Copy number variations: reliable diagnostic markers for Prader-Willi patients

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Background
Syndromic obesity is characterized by a specific set of associated clinical features, but the similarity between different obesity syndromes can make it hard to diagnose accurately. Prader-Willi syndrome (PWS) is the most common type of syndromic obesity. A variety of molecular and cytogenetic techniques may be needed for the diagnosis of obesity. Multiplex ligation-dependent probe amplification (MLPA) helps by allowing the study of multiple specific genetic regions at once. This makes it an effective screening tool for large groups of patients who may have deletions or duplications in specific genes.
Objective
We aim to establish a precise diagnostic scheme for early diagnosis that yields proper early intervention to prevent the development of morbid obesity and intellectual disability, which render a great burden on both health services and the families of the patients.
Materials and methods
Combined cytogenetic fluorescence in-situ hybridization and methylation studies using MLPA was conducted on 20 patients who were clinically suspected to have PWS.
Results and conclusion
We analyzed 20 suspected PWS cases descending from 18 unrelated families and 20 healthy controls matching age and sex, using MS-MLPA PWS/AS probemix (MRC-Holland) enables the identification of copy number variations or abnormal methylation patterns.
One patient (P18) had PW deletion, which was evident by the reduced copy number ratio, and uniparental disomy was evident in two unrelated patients (P2 and P12). The results indicate that our study identified one typical PWS deletion and two uniparental disomy cases among three patients from distinct families, suggesting that atypical deletions are infrequent in this cohort. This research represents the first exploration of PWS in Egyptian patients using MLPA.

Keywords:
multiplex ligation-dependent probe amplification, obesity, Prader-Willi, uniparental disomy

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Introduction

Obesity is characterized by an excessive accumulation of body fat that poses health risks. It is commonly evaluated using BMI, calculated by dividing a person's weight by the square of their height. The condition can arise from single-gene mutations or genomic copy number variations (CNVs), including duplications or deletions. These genetic factors can play a role in both syndromic and nonsyndromic obesity [1,2]. Syndromic obesity includes rare genetic disorders where obesity is one of multiple signs, frequently accompanied by developmental delays and intellectual disabilities [3,4].

Prader-Willi syndrome (PWS) (OMIM# 176270) is the predominant form of syndromic obesity, affecting about one in 10 000 individuals, although many cases go undiagnosed. Diagnosis is usually established in early infancy, often prompted by signs of poor feeding and hypotonia, which usually appear before the development of obesity and hyperphagia [5].

The major clinical features of the syndrome include a recognizable pattern of dysmorphic features with a combination of physical, cognitive, and behavioral/psychiatric disturbances [6,7]. PWS is not widely known in Egypt, and there is a very evident lack of research about it. It is usually undiagnosed, and this results in faulty or unsatisfactory management of

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affected individuals. PWS results from the lack of paternally active genes in the 15q11-q13 region [8,9].

About 70% of individuals with PWS show a deletion in the 15q11-q13 region of the paternally inherited chromosome 15. About 25% have maternal uniparental disomy (m-UPD), while 5% have an imprinting sequence variant, and about 1% have a structural rearrangement affecting the 15q11.2-q13 region [9,10]. In PW patients, only the methylated allele(s) of the small nuclear ribonucleoprotein polypeptide N (SNRPN) gene are present in the promoter region [11,12]. Multiplex ligation-dependent probe amplification (MLPA) offers a most cost-effective, faster alternative to microarray-based methods and allows for simultaneous analysis of CNVs and abnormal DNA methylation across multiple target loci [4,13,14].

Many studies suggested that the main problem in PWS is cognitive and behavioral, and that the clinical picture improves tremendously through early diagnosis and behavioral modification. A review of cognitive abilities in 575 individuals with PWS found that only 5% had a normal IQ [15]. Additionally, 70–90% of PWS patients exhibit behavioral disorders from childhood, including poor impulse control, frequent outbursts of anger, manipulative behaviors, and compulsions such as excessive food-seeking and skin picking [16]. Other microdeletion syndromes with similar phenotypic features also show high obesity rates, making clinical diagnosis challenging and often lacking a clear diagnostic pathway. Examples are Temple syndrome (OMIM # 616222) [17], Beckwith-Weidman (OMIM # 130650, BWS) [18–20], and 6q16 deletion syndrome (OMIM 603128) [21].

