# Prader-Willi/Angelman Syndrome: a comparison study of MS-PCR and MS-MLPA

**Authors:**

Angela Brown[1]

Kimberley Flintoff[1]

Clive Felix[1]

Carmel Gillman[1]

Mansour Zamanpoor[1]

Donald R. Love[2,*]

**Affiliations:**

[1] Wellington Regional Genetics Laboratory, Wellington Hospital, Wellington, New Zealand

[2] Diagnostic Genetics, LabPlus, Auckland City Hospital, Auckland, New Zealand

*** Corresponding author:**

Donald R. Love

Diagnostic Genetics, LabPlus
PO Box 110031, Auckland City Hospital, Auckland 1148, New Zealand
Email: donaldl@adhb.govt.nz
Ph:+64-9-3074949 (ext. 22013)
Fax: +64-9-3074939 (ext. 22031)

**Abstract**

**Background:** Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are well-characterised conditions in which the phenotypes and genes responsible are distinct, but they share a common genetic mechanism. PWS is due to a lack of paternally-expressed genes located to human chromosome region 15q11-q13, and AS is due to a lack of maternally-expressed genes located to the same region. There are a variety of testing strategies available to determine if a patient has either of these syndromes.

**Methods and results:** In this study, we tested two of the methods used in clinical laboratories to confirm a diagnosis of PWS and AS: methylation sensitive PCR (MS-PCR) and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). Thirty samples which had previously been tested for either PWS or AS by MS-PCR were blinded and tested prospectively by MS-MLPA.

**Conclusions:** Both tests showed complete concordance with respect to confirming a clinical diagnosis. Of the three principal mutation mechanisms underpinning PWS/AS, MS-PCR cannot resolve any of them while MS-MLPA can detect deletion events; neither can differentiate between uniparental disomy or point mutations in the imprinting centre. Both techniques provide an accurate confirmation of a clinical diagnosis with the possibility of a quick turnaround time of approximately two days. Therefore, either technique would be of benefit in a routine diagnostic laboratory.

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**Keywords:** Angelman Syndrome; Deletions; Imprinting; Prader-Willi Syndrome; Uniparental Disomy

 

## 1. Introduction

Prader-Willi syndrome (PWS; OMIM 176270) and Angelman syndrome (AS; OMIM 105830) are two clinically distinct syndromes which can be caused by a variety of genetic mechanisms that are localised to human chromosome 15q11-q13. PWS was first described in the literature in 1956 [1]. In infancy, PWS patients generally present with hypotonia, poor sucking and feeding difficulties. Other manifestations include developmental delay, short stature, behavioural abnormalities, hypopigmentation, hypogonadism, a characteristic facial appearance and hyperphagia which results in childhood onset obesity [2-4]. Angelman syndrome is a neurodevelopmental disorder characterised by severe developmental delay, intellectual disabilities, electroencephalogram (EEG) abnormalities and seizures, partial or a complete lack of speech, ataxia and dysmorphic facial features. The behavioral features of AS include a happy demeanor, easily provoked laughter, short attention span, hypermotoric behavior, mouthing of objects, sleep disturbance, and an affinity for water [5,6].

High resolution chromosome banding of both PWS and AS patients led to the identification of a small interstitial deletion located to the proximal long arm of chromosome 15 at 15q11.2-q13.3 [4]. Further molecular studies have shown a variety of mutation events that underlie each of these syndromes. The principal outcome of these events is the lack of expression of genes of either paternally-expressed or maternally-expressed genes for PWS and AS patients, respectively. The mutation spectrum underlying PWS encompasses a paternally-derived deletion (65-70%), maternal uniparental disomy (UPD) (20-30%), a point mutation in, or deletion of, the imprinting centre (IC) (2-5%) or a chromosomal translocation involving the imprinting centre (<1%) [4]. The majority of AS cases are the result of a maternally-derived deletion (~70%), paternal UPD (~1-2%), a mutation of the *UBE3A* gene (~2-5%) [7], and a point mutation in, or deletion of, the IC which accounts for 5% [8]; Figure 1 illustrates the different mechanisms which can cause PWS or AS. In terms of the genetic counselling of parents of an affected child (whether PWS or AS), the recurrence risk is <1%, except for those cases in which deletions in the IC are found where the recurrence risk is up to 50% [9].

