The role of molecular approach in the diagnosis of Prader-Willi syndrome : the experience of a Tunisian laboratory

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ABSTRACT

Prader Willi syndrome (PWS) is a rare genetic disorder that affects an estimated 1 in 10,000 to 30,000 people worldwide. It is a complex multisystemic condition. PWS is a model of genomic imprinting. The underlying genetic disorder is a function loss of 15q11-13 region of paternal origin due either to a microdeletion of paternal origin, a uniparental disomy (UPD) of maternal origin or more rarely to a mutation of the imprint center located near the region 15q11-13. In this study, we discuss the optimal strategy for the genetic diagnosis of PWS by comparing cytogenetic techniques and molecular analysis and their contributions to the various mechanisms of PWS. This molecular genetic test should be proposed in the face of any clinical suspicion of PWS.

Key words : Prader willi syndrome, genomic imprinting, maternal disomie, paternal deletion, molecular analysis.

RÉSUMÉ

Le syndrome de Prader Willi (PWS) est une maladie génétique rare qui touche environ 1 personne sur 10 000 à 30 000 dans le monde. C'est une condition multisystémique complexe. PWS est un modèle d'empreinte génomique. Cette anomalie d'empreinte est une perte de fonction de la région 15q11-13 d'origine paternelle due à une microdélétion d'origine paternelle, une disomie uniparentale (UPD) d'origine maternelle ou plus rarement à une mutation du centre d'empreinte située à proximité de la région 15q11-13. Dans cette étude, nous discutons la stratégie optimale pour le diagnostic génétique de PWS au moyen de techniques cytogénétiques et d'analyse moléculaire et de leurs contributions aux divers mécanismes de PWS. Ce test génétique moléculaire devrait être proposé face à toute suspicion clinique de SPW.

Mots-clés : syndrome de Prader Willi, anomalie d'empreinte, disomie maternelle, délétion paternelle, analyse moléculaire.

INTRODUCTION

Prader Willi Syndrome (PWS) is a rare genetic disorder. It is an important cause of genetic retardation and one of the main causes of syndromic obesity in children (1). It is characterized by hypothalamic-pituitary dysfunction causing a major hypotonia during the neonatal period and the first two years of life. From childhood to adulthood, the major call signs are: hyperphagia leading to morbid obesity, learning difficulties, behavioral disorders and even major psychiatric disorders (2)(3). SPW is a model of genomic imprinting. The underlying genetic disorder is a function loss of 15q11-13 region of paternal origin due either to a microdeletion of paternal origin, a uniparental disomy (UPD) of maternal origin or more rarely to a mutation of the imprint center located near the region 15q11-13 (3). We present in this work an optimal strategy for the genetic diagnosis of PWS. Indeed, we provide molecular diagnostic evidence of PWS in 15 patients, specifying the underlying molecular mechanisms and the specific genetic counseling that breaks down and the procedures for prenatal diagnosis.

