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Prader–Willi Syndrome, from Molecular Testing and Clinical Study to Diagnostic Protocols

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1. Introduction

Prader-Willi syndrome (PWS) is a complex and fascinating human disease, whose pathophysiological characteristics are still the targets of research in teams that can afford multidisciplinary approaches for seeking the link between the genetic, epigenetic and phenotypic aspects. The genetic complexity of the PWS chromosomal region, 15q11-q13, relies on the multiple clustered imprinted genes, alternative splice variants, gene duplications and variant copies, that control the epigenetic phenomenon of the imprinting itself. These DNA and transcriptome levels are matched by the wide variety of phenotypes that involve multiple organ systems and the complexity of brain functions influenced by the expression of the PWS critical genes.

In this review a general description of the clinical diagnostic criteria will be linked with the most recent knowledge described for the structure of the 15 critical chromosomal region and candidate genes, as well as the model mechanisms explaining the interaction of the cis- and trans- genetic factors and the epigenetic ones during the establishment and maintenance of the imprinting marks that define the parental characteristic contribution to the critical genes expression. This review aims at explaining the criteria of molecular diagnosis and genetic counseling based on the techniques that are currently used and that will be used in the future approaches for the improvement of the diagnosis and treatment schemes.

PWS has been initially linked with its main characteristic phenotype, the obesity, and therefore was the first described genetic human obesity syndrome. The main etiology of this disease included: gross hyperphagia, hypogonadism and growth hormone deficiency, indicating hypothalamic dysfunction. A neurodegenerative aspect was also appreciated as a major contributor to the complex PWS phenotype. Recent epidemiological study proved that PWS is a rare disease with an estimated incidence of about 1 in 25 000 births, and a population prevalence of about 1 in 50 000 (Buiting, 2010). An interesting feature linked with the diagnosis and the treatment impact is that this syndrome develops during late development of neonate. Initially, the signs like hypotonia had not suggested a suspicion for PWS, nor the consideration of further clinical and molecular cytogenetic investigations, until

the moment when the feeding habits started to restore but meantime to initiate an uncontrolled rate of weight gaining leading to the obesity state and the linked illness conditions. The wrong concept according to which the genetic disorder involves solely genetic modifications that may be monitored at the chromosomal level has not been proved by the classical cytogenetic approaches : the affected child commonly presents a normal karyotype and rarely a translocation or a gross deletion; this fact was misleading the medical geneticians towards wrong conclusions and hence incorrect strategy for treatment and social integration.

The initial genetic investigations on PWS and its sister syndrome, the Angelmann syndrome (AS), have driven the research towards the first proof of the epigenetic regulation of the gene function: AS was detected by the same genetic defect, a deletion on the same critical 15q11.q13 region, however it was defined by quite different phenotypes. This fact lead to the conclusion that beyond the identical affected chromosomal region there were involved certain other factors, such as the parental allele contribution to the candidate genes expression. Once the specific epigenetic marking of the allele for their expression was discovered in terms of the DNA methylation, the corresponding histone modifications and RNA processing, the research in this domain has been oriented towards new clues that could link the two types of hereditary information: genetic information, defined by either deletions as cis-acting factors and those coded by the single nucleotide polymorphisms (SNPs) as trans-acting factors, and the epigenetic information used in the process of imprinting. Both genetic and epigenetic approaches resulted finally in a more correct estimation of the types of defect frequencies and focused also on the right moment for diagnosis, and the right tissue type for the imprinted gene expression. Once the molecular mechanism of the defect establishment and maintenance is designed based on aberrant germ cells reprogramming during the parental meiosis, then after fertilization, during embryogenesis and fetal development, an improvement of the genetic counseling activities may be envisaged in the future for the patient and genitors' benefit.

