect the template in these samples and the peak sizes were not changed in comparing undigested vs. digested template. In addition, comparing the normalized peak heights [ratio of the height (or area) of the peak of interest divided by the average of the closest smaller control peak and the closest larger control peak] between PWS UPD subjects, the peak sizes were similar to peak heights in undigested samples from control individuals and twice the height of peaks from samples of PWS subjects with deletions, whether digested or undigested, thus providing evidence of UPD (data not shown). However, MLPA alone can not distinguish between an imprinting center (IC) defect and UPD if the IC defect is not a detectable microdeletion using this probe set.

AS subjects with a typical deletion are missing the maternal methylated copy of these sequences; therefore, *HhaI* will completely digest the template without amplification after digestion (Figure 1C, peaks labeled "a"). Methylation analysis in AS deletion subjects resulted in peak heights (or areas) from undigested templates which were approximately 50% the size of normalized values from control individuals (see Fig 1A). Methylation analysis of AS subjects due to pUPD resulted in peak heights (or areas) of the methylated sequences that were similar to those of undigested control samples (data not shown). However, both alleles in samples from AS pUPD subjects were unmethylated and completely digested resulting in no amplicons produced after PCR.

Copy number analysis

The mean normalized ratio of all deleted probes in the PWS/AS critical region between BP2 and BP3 for our subjects with PWS having the typical type I or type II deletion was 0.53 +/− 0.09. Based on the mean normalized ratio data from the typical deletion subjects we chose the mean +/− two standard deviations (i.e., 0.35 to 0.71) as the critical range to determine copy number status. Normalized mean probe intensities within this range was calculated to indicate the presence of a single allele. The range of values from all of our subjects known to be hemizygous for these probes fell within two standard deviations of the mean.

The MLPA analysis correctly identified the genetic subtypes of subjects with PWS. Individuals with a type I deletion have a paternal deletion which includes *CYFIP1* and *TUBGCP5*. Therefore, the normalized peak heights (or areas) are approximately 50% in the PWS subjects with type I deletions (mean normalized ratio of 0.52 +/− 0.07) compared to individuals

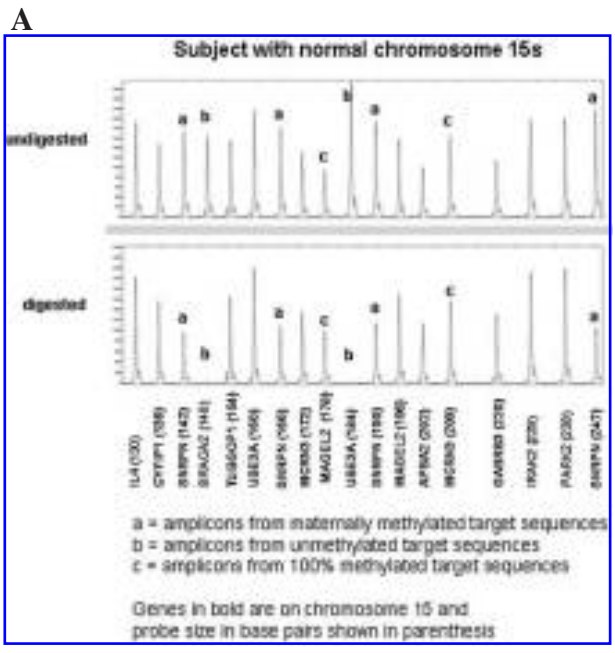

FIG. 1. Representative MLPA patterns following capillary electrophoresis: a = amplicons from maternally methylated target sequences, b = amplicons from unmethylated target sequences. c = amplicons from 100% methylated target sequences, Probes in bold are on chromosome 15. **A**: Subject with normal chromosome 15s. **B**: Subject with Prader-Willi syndrome with a typical 15q11-q13 deletion. **C**: Subject with Angelman syndrome with a typical 15q11-q13 deletion. **D**: Subject with Prader-Willi syndrome due to maternal uniparental disomy 15. **E**: Subject with Angelman syndrome due to paternal uniparental disomy 15.