Materials and methods

We conducted an analytical and descriptive cross- sectional study of 15 Tunisian patients presenting with PWS. The 15 patients were referred to our laboratory for investigation of neonatal hypotonia during the time period between 2010 and 2015 by the neonatology and pediatric departments of Farhat Hached University Hospital in Sousse, Monastir maternity and neonatology center, the department of pediatrics of Monastir and the department of pediatrics of the hospital Ibn El JAZZAR of Kairouan. Patients previously diagnosed with 15q11q13 deletion by in situ fluorescence hybridization (FISH) were not included in this work. The DNA of the patients was extracted from 3 ml of peripheral venous blood sample taken from a tube containing an anticoagulant "EDTA". The extraction was carried out using QIAGEN's "FexiGeneDNA kit" from the blood leukocytes according to the manufacturer's recommendations. Molecular analysis was carried out in accordance with international recommendations by first using the MS-MLPA technique (Methylation Specific Multiplex Ligation-Dependent Probe Amplification) which is a semi-quantitative technique. It is a variant of the MLPA technique which allows the simultaneous detection of variations in the number of copies of several specific sequences of the genome. The MS-MLPA is distinguished from MLPA by its property of detecting methylation abnormalities in specific sites of the genome. This is accomplished by the addition of a methylation-sensitive restriction enzyme digesting step that does not cleave the DNA when the cytosines of its restriction site are methylated. Thus, once hybridized to the patient's genome, the probes carrying the unmethylated restriction site will be digested by the enzyme and thus will not be amplified. Conversely, when the restriction site contains methylated cytosines, the probe will not be cleaved and amplified. Thus, we obtain for the probes which target regions differentially methylated according to their parental origin, an amplification product which corresponds to half of that which is expected. This allows us to study the methylation profile of each of these regions and detect any changes. While MLPA detects 15q11q13 deletions, MS-MLPA detects methylation abnormalities (maternal disomy and mutation of the imprint center). To confirm a DUP, we use a second technique, the primed PCR triplet (TP-PCR), which is a variant of the fluorescent PCR. This technique allows to confirm the uniparental disomy by analysing the microsatellite markers type STR (Short Tandem Repeat). STRs are short DNA-based repeats, distributed throughout the genome, and the size of which may vary from one individual to another within the population. Since these repetitions are transmitted in a Mendelian way, each individual has 2 alleles of each repetition, one transmitted by the chromosome of paternal origin and the other by the chromosome of maternal origin. The analysis of several microsatellite-type STR markers located in 15q11q13 enabled us to determine in some of our patients whether they received a chromosome 15 from each parent (two-parent origin) or both of its chromosomes are derived from the same parent (UPD). The fluorescent PCR products resulting from the MLPA, MS-MLPA and TP-PCR reactions are analyzed by the ABI 310 and 3500 genetic analyzers. The results of MLPA and MS-MLPA are analyzed by the "GeneMarker" software. The results of TP-PCR are analyzed by the "Genotyper®" software.

Results

The admission pattern consisted for our patients in generalized hypotonia, associated or not with facial dysmorphy, obesity, anomaly of the external genital organs, hyperphagia and psychomotor retardation. Ten of our patients were male compared to only five girls. A maternal age of more than 35 years was observed in 6 mothers (Table 1).

Tableau 1 : Table summarizing the clinical and genetic characteristics of our 15 patients.

Patient	Sex	Mother's Age	Antenatal Ultrasound	Terme	Age Of Diagnosis	Birth Weight	Clinical Signs			Mechanism
							Global hypotony	Facial dysmorphism	Hypogonadism	
1	M	24 years	Hydramnios	36 weeks + 3D	First hour of life	2500g	+	+++	+	Paternal deletion
2	M	43 years	oligohydramnios, IUGR	36 weeks	First hour of life	1930g	+	+/-	+	Maternal disomy or mutation of impression center
3	M	28 years	Normal	35 weeks + 2D	First hour of life	2090g	+	+/-	-	Paternal deletion
4	F	35 years	Normal	38 weeks + 5D	First hour of life	2750g	+	+++	+	Maternal disomie
5	F	24 years	Hydramnios	41 weeks	20 days	2200g	+	+	-	Paternal deletion
6	M	36 years	Normal	34 weeks	1 months et 4 days	2330g	+	-	-	Maternal disomie
7	M	36 years	Normal	39 weeks	17 days	2400g	+	-	-	Maternal disomie
8	M	40 years	Normal	42 weeks	First hour of life	2800g	+	+/-	+	Maternal disomy or mutation of impression center