Some of these syndromes are now understood to be caused by haploinsufficiency of a single gene within the critical deletion region. For instance, the deletion of 6q16 is probably due to haploinsufficiency of the single-minded 1 gene, which functions as a transcription factor. In contrast, the obesity susceptibility associated with WAGR syndrome is primarily linked to haploinsufficiency of the brain-derived neurotrophic factor gene, which is involved in brain function [21].

This research aims to establish a precise diagnostic scheme for early diagnosis that yields proper early intervention to prevent the development of morbid obesity and intellectual disability, which render a great burden on both health services and the families of the patients.

Materials and methods

This study included 20 suspected PWS cases descending from 18 unrelated families and 20 healthy controls matching age and sex. They were enrolled in the Clinical Genetics Department, National Research Centre, Egypt. Their age ranged from 6 to 15 years (mean±SD=7.67±4.48). Patients were comprised based on the presence of the essential clinical disease manifestations. Meticulous medical history, evaluation of demographic data, history of present illness, and disease progression were documented. Informed written consent was obtained from the parents, and the study received approval from the ethical committee of the National Research Centre.

The following cytogenetic techniques were accomplished:

(1) Karyotype: using the conventional G-banding technique.
(2) Fluorescence in-situ hybridization (FISH) technique.

FISH technique was performed on metaphase chromosomes or interphase nuclei from peripheral blood samples, following modifications of Pinkel *et al.* [22] and the manufacturer's instructions, using Prader-Willi/Angelman (SNRPN) Region Probe15q11.2, Red 15qter, 15q26.3 Green, 50 metaphases were analyzed to confirm the deletion of chromosome 15.

(1) MS-MLPA technique.

MS-MLPA was conducted using the "SALSA MS-MLPA probemix ME028-C1 Prader-Willi/Angelman PWS/AS" from MRC-Holland to analyze CNVs and methylation patterns.

DNA extraction

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For DNA denaturation, 1.5 μl of SALSA MLPA buffer was added, followed by 1.5 μl of methylation of multiplex ligation-dependent probe amplification (MS-MLPA) probes (1 fmol each) to the denatured DNA from each patient. After incubating for 1 min at 95°C, the probes were hybridized to their targets for 16 h at 60°C. Following this hybridization, the mixture was diluted with water and 3 μl of ligase buffer A to achieve a final volume of 20 μl, which was then split into two tubes. One tube was incubated at 49°C with 0.25 μl of ligase-65 (MRC-Holland), 5 U of HhaI, and 1.5 μl of ligase buffer B in a total volume of 10 μl, while

the other tube received water in place of HhaI. After ligation and digestion at 49°C for 30 min, the mixture was heat inactivated at 98°C for 5 min. The ligation products were subsequently PCR amplified by adding 5 μl of the ligation mixture to 20 μl of the PCR mixture, which contained PCR buffer, SALSA polymerase, and PCR primers (one unlabeled and one D4-labeled), at 60°C.

The thermocycler program included 30 min at 48°C (for ligation and HhaI "restriction endonuclease enzyme" for digestion), 5 min at 98°C (for enzyme heat inactivation), and a pause at 20°C before removing the tubes from the thermocycler.

Fragment separation by capillary electrophoresis

In the ABI-Prism genetic analyzer sequencing plate, 0.7 μl of each PCR reaction was placed in a different well and mixed with 0.2 μl LIZ GS 500 size standard dye and 9 μl HiDiformamide. The plate was sealed and heated at 86°C for 3 min, then cooled at 4°C for 2 min before injection into the sequencer.

Statistical analysis

Coffalyser Net was utilized to analyze the MS-MLPA data. MS-MLPA analysis involves two main steps: first, determining copy numbers by normalizing the measured fluorescence of each probe within the sample to obtain accurate data. Second, the methylation profile is assessed to quantify the percentage of methylation for specific probe sets in the sample. The ratio obtained for a single probe is then compared between the digested and undigested samples from PWS deletions, which would show a ratio of 0.5 to 0.5 due to the control remaining uninfluenced by digestion due to the absence of unmethylated paternal sequences. To assess the methylation profile of a test sample, it is compared to reference samples. Patients with m-UPD will also show a ratio of 1 to 1, as two duplicates are maternally methylated and thus remain undegraded.

Results and discussions

We analyzed 20 suspected PWS cases and 20 healthy controls. Twelve were the offspring of consanguineous marriage (60%). Their age ranged from 6 to 15 years (mean±SD 7.67±4.48 years). They were 12 males and eight females (1.5 : 1). The most common features were hypotonia, feeding difficulties, and overweight, followed by delayed milestones, dysmorphic features, behavioral problems, and abnormal genitalia as shownin Table 1.