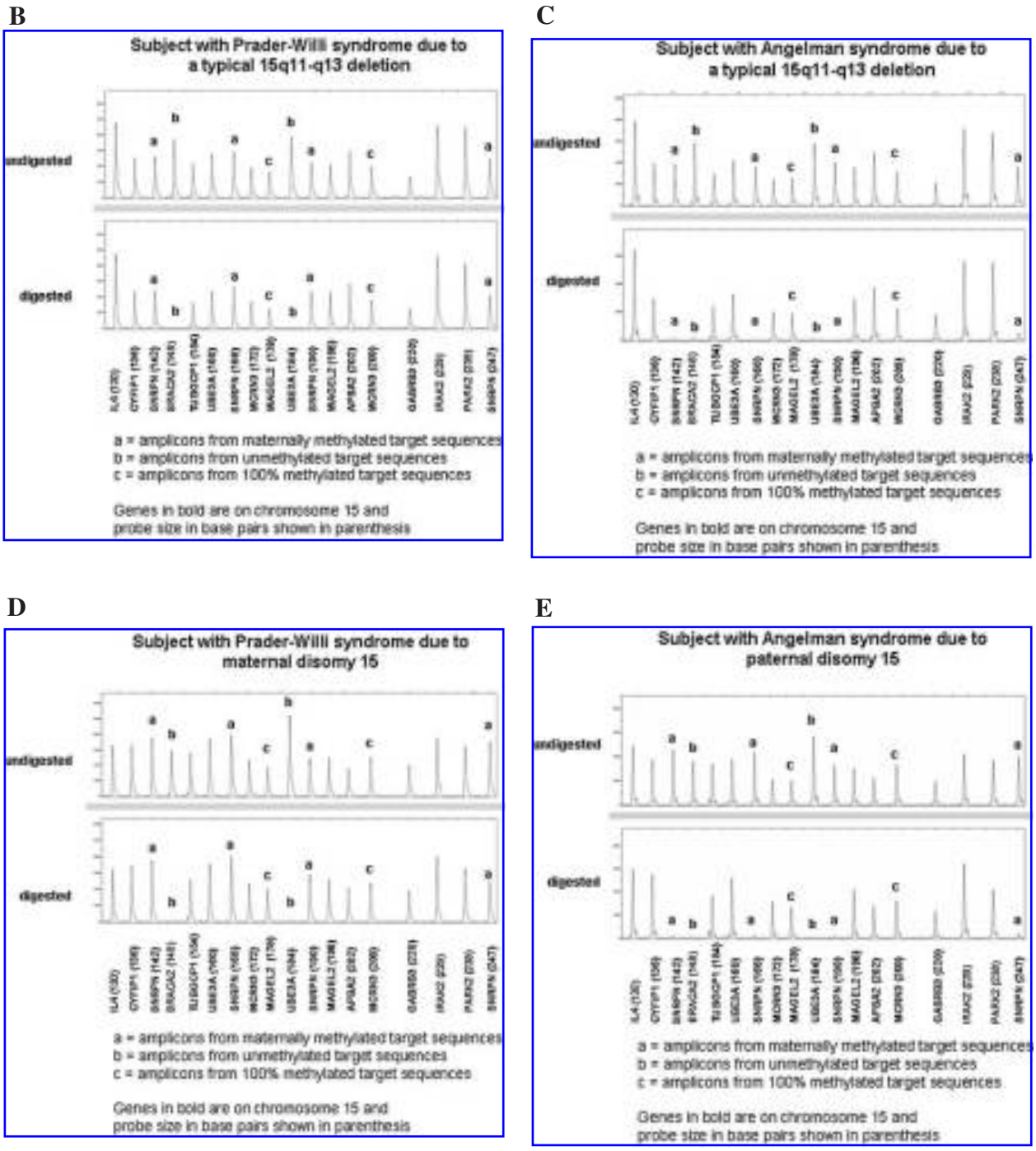

FIG. 1. (*Continued*).