9	F	NP	NP	41 weeks	8 months	2400g	+	-	-	Paternal deletion
10	F	31 years	NP	Full-term	1 year and 4 months	2700g	+	+	-	Paternal deletion
11	M	NP	IUGR	36 weeks + 6D	8 months	NP	+	-	-	Paternal deletion
12	M	31 years	Hydramnios	38 weeks	4 years	2280g	+	+	+	Maternal disomy or mutation of impression center
13	M	NP	NP	NP	6 months	NP	+	+	+	Paternal deletion
14	F	44 years	IUGR	post-term	14 years	2000g	+	-	-	Maternal disomie
15	M	40 years	IUGR	Post-term	11 months	2900g	+	+	+	Maternal disomy

Abbreviations :
M: Male, F: Female, g : gram, H: hour, D: day, IUGR: Intrauterine growth restriction
+ present, +++ net, +/- discreet, - absent
NP : not precised

We detected a 15q11q13 deletion of paternal origin in 7 patients (46.6%) and maternal disomy in 5 other patients (33.33%) (figure 1) (Table 1).

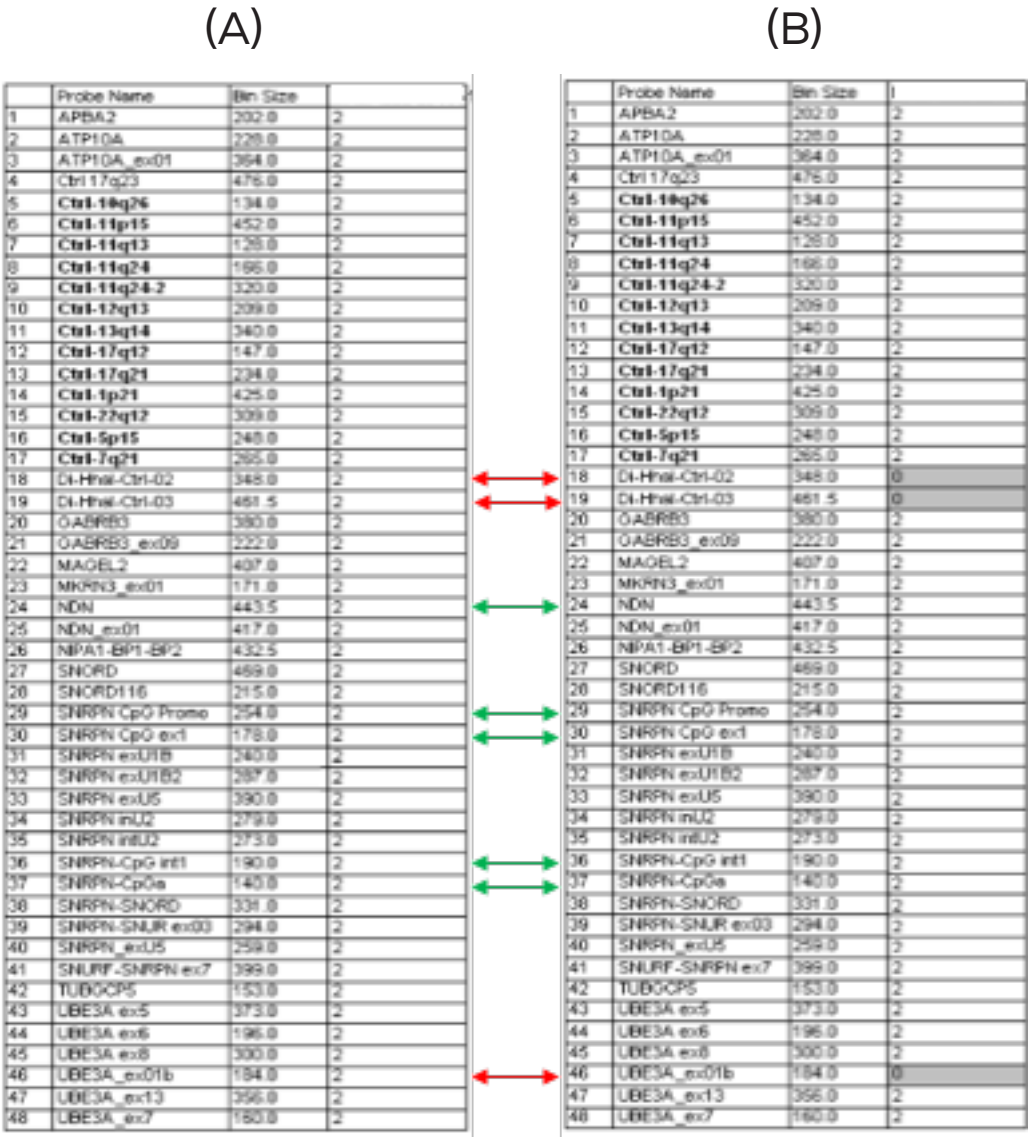
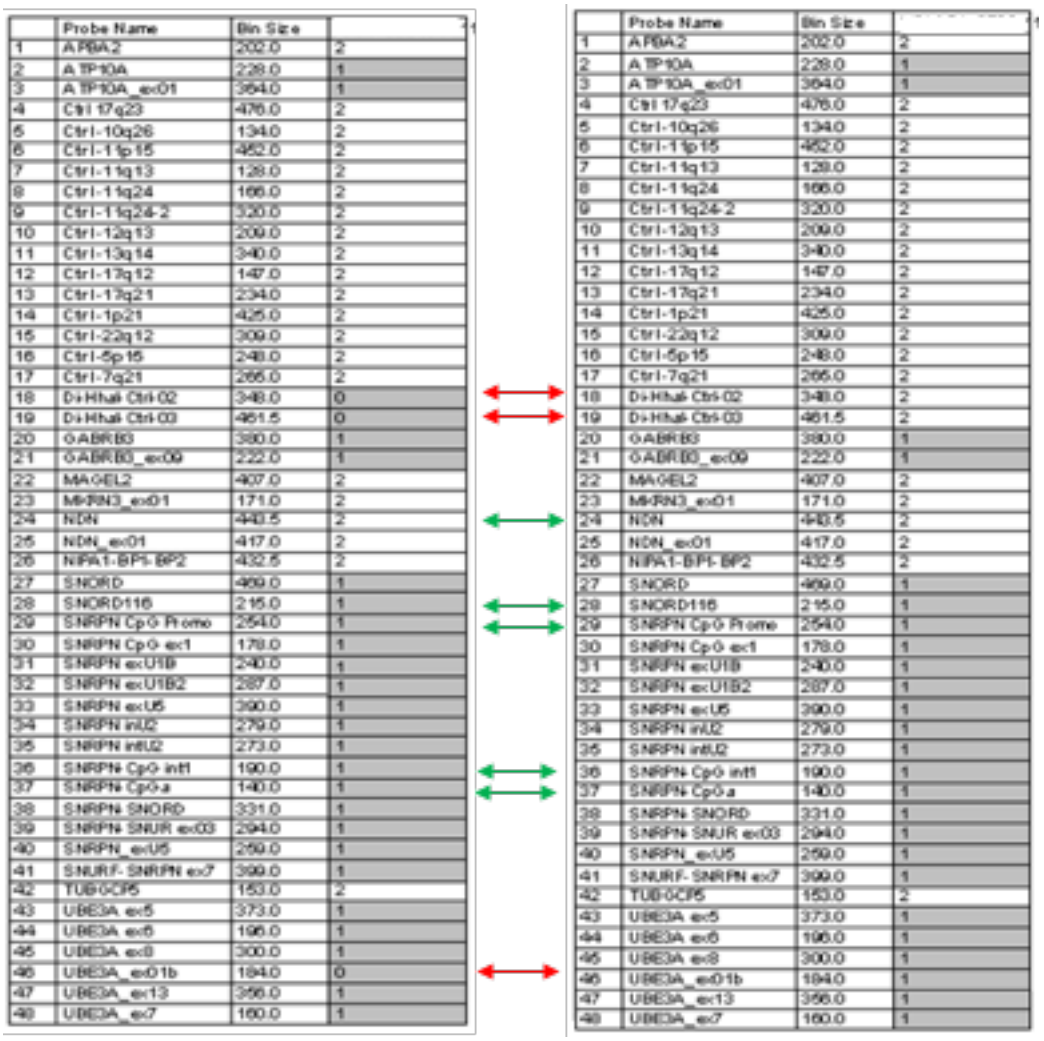