Deletions in PWS and AS are subdivided into two main subgroups, termed class I or class II depending on where the breakpoints occur, which are flanked by low-copy repeats (Figure 2). Approximately 40% of all deletions are Class I that typically span from breakpoint 1 (BP1) to breakpoint 3 (BP3), resulting in an approximate 6Mb deletion. The remaining deletions, termed Class II, are smaller (~5.3Mb) and lie between breakpoint 2 (BP2) and breakpoint 3 (BP3) [10,11]. Rarely, other deletions have been detected that involve alternative breakpoints (BP4, BP5 and BP6), resulting in larger deletions [12]. In addition, chromosomal translocation events may lead to novel breakpoints.

The PWS and AS critical regions lie between BP2 and BP3, and were the first known examples of human diseases involving imprinted genes [7]. Some genes in this region (e.g. the *P* gene also known as *OCA2* and the cluster of three *GABA* receptor genes) are biparentally expressed [13,14]. Between BP2 and BP3 the 'PWS paternal-only expressed region' contains five protein coding genes (*MKRN3*, *MAGEL2*, *NDN* and the bicistronic *SNURF-SNRPN*) [15] and multiple copies of the so-called C/D box small nucleolar RNAs (snoRNAs) or SNORDS (*SNORD64*, *SNORD107*, *SNORD109A*, *SNORD115* and *SNORD116*) [14]. The 'AS region' contains the preferentially maternally expressed genes *UBE3A* and *ATP10A* [15]; see Figure 2.

 