Karyotype analyses of 20 collected samples of clinically evaluated patients for PWS were carried out, followed by FISH analysis. FISH analysis showed that 17 out of 20 patients were found negative and three patients were found positive (Fig. 1).

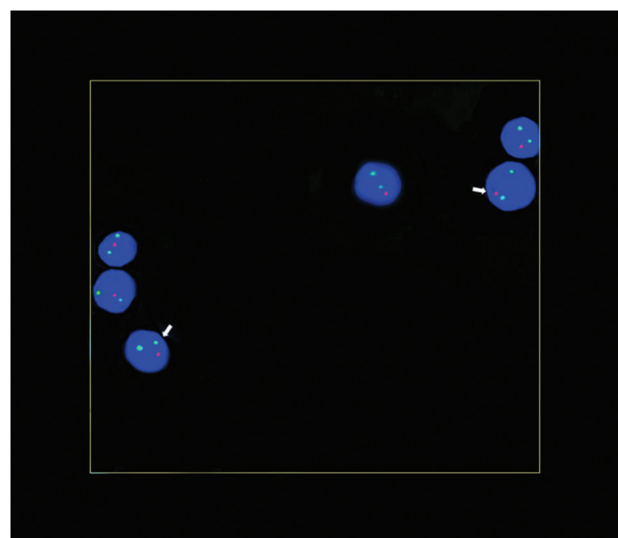
The patients were diagnosed utilizing MS-MLPA PWS/AS probemix (MRC-Holland) to the identify CNVs or abnormal methylation patterns. MLPA studies were done for patients and controls. DNA was degraded in one patient. The results are summarized in Table 2. One patient (P18) had PW deletion evident by reduced copy number ratio (ratio=0.5) of 33 MS-MLPA compared to control (ratio=1) and methylation pattern of 100% for the five methylation-specific probes (four SNRPN and one MAGEL2 probe) compared to the reference sample's 50% methylation (Figs 2 and 3).

MS-MLPA profile of PW deletion was not detected in the rest of the 19 patients. UPD was evident in two

Table 1 The clinical picture of the studied patients with Prader-Willi syndrome

	Age	Percentage
	6–15 years	7.67±4.48
Sex male : female	12 : 8	1.5 : 1
Consanguinity	12	60
Hypotonia	20	100
Feeding problems	20	100
Overweight	20	100
Delayed milestones	19	95
Dysmorphic features	18	90
Behavioral problems	17	85
Abnormal genitalia	14	70

Figure 1



Fluorescence in-situ hybridization (FISH) using Prader-Willi Angelman region probe showing deletion in 15q11.2 (one red signal) and the internal control probe 15q26.3 (two green signals).

Table 2 Results of methylation of multiplex ligation-dependent probe amplification technique for 20 patients with Prader-Willi syndrome