Figure 1 : MS-MLPA profiles before (A) and after (B) enzymatic digestion indicating a maternal disomy.
P : paternal, M : maternal
The 3 red arrows indicate the methylation-sensitive probes or digestion controls reduced from 2 copies to no copy after digestion thus validating the digestion reaction. The green arrows indicate the differentially methy-

lated probes remaining undigested, which clearly indicates the presence of two methylated copies corresponding to either 2 maternal copies or a maternal copy and a paternal copy with mutation of the impression center.

In 3 newborn babies (# 2, # 8 and # 12), disomy remains the most likely genetic mechanism, although the latter could not be confirmed by TP-PCR, in the absence of parental DNA. Our patients with paternal deletion (#1, #3, #5, #9, #10, #11, and #13) presented themselves with a clinical picture strongly suggestive of the SPW. Indeed, in addition to hypotonia, facial dysmorphism and hypogonadism are noted.
Children with maternal disomy (#4, #6, #7, #14, #15) have a lighter phenotype with very discrete or absent facial dysmorphism, less severe hypotonia, absence of hyperphagia and better development.
The MLPA technique allowed delimiting the size of the deletion for these patients with paternal deletion.
Patients number #1 and #13 carry respectively a 2,7 Mega bases deletion and a 1,7 Mega bases deletion and present a complete and typical clinical picture.
Despite the fact that patients number #5 (figure2) and #10 carry the same 2,7 Mega bases deletion containing the same genes as patient #1, they don't present the identical clinical signs.

(A) (B)



	Probe Name	Bin Size	
1	APBA2	202.0	2
2	ATP10A	228.0	1
3	ATP10A_ex01	364.0	1
4	Ctrl 17q23	476.0	2
5	Ctrl-10q26	134.0	2
6	Ctrl-11p15	462.0	2
7	Ctrl-11q13	128.0	2
8	Ctrl-11q24	166.0	2
9	Ctrl-11q24-2	320.0	2
10	Ctrl-12q13	209.0	2
11	Ctrl-13q14	340.0	2
12	Ctrl-17q12	147.0	2
13	Ctrl-17q21	234.0	2
14	Ctrl-1p21	425.0	2
15	Ctrl-22q12	309.0	2
16	Ctrl-5p15	248.0	2
17	Ctrl-7q21	266.0	2
18	D+Hhal Ctrl-02	348.0	0
19	D+Hhal Ctrl-03	461.5	0
20	GABRB3	380.0	1
21	GABRB3_ex09	222.0	1
22	MAGEL2	407.0	2
23	MKRN3_ex01	171.0	2
24	NDN	448.5	2
25	NDN_ex01	417.0	2
26	NIPA1-BP1-BP2	432.5	2
27	SNORD	469.0	1
28	SNORD116	215.0	1
29	SNRPN Cp G Promo	254.0	1
30	SNRPN Cp G ex1	178.0	1
31	SNRPN exU1B	240.0	1
32	SNRPN exU1B2	287.0	1
33	SNRPN exU5	390.0	1
34	SNRPN inU2	279.0	1
35	SNRPN inU2	273.0	1
36	SNRPN Cp G int1	190.0	1
37	SNRPN Cp G a	140.0	1
38	SNRPN SNORD	331.0	1
39	SNRPN SNUR ex03	294.0	1
40	SNRPN_exU5	259.0	1
41	SNURF-SNRPN ex7	399.0	1
42	TUBGCP5	153.0	2
43	UBE3A ex5	373.0	1
44	UBE3A ex6	196.0	1
45	UBE3A ex8	300.0	1
46	UBE3A_ex01b	184.0	0
47	UBE3A_ex13	356.0	1
48	UBE3A_ex7	160.0	1