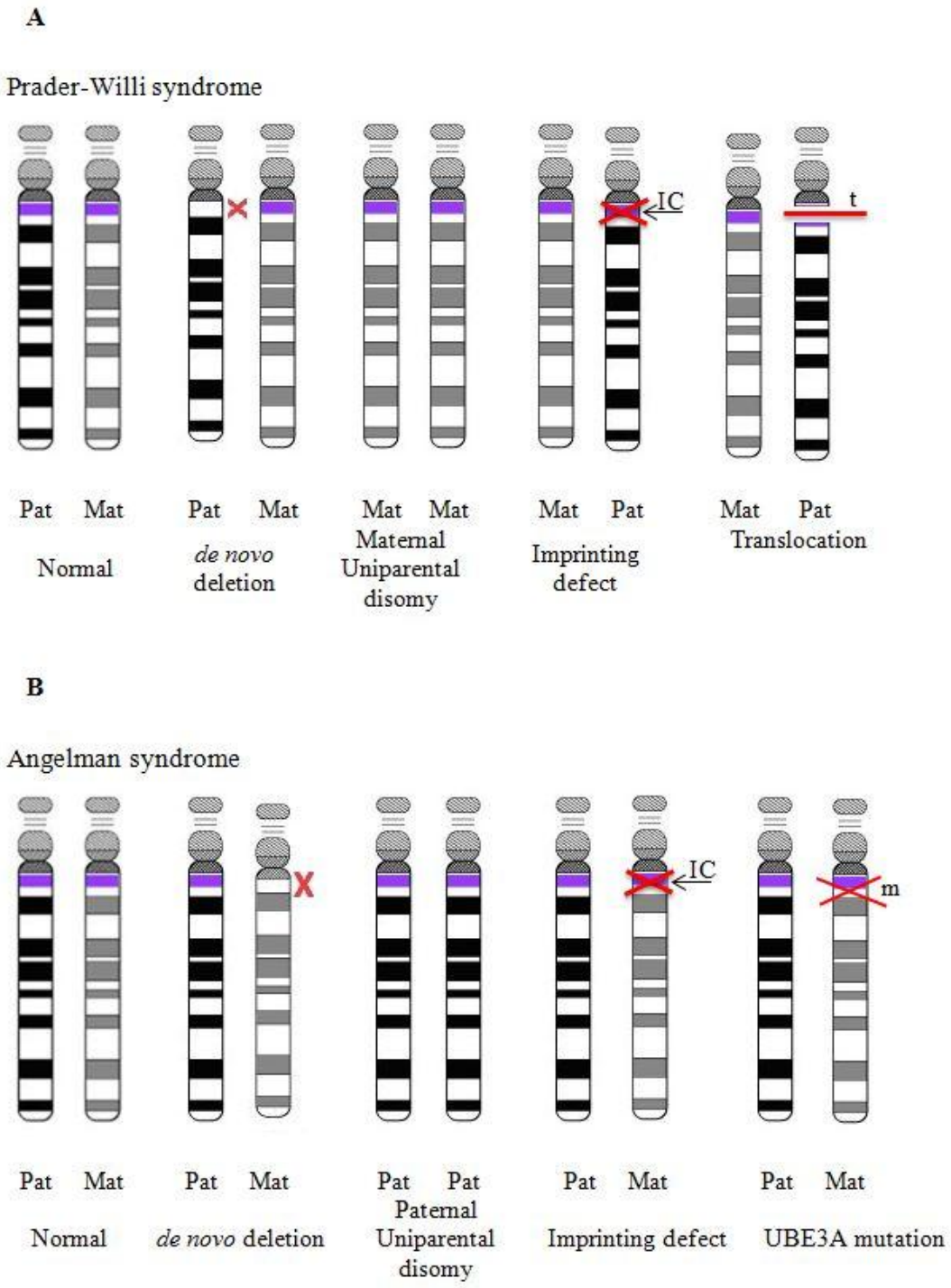


**Figure 1.** Molecular defects in PWS and AS.

**Panel A.** The chromosome pair on the far left shows the scenario in a normal individual; the second pair shows PWS due to a paternal deletion; the third pair shows PWS due to maternal UPD; the fourth pair shows PWS due to a mutation or a deletion of the IC on the paternally-derived chromosome; the final pair shows PWS due to disruption by a translocation involving the IC of the paternally-derived chromosome.

**Panel B:** The chromosome pair on the far left shows the scenario in a normal individual; the second pair shows AS due to a maternal deletion; the third pair shows AS due to paternal UPD; the fourth pair shows AS due to an imprinting defect on the maternally-derived chromosome; the final pair shows AS due to a mutation in the *UBE3A* gene on the maternally-derived chromosome.

The PWS/AS critical regions are highlighted in purple. Mat and pat refer to maternal and paternal, respectively. M refers to mutation and t refers to translocation.

 