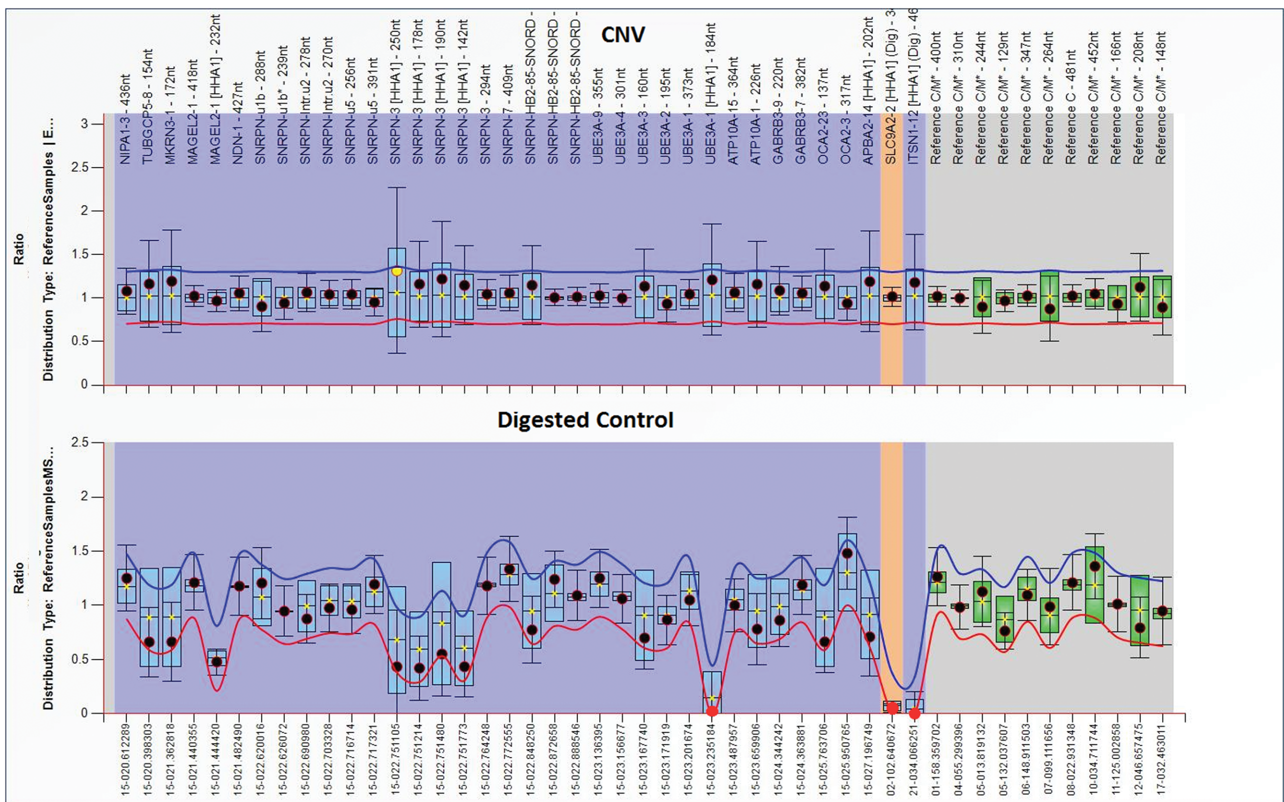
Patients	Copy number variations (CNV) analysis	Methylation pattern
P1	Normal CNV	Normal
P2	Normal CNV	UPD
P3–P11	Normal CNV	Normal
P12	Normal CNV	UPD
P13–P17	Normal CNV	Normal
P18	PW deletion	PW deletion
P19 and P20	Normal CNV	Normal

PW, Prader-Willi; UPD, uniparental disomy.

unrelated patients (P2 and P12) who showed a 100% methylation pattern of the five methylation-specific probes in comparison to the normal 50% methylation pattern in control samples with no changes in CNV profiles (Fig. 3). Large deletions of the 15q11 region (covered by 33 MLPA probes) on the maternal chromosome and/or aberrant methylation (covered by five methylation-specific MLPA probes) of the 15q11 locus cause PWS. The MS-MLPA reaction was repeated twice for samples that previously showed ambiguous results. For example, the P1 sample showed double deletion of SNRPN5 [250 nucleotides (nt) probe] and SNRPN-u1b (287 nt probe) probes only plus two other samples (P5 and P6), which showed deletion of SNRPN-3 (294 nt probe) only. Upon MS-MLPA reaction repetition, the three samples were found to have normal CNV and methylation profiles. Other ambiguous results were obtained for two patients (a male and a female) descending from the same family, where the male showed a 75% methylation pattern while the female showed normal MS-MLPA profile. Upon repetition of these two samples, similar results were obtained.