with two normal chromosome 15s for the BP1 region (1.01 +/− 0.12, Figure 2A and 2B). In PWS subjects with a type II deletion in which copy numbers of *CYFIP1* and *GCPTUB5* are unaffected, the normalized peak heights (or areas) are approximately the same when comparing the PWS type II subjects (1.03 +/− 0.09) with individuals with normal chromosome 15s for the BP1 region (1.01 +/− 0.12). PWS subjects with mUPD have two maternal copies of chromosome 15 and the copy number analysis was the same when comparing subjects with normal chromosome 15s for BP1 region (Figure 2A and 2C). All amplicons generated from the region between BP2 and BP3 were hemizygous in all subjects with a typical deletion. When comparing normalized peak heights (or areas) to samples from control subjects, the peaks in the PWS deletion subjects (0.53 +/− 0.09) were approximately 50% of the peak size in control subjects (0.98 +/− 0.13) reflecting a single allele at each

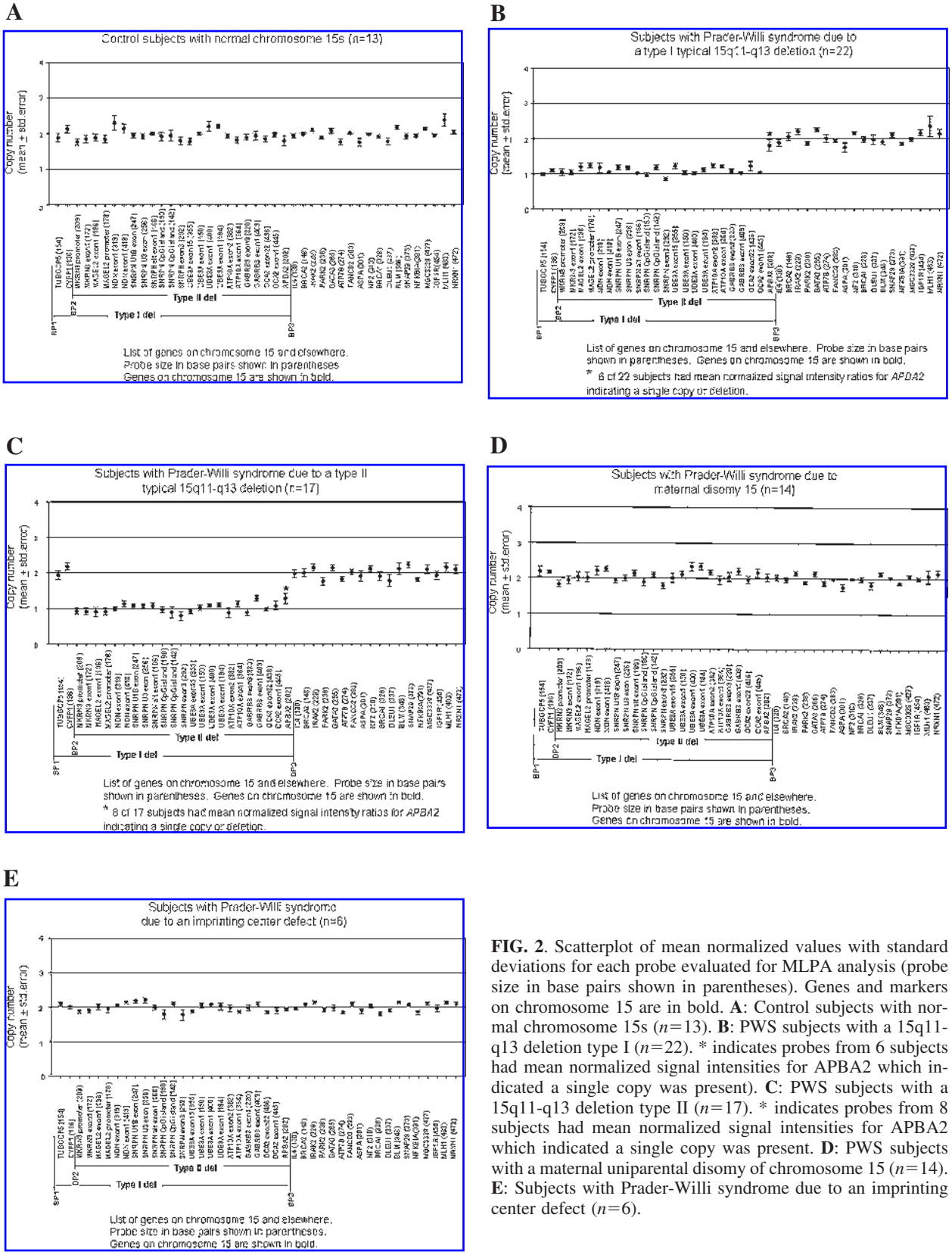

FIG. 2. Scatterplot of mean normalized values with standard deviations for each probe evaluated for MLPA analysis (probe size in base pairs shown in parentheses). Genes and markers on chromosome 15 are in bold. **A**: Control subjects with normal chromosome 15s ($n=13$). **B**: PWS subjects with a 15q11-q13 deletion type I ($n=22$). * indicates probes from 6 subjects had mean normalized signal intensities for APBA2 which indicated a single copy was present). **C**: PWS subjects with a 15q11-q13 deletion type II ($n=17$). * indicates probes from 8 subjects had mean normalized signal intensities for APBA2 which indicated a single copy was present. **D**: PWS subjects with a maternal uniparental disomy of chromosome 15 ($n=14$). **E**: Subjects with Prader-Willi syndrome due to an imprinting center defect ($n=6$).

locus between BP2 and BP3 (Figure 2A, 2B and 2C). PWS subjects with mUPD will have two copies of all genes examined so normalized peaks heights (or areas) should be similar when comparing with control subjects (Figure 2A and 2D). Interestingly, the location of the chromosome break at BP3 was distributed into two groups (i.e., those with copy numbers indicating the presence or absence of *APBA2*). Six of 22 PWS type I deletion subjects and 8 of 17 PWS type II deletion subjects had signal intensities consistent with a deletion of the *APBA2* gene located at 27.0 Mb. Therefore, breaks at BP3 appear to occur at one of two sites; one located between 25.8 Mb and 27.0 Mb and the other distal to 27.0 Mb.