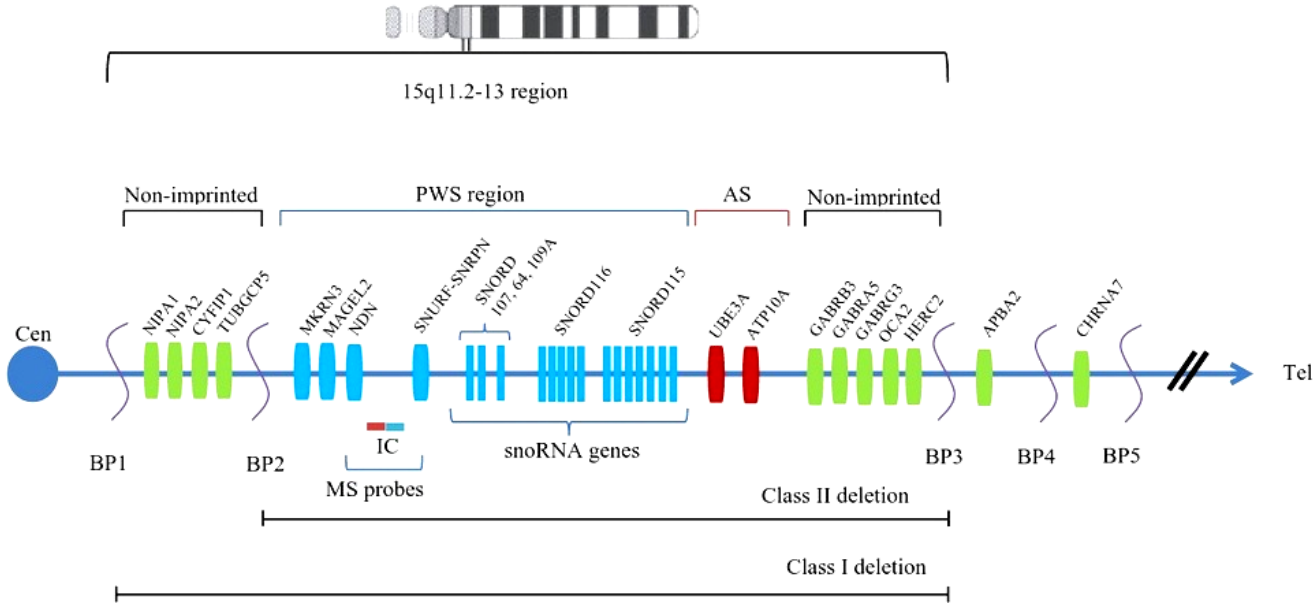


**Figure 2.** Genes within the PWS and AS critical region.
The green boxes represent the non-imprinted/biallelically expressed genes; the blue boxes indicate paternally expressed genes; the red boxes indicate the maternally expressed genes. The regions of the class I and class II deletions are indicated. The arrow shows the orientation of transcription. IC shows the imprinting centre. BP1-BP5 indicates the breakpoint regions 1-5. The region marked 'MS probes' indicates the binding region for the methylation specific probes and primers for MS-MLPA and MS-PCR, respectively.

Despite the fact that the PWS critical region has been well characterised, the exact function of each of the genes in determining the PWS phenotype remains to be elucidated [16]. The SNURF-SNRPN transcript was initially considered to be a strong PWS candidate gene, but subsequent evidence from patients with balanced translocations has excluded it as a primary candidate [17,18]. There is evidence to suggest that the loss of the paternally-expressed snoRNA gene cluster, *SNORD116* may have a significant role in many of the features of the PWS phenotype [19,20].

The candidate gene for AS was initially narrowed down to *UBE3A* by the study of rare patients with atypical deletions and the identification of an inversion breakpoint within this gene [21]. The imprinted *UBE3A* gene exhibits maternal-only expression in specific cell types in the brain, but exhibits biallelic expression in other cell types [22]. *UBE3A* functions both as an E3 ligase in the ubiquitin proteasome pathway and as a transcriptional coactivator [23].

There are a number of techniques that can confirm a diagnosis of PWS or AS. The detection of abnormal parent-specific methylation within the PWS and AS critical region has been reported in more than 99% of individuals with PWS and approximately 80% of individuals with AS [9]. The more commonly used methylation assessment techniques include methylation-sensitive PCR (MS-PCR), methylation-specific multiplex ligation-dependant probe amplification (MS-MLPA) and Southern blot analysis. The latter has largely fallen out of favour due to the need for large amounts of high molecular weight DNA and the time and technical commitments that are required.

MS-PCR is a targeted molecular assay that examines the methylation status of the *SNRPN* gene; however, MS-PCR does not

 

provide information regarding the disease mechanism. Therefore, follow-up studies are required to resolve the specific mutation resulting in an aberrant methylation profile. In contrast, MS-MLPA has the ability to determine aberrant methylation status and to attribute it to a deletion of the *SNRPN* locus. In the event of aberrant methylation but a normal diploid complement of the *SNRPN* locus, then haplotype studies are sometimes undertaken to discriminate between UPD and an imprinting defect.