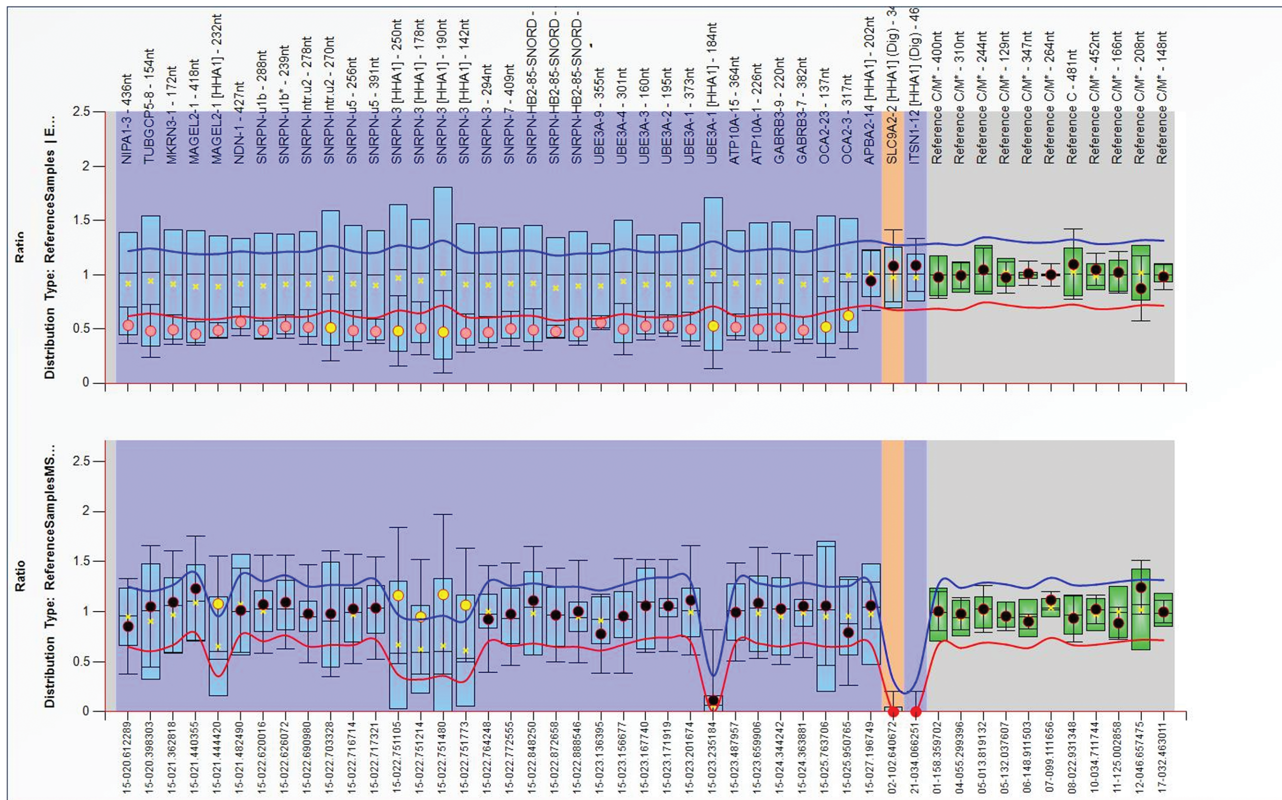
The age of diagnosis of PWS is often delayed to early childhood because the clinical findings at the earlier ages are relatively nonspecific, and the dysmorphism is usually subtle [23,24]. This study involved children aged 6–15 years. While PWS is commonly linked with obesity, children with PWS usually face feeding problems and poor growth during their first year. Newborns with PWS often have weak muscle tone, may cry less, have difficulty sucking, and tend to sleep a lot. They also reach developmental milestones later

Figure 2



Distribution frequency curves of control DNA analyzed with Prader-Willi/Angelman multiplex ligation-dependent probe amplification (MLPA) probemix for copy number variations (CNV) analysis (upper) and methylation analysis (lower). The figure shows probes arranged according to their chromosomal location (horizontal) versus the copy number ratio for each probe (vertical). The 33 MLPA probes (blue bars) and 11 reference probes (green bars) show an average copy number ratio of 1. In the methylation analysis (lower graph), complete digestion by *HhaI* is indicated by the absence of digestion control probes (red circles) and 50% methylation pattern of the five methylation-specific probes (red arrows) of average ratio=0.5.

Figure 3



Distribution frequency curve of DNA of patient 18 analyzed with Prader-Willi/Angelman multiplex ligation-dependent probe amplification (MLPA) probemix for copy number variations (CNVs) analysis (upper) and methylation analysis (lower). The figure shows probes are arranged according to their chromosomal location (horizontal) versus the copy number ratio for each probe (vertical). In CNV analysis (upper graph), the 33 MLPA probes (blue bars) show an average copy number ratio of 0.5 versus an average copy number ratio of 1 for the 11 reference probes (green bars). In the methylation analysis (lower graph), complete digestion by *HhaI* is indicated by the absence of digestion control probes (red circles) and the 100% average methylation pattern of the five methylation-specific probes (yellow circles).

than usual. After their first year, they start experiencing extreme hunger, and by school age, managing their constant food-seeking behavior becomes increasingly challenging [25].

Although it is reported that PWS affects both males and females equally and is found across all races and ethnicities [26], the suspected PWS cases of the current study included 12 males and eight females (1.5 : 1) showing a tendency to suspect the diagnosis of PWS more in the males. Twelve (60%) cases (out of 20) of the current study were the offsprings of consanguineous marriage suspecting a possible link between affection with PWS and the consanguinity factor. This is in agreement with the study of Shawky *et al.* [27], which found that chromosomal and microdeletion syndromes, such as PWS, are caused by a deletion on chromosome 15, specifically in the 15q11-q13 region were more prevalent in the offsprings of consanguineous marriages in Egypt.