Copy number analysis in subjects with AS are similar to that of PWS subjects. AS subjects with a type I deletion are hemizygous for all amplicons between BP1 and BP3 (data not shown). AS subjects with a type II deletion are biallelic for *CYFIP1* and *TUBGCP5* and hemizygous for the sequences between BP2 and BP3. Furthermore, AS subjects with pUPD have two copies of amplicons and peak sizes were similar to those seen in control subjects.

OTHER CHROMOSOME 15 ABERRATIONS

Several subjects were included in our analysis having chromosome 15 aberrations other than the typical deletion seen in PWS or AS (Table 1). Six PWS individuals with IC defects were analyzed. As expected, the PWS subjects with IC defects were biallelic for the analyzed sequences, except for one subject who had single copy probes for *SNRPN* CpG island (142) and *SNRPN* exon 3 (292). These two probes are located near the start codon of SNRPN in regions known to influence methylation establishment and maintenance. Methylation analysis of subjects with IC defects indicated both chromosomes were methylated (maternal imprint) and similar to PWS subjects with mUPD. Furthermore, the methylation analysis with undigested DNA produced peak sizes comparable to those seen in control subjects which indicated the presence of two alleles. Peak sizes did not change after digestion in samples from subjects with IC defects and showed approximately twice the peak size of normalized digested control DNA. In addition, microsatellite analysis in all of these subjects indicated biparental inheritance of informative markers from chromosome 15, thus excluding the possibility of mUPD.

Three subjects were studied with translocations involving chromosome 15 [one 3y/o male with a PWS phenotype and a balanced t(15;19) previously reported, (Sun et al. 1996) one 1y/o female with hypotonia and developmental delay with a Robertsonian t(15;15) and one adult female presenting with miscarriages and a balanced t(10;15)] (Table 1). The results from MLPA analysis of each these subjects were similar to those from control subjects. MLPA detected no alterations in methylation patterns nor in copy number consistent with the cytogenetic analysis.