	Probe Name	Bin Size	
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11	Ctrl-13q14	340.0	2
12	Ctrl-17q12	147.0	2
13	Ctrl-17q21	234.0	2
14	Ctrl-1p21	425.0	2
15	Ctrl-22q12	309.0	2
16	Ctrl-5p15	248.0	2
17	Ctrl-7q21	266.0	2
18	D+Hhal Ctrl-02	348.0	2
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45	UBE3A ex8	300.0	1
46	UBE3A_ex01b	184.0	1
47	UBE3A_ex13	356.0	1
48	UBE3A_ex7	160.0	1

Figure 2 : MS-MLPA profiles before (A) and after (B) enzymatic digestion indicating a paternal deletion.
The 3 red arrows indicate the methylation-sensitive probes or digestion controls reduced from 2 copies to no copy after digestion thus validating the digestion reaction.
The green arrows indicate the differentially methylated probes found in a single copy before and after digestion, which indicates the presence of a single maternal methylated copy and thus the presence of a paternal deletion.

They only have hypotonia and facial dysmorphism (figure 3). Patients number #9 and #11 carry a 2,7 Mega bases deletion as well and have only hypotonia.
Patient number #3 carries a 1,4 Mega bases deletion with a less severe phenotype consisting of hypotonia.

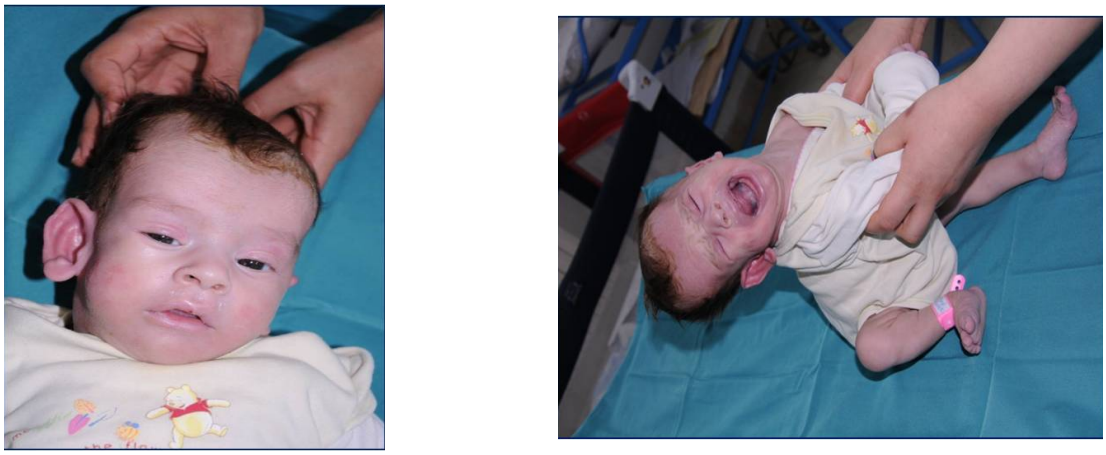

Figure 3 : facial dysmorphism and general hypotonia of patient #5.
A broad front, a bitemporal retraction, almond-shaped eyes, low implanted ears, a thin upper lip, a micro-retrognathism, base of the nose wide and flattened, an accentuation of the nasolabial folds.

DISCUSSION

Once the diagnosis of PWS is performed, it is essential to establish a genetic diagnosis of certainty not only for the affected child ,so that he may benefit from specific and early care, but also for the family because the risk of recurrence depends on the underlying genetic mechanism (5).
Three main mechanisms are involved: paternal 15q11q13 micro-deletion involved in 70% of cases of SPW, maternal uniparental disomy found in 25 to 30% of cases and much more rarely in 1 to 2% of cases a mutation Of the impression center (6) (7) (8) (Figure 4).