The most common features of the cases in the current study were hypotonia (100%), feeding difficulties (100%), and overweight (100%), followed by delayed milestones (95%), dysmorphic features (90%), behavioral problems (85%), and abnormal genitalia (70%). These are the cardinal features to clinically suspect the diagnosis of PWS. The percentages of most of these features in the cases of the current study were comparable to those of the study of Lu *et al.* [28], which included 104 PWS patients. In this study, certain symptoms were nearly universal or very common at different ages for those with PWS; feeding problems (94.23%), hypotonia (100%), and cryptorchidism (90% in males) were nearly widespread during the neonatal period. Motor delay (100%), sticky saliva (79.41%), and short stature (85.29%) were prevalent from infancy. Speech delay (74.70%), learning disabilities (97.59%), and hyperphagia (73.49%) were usual during childhood. In older patients, incomplete or delayed puberty (81.82%), hypogonadism (90.91%), and lack of satiety (86.67%) were frequently observed. This is similar to previously reported findings [26,29].

PWS results from the absence of genes in the 15q11-q13 region that should be expressed from the father's

side. This loss can happen either through a deletion of the paternal chromosome or by obtaining a couple of copies of chromosome 15 from the mother, both of which are typically inactive on the m-UPD 15 [2,30]. Newly formed paternal deletions account for around 70% of PWS, while atypical deletions in the 15q11-q13 region make up ~5% of cases. m-UPD of chromosome 15 is observed in around 25% of PWS [30]. Samples of 20 PW patients and 20 healthy controls were analyzed using MS-MLPA PWS/AS kit to determine PWS's underlying genetic makeup. To identify copy number changes in the PWS region, the probemix includes 33 probes targeting sequences in or near the critical 15q11-q13 region of chromosome 15. This region contains multiple paternally expressed genes, including both coding and noncoding genes (e.g. small noncoding RNAs) [2]. Large deletions of the 15q11 region (covered by 33 MLPA probes) on the paternal chromosome and/or aberrant methylation (covered by five methylation-specific MLPA probes) of the 15q11 locus cause PWS. The addition of 11 reference probes for genes situated outside the PWS/AS region helps ensure accurate CNV analysis within the sample. Typical PWS deletions involve the absence of most of the signals of the PWS region, as seen in the CNV analysis of the P18 sample (Fig. 2). The remaining 19 patients have shown normal CNV patterns similar to healthy control samples (Fig. 1). Interestingly, typical PWS deletion represents 5% of our PWS Egyptian cohort.

To examine CpG island methylation of the 15q11 region, the *HhaI* digestion reaction on five MS-MLPA probes (four SNRPN and one MAGEL2 probe) is compared to results obtained on DNA samples from healthy controls. Digestion control probes ensure a complete digestion reaction where 90–96% of their peak height is lost after digestion. The methylation profile was normal (50% methylation of the five methylation-specific probes) except for three patients (P2, P12, and P18) who showed a 100% methylation pattern in comparison to the normal 50% methylation pattern in the control sample (Figs 1–3). In P18, the methylation profile confirms the PWS deletion detected by the CNVs analysis (Fig. 2). In P2 and P12, m-UPD is confirmed given the aberrant methylation profile with the normal CNVs pattern (Fig. 3). Conclusively, UPD represented 10% of our PWS Egyptian cohort.

In the first report, we reported small, atypical deletions that were observed in three patients (P1, P5, and P6), which are rare and should be confirmed in paternal samples. Nonetheless, when the three samples were repeated twice, they showed normal CNVs. Given that the same probe was observed to be deleted in two of those samples, we suspected probe failure, which was confirmed by reaction repetition. Other confusing results were obtained for two patients (a male and a female) descending from the same family, where the male showed a 75% methylation pattern while the female showed normal MS-MLPA profile. Upon repetition of these two samples, similar results were obtained, which provided interest for the acquisition and analysis of their paternal samples.

Conclusion

The findings suggest that one typical PWS deletion and two UPD cases were identified in three patients from different families, indicating that atypical deletions are rare in this cohort. This study serves as the first investigation of PWS in Egyptian patients using MS-MLPA.

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Conflicts of interest

There are no conflicts of interest.

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