Two female subjects were studied (5 y/o female and 15 y/o female both presenting with developmental delay and/or learning problems) with partial proximal long arm duplications of chromosome 15. Neither subject was diagnosed with PWS but both had a cytogenetic duplication of the 15q11-q13 region. However, the precise distal location of the duplication was not known. In our study, the size of the probes between BP1 and BP3 were approximately 50% larger in the 5 y/o female compared with control subjects. This observation reflected three copies of genes between BP1 and BP3 in this subject compared to two copies in the control subjects. MLPA results showed that the second subject (15 y/o female) had a duplication involving 15q11.1-q11.2 but did not include the 15q12 region (e.g., *OCA2* exon 1) further supported by FISH analysis with the SNRPN probe (not duplicated, data not shown). The distal sequences on chromosome 15 (e.g., *BLM* and *IGFIR* genes located at 15q26) were similar (not duplicated) to those of control subjects providing supporting evidence for interstitial duplications. Additionally, methylation analysis of these two subjects was normal and consistent with their previous cytogenetic diagnosis.

We analyzed a unique subject with PWS due to a type II deletion but in addition had a proximal duplication involving the 15q11 region (Butler et al. 2002). All probes within the BP2 to BP3 region had peak sizes at approximately 50% (mean normalized ratio of 0.61 +/− 0.14) compared to those sequences seen in the control subjects. However, *CYFIP1* and *TUBGCP5* had peak sizes of 50% greater (1.56 +/− 0.18) than those of control subjects reflecting three copies of these sequences in our subject relative to control subjects consistent with the previous analysis. The methylation analysis was consistent with PWS and due to a deletion, while the proximal duplication was familial (i.e., father had the same 15q11 duplication) (Butler et al. 2002).

We also studied a PWS infant female with a deletion extending beyond BP3 involving the 15q14 band. The MLPA results for this subject were the same as seen in subjects with a type I deletion. The most distal sequence in the 15q11-q13 region analyzed with the MLPA kit is *APBA2*, thus further assignment of breakpoints in the 15q14 band cannot be assessed with this kit due to the absence of probes in this band region. However, microsatellite analysis with parental DNA using the microsatellites D15S165 and D15S144 (located at 29.1 and 31.4 Mb, respectively) showed a paternal deletion indicating that the deletion extended at least to the 15q14 band (data not shown).

We analyzed additional individuals with chromosome 15 abnormalities including an extra pseudo-isodicentric (marker) chromosome 15 (10 y/o male with autism and 25 y/o unaffected female with miscarriages) identified with FISH analysis and two subjects with ring chromosome 15 (4 y/o male with developmental and speech delay and growth retardation (previously reported (Butler et al. 1988)) and DNA from the second male was obtained from the Coriell cell repository, Camden, New Jersey, GM10173) (Butler et al. 1988). The MLPA analysis of the individuals with marker chromosome 15s showed that the 10 year old male with autism had 4 copies of probes from the entire 15q11-q13 region (mean normalized ratio of 1.90 +/− 0.19) indicating a large marker chromosome 15 reported in individuals with autism. The MLPA analysis of the 25 year unaffected female indicated no increase in copy number for available probes from 15q11-q13 supporting a small marker chromosome involving only 15q11. The lack of MLPA probes in the proximal 15q11 region prevented an informative analysis of this subject. Both males with ring chromosome 15 had normal copy numbers for all probes in the 15q11-q13 region but a normalized ratio of 0.57 for *IGF1R* located at 97.0 Mb but *BLM* located at 89.1 Mb was not deleted indicating a

break occurred between these two genes resulting in a deletion of the distal long arm of chromosome.[15]

We analyzed a female infant with a 15q26.1 deletion reported previously (Roback et al. 1991). The analysis of the sequences located at 15q11-q13 and methylation analysis were similar to those of control subjects. However, the size of peaks from probes located at 15q26 (*BLM* and *IGF1R*) were approximately 50% (0.56 +/− 0.001) compared to control subjects (1.04 +/− 0.08) consistent with a deletion of this region.

One subject, labeled as having an atypical deletion (Table 1), was previously reported with a small deletion including the D15S63 locus and *SNRPN* by FISH analysis. This subject had biparental inheritance of other microsatellites in the 15q11-q13 region (Butler et al. 1996). We identified a small deletion using the MLPA system (*SNRPN*, probe numbers 142, 190 and 292; data not shown) consistent with the previous report indicating a deletion of 100–200 kb including *SNRPN*.