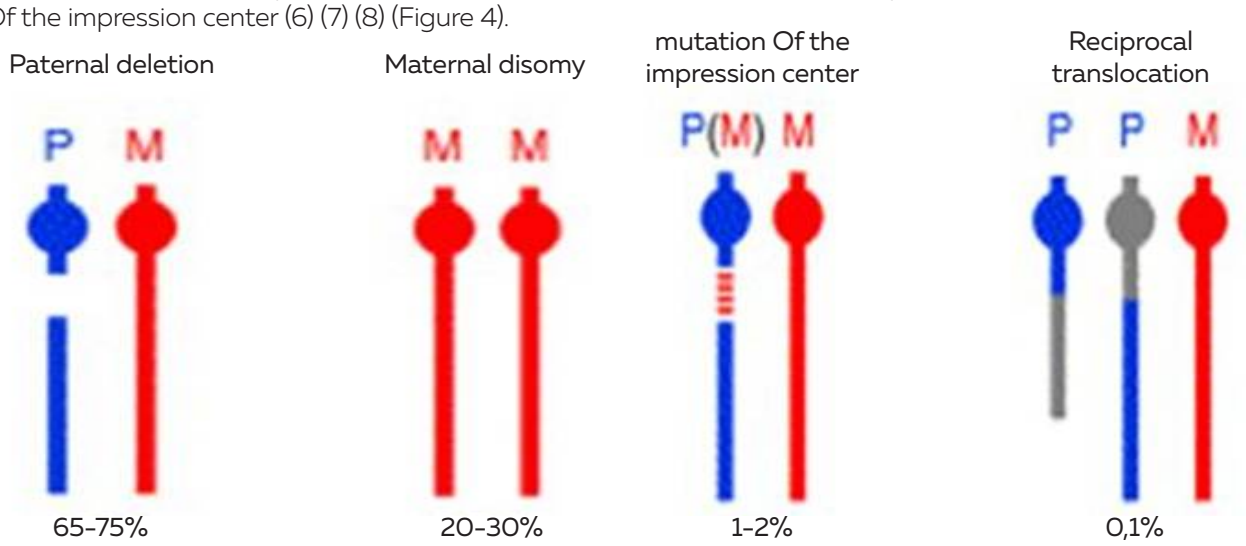

Figure 4 : Molecular mechanisms involved in PWS.

Confrontation of techniques: Since maternal uniparental disomy is involved in almost a third of the causes of SPW, it can only be confirmed by a molecular study. This UPD can in no case be detected by a cytogenetic technique (karyotype + FISH), the latter being able to demonstrate only the deletions. The diagnostic approach should therefore not be limited to cytogenetic analysis and molecular analysis is necessary. In fact, in the case of UPD or mutation of the imprinting center, the FISH will reveal two copies of the SNRPN gene, which can be wrongly excluded from the diagnosis (table 2). The optimal diagnostic approach and according to the international recommendations (5) consists in using directly the study by molecular genetics which makes it possible to confirm or invalidate the diagnosis of PWS whatever is its mechanism. The MLPA and MS-MLPA techniques have the possibility to detect in a single step the methylation disorders and their probable causes, namely deletions, rare duplications, UPD and mutations of the center of imprint (7). They have the advantage of being simpler and quicker to realize with a lower cost and a small amount of DNA required (9). Analysis by MLPA also allows delimiting the exact size in the case of deletion compared to the FISH which uses a single probe of a predefined size. Moreover, those cytogenetic techniques (karyotype and FISH) require costly cell culture with a high cost. Moreover, there is a difficulty in detecting nested deletions of smaller size than the FISH probe (8) (10). While cytogenetic techniques may be faulty in the diagnosis of PWS, they are essential to define the risk of recurrence in the event of future pregnancies in case of deletion (8) (11). Indeed, a deletion can be transmitted by one of the parents, in particular in the case of PWS by the

father. This deletion in the father will define a risk of recurrence which will be 50% for each pregnancy (4) (11).