The purpose of this study was to compare the performance of MS-PCR and MS-MLPA assays in a cohort of patients referred for PWS/AS testing in order to assess concordance as well as the advantages and disadvantages of these laboratory techniques.

## 2. Materials and Methods

### *2.1. Genomic DNA extraction*

DNA from 30 individuals which had been referred to LabPLUS with a clinical suspicion of PWS or AS were tested using MS-PCR. Subsequent to this analysis, these DNA samples were blinded and tested by MS-MLPA. The samples included nine patients diagnosed with PWS, eight with AS and twelve which were determined to have a normal result by MS-PCR. The data reported here involved the confirmation of a clinical diagnosis on the referral to our public hospital laboratory following routine informed consent procedures, and so are excluded from formal ethics committee approval.

### *2.2. MS-PCR*

For this technique, bisulfite conversion was performed using the Qiagen EpiTect Bisulfite Kit (Bio-Strategy Ltd, Vic, AU). Primer sequences PWAS-MF1, PWAS-MR1 which bind to the methylated strand and PWAS-PF2 and PWAS-PR2 which bind to the unmethylated strand were obtained from Emory Genetics (GA, USA); see Table 1 for primer sequences. SNRPNmethv2F and SNRPNmethv2R, which act as an internal control to determine if bisulfite conversion has been successful, were obtained from Integrated DNA Technologies (IA, USA). In addition, SNRPN PCR was performed on non bisulfite-treated DNA to exclude the presence of polymorphisms which could give rise to a false negative SNRPN result. The MS-PCR assay followed the method described by Askree *et al* [24] using the primers described above. The subsequent amplicons were electrophoretically separated in an E-gel. Normal, PWS positive and AS positive controls were included in each assay.

**Table 1.** Details of the primer sequences used in the MS-PCR method in this study.

| Primer name | Sequence | Size (bp) |
|---|---|---|
| PWAS-MF1 | 5'-TAAATAAGTACGTTTGCGCGGTC-3’ | 174 |
| PWAS-MR1 | 5'-AACCTTACCCGCTCCATCGCG-3’ | |
| PWAS-PF2 | 5'-GTAGGTTGGTGTGTATGTTTAGGT-3’ | 100 |
| PWAS-PR2 | 5'-ACATCAAACATCTCCAACAACCA-3’ | |
| SNRPNmethv2F | 5'-GGAGGGAGCTGGGACCCC-3’ | 242 |
| SNRPNmethv2R | 5'-CTCCCCAGGCTGTCTCTTG-3’ | |

 

*2.3. MS-MLPA*

A commercial MS-MLPA kit (ME028, MRC-Holland, Amsterdam, The Netherlands) was used in this study. This probe mix contains 32 probes specific for sequences in or near the PWS/AS critical region which provide information on the copy number within this region. Five of these probes are specific for an imprinted sequence and contain a recognition site for *Hha*1. Table 2 lists the sequences of the five methylation-sensitive probes used in this assay. These probes are used to detect the presence of aberrant methylation patterns. In addition, there are 14 reference probes for genes located outside the PWS/AS region and three probes are used to confirm complete digestion by the *Hha*1 enzyme has occurred within the reaction. The manufacturer's protocol was followed for all steps including DNA preparation, hybridisation, ligation and PCR. Capillary electrophoresis was performed using the ABI 3130 (Applied Biosystems, Foster City, CA, USA). Data was analysed using GeneMarker Software V2.6.7 (SoftGenetics, PA, USA). A panel of six genomic DNA reference samples were used, including 07/236 (PWS paternal deletion-unbalanced chromosome translocation between chromosome 15 and chromosome 19), 07/238 (PWS maternal UPD), 07/240 (PWS paternal deletion class I), 07/230 (AS maternal deletion, class I), 07/232 (AS UBE3A point mutation) and 07/234 (AS paternal uniparental disomic or imprinting centre defect). These samples were sourced from NIBSC (Hertfordshire, UK).