DISCUSSION

The diagnosis of epigenetic disorders is often time consuming and expensive. We have examined MLPA analysis of methylation and copy number of individuals with various chromosome 15 abnormalities. The multiplex reaction contains 43 pairs of primers designed to be separated by capillary electrophoresis into individual peaks. The MLPA kit contains four pairs of primers which amplify fragments of DNA which are ligation independent. These peaks will be high relative to other peaks if the amount of input DNA is low, making the reliability of the reaction questionable. In addition, the kit contains three pairs of primers which are ligation-dependent and will not amplify if the ligation reaction failed or if insufficient DNA was added, making the reaction unreliable. Furthermore, the MLPA kit contains primers specific for target sequences not on chromosome 15 that are either completely methylated or completely unmethylated. These allow for assessment of the quality of the effectiveness of *HhaI* digestion. We found these kit components to be helpful, especially while optimizing the reactions during our first trials. When managed correctly, the PCR reaction results in peaks whose normalized height (or area) is reflective of the starting copy number identified for each probe. Hence, comparison between diploid control subjects allows for rapid and generally reliable detection of deletions and duplications of the 15q11-q13 region and the 15q26 region of chromosome 15.

The assay includes 5 pairs of primers from chromosome 15 which are specific for maternally methylated sequences located in the *SNRPN* or *NDN* promoter. Comparison of the size of the amplified sequences between reactions using *HhaI* digested template and undigested template allowed for a remarkably robust assessment of methylation in subjects with deletions or with uniparental disomy 15.

The assay can distinguish between subjects with deletion or nondeletion (UPD or IC defects) status by first establishing a disorder-specific pattern of methylation (AS or PWS) and then by comparison of relative peak sizes (copy number). If a single copy is present the subject has a deletion while two copies represent either UPD or an IC defect not involving the marker. Subjects with PWS or AS due to UPD or IC defects (nondeletion) can not be distinguished with this assay. However, microsatellite analysis of subject and parental DNA can establish if biparental inheritance occurred in which case the subject would have an IC defect (microdeletion or epimutation) if the DNA methylation pattern is consistent with PWS or AS. If microsatellite analysis indicates uniparental inheritance, then UPD is the cause of the syndrome.

Interestingly, we observed a reduction in copy number for repeated measurements for two probes (*SNRPN* probes sizes 142 and 292) in one of our subjects with an IC defect. These two probes are located near the transcription start site for *SNRPN* and exon 3, respectively. Both loci are known to contain sequences which are important for establishing and maintaining proper methylation of the IC (Kantor et al. 2004). Although this kit is not intended for single probes to be diagnostic, if single probes consistently indicate a reduction in copy number it warrants further consideration.

We used the kit to evaluate multiple other chromosome 15 abnormalities. The MLPA analysis did reveal changes in copy number in the 15q26 band (e.g., *IGF1R*) for the subjects with ring 15 and in one of two subjects with nonmosaic marker 15 chromosomes. We analyzed an individual with a 15q26 deletion and two probes from the kit (i.e., *BLM* and *IGF1R*) had reduced intensity correctly indicating a deletion.

Limitations of the kit were illustrated in two subjects. One individual had a small marker 15 chromosome involving only the 15q11 region but no kit probes are included centromeric to BP1. The distal breakpoint could not be determined in a second subject with PWS with a large deletion involving the proximal 15q region and extending to the 15q14 band because no DNA probes in this kit exist beyond *APBA2* located at 27.1 Mb.

In conclusion, methylation-specific multiplex ligation-dependent probe amplification is a cost and time efficient, reliable technique for analysis of the long arm of chromosome 15 with generally good coverage of probes in the 15q11-q13 region. MLPA can be used to determine methylation patterns and copy number changes in DNA derived from blood or buccal samples from patients with PWS and AS. Used together with microsatellite analysis, MLPA can distinguish between all of the genetic subtypes associated with PWS and AS. Once optimized, MLPA may be the technique of choice for determining the status of the highly variable long arm of chromosome 15 and particularly useful when parent DNA or FISH data are not readily available.

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