Characteristics of our study population : Ten of our patients are male (two-thirds) versus only five female. Most studies agree on the lack of predilection for sex. This male predominance would be justified by a greater ease in detecting the signs of hypogonadism in boys (13). This hypogonadism is of central origin. It is characterized in boys by unilateral or bilateral cryptorchidism and scrotal hypoplasia, which are quasi-constant. A micropenis, a hypo-pigmented and slightly wrinkled scrotum are less frequently reported (8). In females, severe absence or hypoplasia of the labia minora and/or the clitoris are noted but the diagnosis remains more difficult (14) (15). In all our patients, neonatal hypotonia was the first clinical sign that made the SPW suspect. This hypotonia manifests itself most often from the prenatal period by a decrease in the active fetal movements (16). This finding may go unnoticed especially if the patient is primitive and the newborn is then diagnosed from the first hours of life to the discovery of neonatal hypotonia (17). This major hypotonia is constant at birth, it is predominantly axial and of varying severity. Facial dysmorphism can be discrete to moderate, often difficult to appreciate in newborns. This dysmorphia associates a narrow forehead, almond-shaped eyes, a fine upper lip, and a mouth in a gendarme's hat or in a circumflex accent (18)(19). During the first 2 years of life, the clinical picture is often marked by dietary difficulties with stagnation by weight. It is not until the second year of life that the appearance of a hyperphagia causing morbid obesity. The clinical picture is most often richer combining a small size, hypogonadism, learning disabilities, behavioral disorders that can go to psychiatric disorders (8) (20)(22). The consequences and management are therefore cumbersome, leading to major difficulties for patients, their families and healthcare teams.

Correlations between genotypes and phenotypes : Patients with paternal deletion (#1, #3, #5, #9, #10, #11, and #13) present a clinical picture strongly suggestive of the SPW (figure 3) while children with maternal disomy (#4, #6, #7, #14, #15) have a lighter phenotype with very discrete or absent facial dysmorphism (figure 5), less severe hypotonia, absence of hyperphagia and better development (9).

Figure 5 : facial dysmorphism of patient #4.

A broad front, a discreet bitemporal retraction, bilateral ptosis more marked on the left, low implanted ears, thin and drooping upper lip and micro-retrognathism.

This variability of the clinical spectrum in terms of severity is explained by the presence in PWS with UPD of two alleles of the 15q11-13 region, whereas the second allele is lacking in the case of deletion. Indeed, the presence of the second allele even in the case of disomy contributes to attenuate the phenotype.
For the patients with paternal deletion, phenotypes present a very large clinical spectrum even for the same detected size deletion. This may be explained by the limits of the MS-MLPA and TP-PCR techniques in spotting the entire deletion and its exact size.
These results also illustrate the need for collaborations between geneticists, pediatricians, neonatologists and endocrinologists by the genotype-phenotype correlations.

CONCLUSION

PWS remains under-diagnosed in routine diagnostic by the strict use of cytogenetic techniques (karyotype and FISH), which as we have documented are unable to detect abnormalities other than deletions and can therefore miss a third of PWS. This work allowed us to conclude on the interest of the molecular test in confirming the diagnosis of SPW and essentially for the incomplete or atypical forms, apart from the deletions. Given its reliability and availability, this molecular genetic test should indeed be proposed in the face of any clinical suspicion of PWS. The results of this study allowed us to provide diagnostic proof of the PWS and to provide genetic counseling adapted to the families concerned. This genetic counseling is an integral part of the care of the patient and his family. It consists in informing parents about the possibility of recurrence of this disease for possible future pregnancies.

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