**Table 2.** Primer sequences for the five methylation-sensitive MS-MLPA probes.

| Gene | Chr. pos. | Start | End | Probe sequence |
|---|---|---|---|---|
| NDN | 15q11.2 | 21482490 | 21482551 | 5'-CCAGACTTTGCTAGTCCTCAGAGACACTGCTGCGA-3'<br>3'-GGGTAGTGGGCAGTGGGATTAGCCTCCCGCAGAGC-5' |
| SNRPN | 15q11.2 | 22751105 | 22751156 | 5'-GGAGGGAGCTGGGACCCCTGCA-3'<br>3'-CTGCGGCAAACAAGCACGCCTGCGCGGCCGC-5' |
| SNRPN | 15q11.2 | 22751214 | 22751271 | 5'-CTGCCGCTGCTGCAGCGAGTCTGGCGCAGAGT-3'<br>3'-GGAGCGGCCGCCGGAGATGCCTGACGCATCTGTCTGAG-5' |
| SNRPN | 15q11.2 | 22751480 | 22751541 | 5'-CACCGATGGTATCCTGTCCGCTCGCAT-3'<br>3'-TGGGGCGCGTCCCCCATCCGCCCCCAACTGTGGT-5' |
| SNRPN | 15q11.2 | 22751773 | 22751832 | 5'-GAAGTGATCGGTATTTAGGGGGTGTTGAGCGCAGGT-3'<br>3'-AGGTGTATAATAGTGACCACTGCGTGGTGGAGCAGGGTAC-5' |

Chromosome positions are based on data retrieved from hg18 / map view build 36.

## 3. Results and Discussion

The concordance between the MS-PCR and the MS-MLPA assay results was 100% (Table 3). The MS-PCR results indicated that, of the thirty samples, nine showed methylation patterns consistent with PWS and eight were consistent with AS. The MS-MLPA assay detected the same outcomes. Additional information provided by the MS-MLPA assay indicated that four of the nine PWS cases were due to a deletion (one class I deletion and three class II deletions), the remaining five arose either from UPD or an imprinting defect. With the eight AS cases, five were shown to be due to deletions (two of which were class I and three were class II deletions), the remaining three arose either from UPD or an imprinting defect.

 

**Table 3.** Comparison of results between MS-PCR and MS-MLPA assays

| Sample number | MS-PCR result | MS-MLPA result | |
|---|---|---|---|
| | | Copy number result | Methylation result |
| 1 | PWS | Deletion (class II) | PWS |
| 2 | AS | Normal | AS |
| 3 | Normal | Normal | Normal |
| 4 | PWS | Normal | PWS |
| 5 | Normal | Normal | Normal |
| 6 | PWS | Normal | PWS |
| 7 | Normal | Normal | Normal |
| 8 | PWS | Normal | PWS |
| 9 | Normal | Normal | Normal |
| 10 | Normal | Normal | Normal |
| 11 | PWS | Deletion (class I) | PWS |
| 12 | AS | Deletion (class II) | AS |
| 13 | Normal | Normal | Normal |
| 14 | AS | Deletion (class I) | AS |
| 15 | PWS | Normal | PWS |
| 16 | AS | Normal | AS |
| 17 | Normal | Normal | Normal |
| 18 | AS | Deletion (class II) | AS |
| 19 | AS | Normal | AS |
| 20 | AS | Deletion (class II) | AS |
| 21 | Normal | Normal | Normal |
| 22 | PWS | Normal | PWS |
| 23 | AS | Deletion (class I) | AS |

 

| | | | |
|---|---|---|---|
| 24 | Normal | Normal | Normal |
| 25 | PWS | Deletion (class II) | PWS |
| 26 | Normal | Normal | Normal |
| 27 | Normal | Normal | Normal |
| 28 | Normal | Normal | Normal |
| 29 | Normal | Normal | Normal |
| 30 | PWS | Deletion (class II) | PWS |

## 4. Discussion

There are a variety of approaches used in laboratories to confirm a clinical diagnosis of PWS or AS. These approaches include MS-PCR, MS-MLPA, Southern blotting, microarray and methylation-specific melting analysis [9, 25,26], each with their own benefits and limitations. In the work described here, we performed a comparative analysis of MS-PCR and MS-MLPA assays on thirty patients who had been referred for PWS or AS testing. The results obtained from the two molecular techniques showed complete concordance, with an identical diagnosis in all thirty cases.

MS-PCR determines the methylation status solely at the *SNRPN* locus. By using this technique, an immediate confirmation can be determined; however, it is not possible to distinguish between deletions, UPD or imprinting defects. To determine the disease mechanism, further testing needs to be performed such as FISH or microarray analysis to determine if a deletion has occurred. If no deletion is detected, then haplotype analysis may be required to determine if UPD is the cause of the syndrome. If still no abnormality is detected then further testing is required to screen for an IC defect, which may involve referral to a specialist laboratory.

By using MS-MLPA, the confirmation of a clinical diagnosis of PWS or AS can be promptly obtained. In addition, the copy number status can be determined concurrently with methylation status, thereby enabling cases with a deletion to be distinguished from those with UPD or an IC defect. Should the methylation probes confirm a diagnosis, but there is no evidence of a deletion, further testing is required as MS-MLPA cannot distinguish between UPD and an IC defect [9]. Table 4 provides a summary of the differences between the two techniques. Given the frequency of a deletion in PWS and AS patients is 65-70% and 70%, respectively, then up to 70% of affected patients by MS-MLPA would not require further testing. In the series of patients tested here, 4/9 PWS patients and 5/8 AS patients were found to have a deletion by MS-MLPA. Thus, nine of the seventeen (53%) affected cases would not require further testing to determine the mutation mechanism if MS-MLPA were used.

 

**Table 4.** Comparison between the MS-PCR and MS-MLPA methodology

| **MS-PCR** | **MS-MLPA** |
|---|---|
| No information of disease mechanism. Determines the methylation status only. Requires further testing such as FISH or microarray to determine if a deletion is present. | Provides limited information on disease mechanism. Determines the methylation status and copy number status. Has the ability to distinguish between class I and class II deletions. |
| Requires further testing such as FISH or microarray to determine if a deletion is present. | Further testing such as FISH or microarray is not required. |
| Cannot distinguish between UPD and imprinting disorders. Thus, microsatellite analysis is required to determine if UPD is the cause. | Cannot distinguish between UPD and imprinting disorders. Thus, microsatellite analysis is required to determine if UPD is the cause. |
| Cannot detect small IC deletions and microdeletions within the SNORD116 cluster. | Can detect small IC deletions and microdeletions within the SNORD116 cluster. |
| If UPD testing is normal, characterisation of the IC is required. | If UPD testing is normal and there is no evidence of a small IC deletion, characterisation of the IC is required. |

MS-MLPA can detect small IC deletions and microdeletions within the *SNORD116* cluster [26]. However, analysis of a very large series of PWS and AS patients with an IC defect has shown that the vast majority of IC defects are primary epimutations that have occurred spontaneously in the absence of DNA sequence changes [27]. Therefore, the additional information gained by MS-MLPA with respect to IC defects would only prove informative in a small number of cases. Although the frequency of IC defects in PWS and AS is low (2-5% for PWS and ~2-4% for AS), it is essential to determine the genetic defect leading to these syndromes. Should an IC deletion be identified, then the recurrence risk may be up to 50% for each of PWS or AS if present in the father or mother, respectively [9]. To determine the

 

recurrence risk for patients with an IC deletion detected by MS-MLPA, testing the parents of the proband would be required to determine whether the deletion is present maternally or paternally.

When a deletion is detected by MS-MLPA, it is possible to determine whether the deletion is class I or class II. This is due to the presence of the two most centromeric probes, NIPA1 and TUBGCP5, which enable distinction between the two classes. In this study, there was one class I deletion and three class II deletions for PWS and two class I deletions and three class II deletions for AS. There have been a few studies that have investigated phenotypic characteristics between PWS patients with class I and class II deletions; however, there appears to be a lack of consensus among them. For PWS, some studies have reported that individuals with class I deletions generally have a more severe phenotype than individuals with the class II deletion [11,28]. Other studies have shown no significant behavioural differences between the class I and class II deletion groups [29-31]. With regard to AS, one study has found no major phenotypic differences between class I and class II deletions; however, their study suggested class I patients may have more severe speech impairment than those patients with class II deletions [32]. Another study showed more severe phenotypes in AS patients with class I deletions than those with class II deletions [8]. Clearly, more studies are needed to determine if there is a difference between the two classes of deletion. Should a phenotype/genotype correlation between the two deletion types be established at some stage in the future, then MS-MLPA offers a greater diagnostic outcome.

Both MS-PCR and MS-MLPA have significant advantages over Southern blotting. First, both MS-MLPA and MS-PCR can be completed in two days whereas Southern blotting can take up to five days. The rapid turnaround of this test is important, especially for new-borns presenting with hypotonia in which the incidence of PWS is high [33]. Secondly, testing can be done on smaller amounts of DNA. Approximately 50ng of DNA is required for MS-PCR [34] and 20-200ng is required for MS-MLPA [25] whereas approximately 10μg of DNA is required for a Southern blot. As this testing is often performed on neonates, the volume of blood obtained may be very limited.

One main concern of MS-MLPA is the presence of single base variations within the probe-binding regions, which have the potential to lead to false positive results. If a deletion is detected by a single probe then it is recommended to confirm this using an alternative method. This situation is overcome with the MS-PCR method described in this paper, as a separate PCR is undertaken using non-bisulfite-treated DNA to ensure that there are no polymorphisms that may lead to false-positive results.

It is important to bear in mind that techniques such as MS-MLPA and MS-PCR only provide information for those regions that are interrogated. Balanced cytogenetic rearrangements such as translocations involving the critical region, or a parent carrying a Robertsonian translocation, have an increased risk of producing a foetus with UPD15. Karyotyping is useful to determine if a structural defect is present as a cytogenetic rearrangement would impact on the recurrence risk. For example, the estimated risk of having a child with UPD is ≤0.5% for a parent who is a carrier of a Robertsonian translocation involving chromosome 15 [36], which is a small, but not negligible risk. Carrier parents may wish to be appraised of this risk if they had previously had an affected child. According to best practice guidelines for the molecular analysis of PWS and AS, cytogenetic studies of parental samples is recommended to investigate the possibility of a balanced chromosomal rearrangement following the

 

confirmation of a clinical diagnosis in an affected child.

In general, the fewer tests required, the more cost-effective the investigation becomes. MS-MLPA is relatively expensive, particularly when the batch size is small as multiple controls are required. MS-PCR on the other hand is less expensive compared to MS-MLPA as it requires little more than a bisulfite modification kit and PCR reagents; however, the outcomes do not allow mutation mechanisms to be determined. The complex nature of this syndrome means that no one test can both confirm a clinical diagnosis and unequivocally identify the relevant mutation event. Therefore, testing strategies vary from lab to lab depending on factors such as available resources and specific laboratory policies.

**Acknowledgments/Disclosure of Interests**

The authors have no conflicts of interest to declare, and alone are responsible for the content and writing of the paper.

 

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