Based on the newly established relationship between genotype-epigenotype and phenotype, new approach of the clinical diagnosis was initiated, that considers actually the dynamics of the epigenome reprogramming and hence the spatio-temporal variation in gene expression, that is imposed by the epigenetic control of cytodifferentiation processes. It became commonly apparent that clinical features of PWS appear during different developmental moments: severe hypotonia and consequently feeding difficulties concomitant with low birth weight- in early infancy, followed by hyperphagia and obesity - starting in early childhood. These general characteristics are accompanied by certain common features that determined the establishment of consensus diagnostic clinical criteria for PWS (Holm et al., 1993; Cassidy and Driscoll, 2009). They include both physical features such as facial appearance (like almond-shaped eyes, triangular mouth and narrow bifrontal diameter), short stature and small hands and feet, and distinctive behavioral traits due to mild and moderate mental retardation (such as temper tantrums, obsessive-compulsive characteristics and psychiatric disturbance as well as motor milestones and language development delay). The score includes also features that appear later during the child development; these are defects in sexual development (genital hypoplasia that may result in hypogonadism in both sexes and incomplete pubertal development and frequent infertility) and the obesity linked

features, among which the most frequent one is the non-insulin-dependent diabetes mellitus (Buiting, 2010).

Structural characteristics of the critical chromosomal 15q11.q13 region explain its genomic instability and its special behavior during the primary imprinting process, during the genitor's meiosis and germ line establishment, followed by the secondary imprinting process, that occurs during the affected offspring PCG (primordial germ cells) determination and germ cells specialization.

The major determinant feature of the critical chromosomal region is the abundant imprinted genes arranged in clusters together with numerous variable sequences (CNVs) (such as interstitial microdeletions, duplications, triplications) that are flanking the critical breakpoints (BPs) defining the around 5 Mb deletions. Due to the presence of these critical instability regions on chromosome 15, another critical region, named IC for imprinting control region, apparently contributed to defects that determines wrong marking of parental alleles and hence their gene expression in all somatic cells of the offspring. The structure and control of functions in this region will be discussed below.

2. PWS genetics

A genetic approach of the PWS includes the description of the candidate genes and their expression which is epigenetically controlled based on the parental contribution. PWS arises when the lack of contribution from the paternally derived chromosome 15q11-q13 occurs (Goldstone, 2003). Normally, candidate genes for PWS in this region are imprinted and silenced on the maternally inherited chromosome. The causes of the lack of contribution are multiple in PWS: either paternal alleles are sequence defective (mutant or missing) or silenced by wrong, repressive epigenetic marking. The imprinting marking determines in normal, healthy individuals, the expression of critical genes only from the paternal allele. Paternally expressed genes are particularly important in hypothalamic development, this fact explaining the spectrum of neuroendocrine disturbances in PWS. These genes are located in the centromeric part of the 15q11q13 region and are as follows: MKRN3, MAGEL2, NDN, C15orf2, SNURF-SNRPN and the C/D box small nucleolar (sno)-RNA genes. The latter genes are represented by numerous, so-called SNORD genes, previously named HB (human brain) II genes (SNORD107, previously named HBII-436; SNORD64, previously named HBII-13; SNORD108, previously named HBII-437; SNORD109A, previously named HBII- 438A; SNORD116, previously named HBII- 85; SNORD115, previously named HBII- 52 and SNORD109B, previously named HBII- 438B) (Buiting, 2010). These genes are differentiated based on their repetitive state: SNORD 115 and SNORD116 genes are present as multiple copy clusters, whereas the other SNORD genes are single copy genes. The snoRNA genes in the critical chromosomal 15 region might have a role in modulating alternative splicing and thus be involved in the modification of mRNA (Cavaille et al., 2000; Bazeley et al., 2008). Recent investigations revealed that SNORD116 gene is the minimal region linked with the PWS phenotypes (Buiting, 2010).

The most frequent genetic causes linked with the paternal contribution are large (a typical 5-7 Mb) chromosomal de novo interstitial deletions (either type I or type II deletions detected in around 70%, of PWS cases) and the double maternal chromosomal contribution by uniparental maternal disomy (UPD), with 22% frequency (Fig.1). With much lower

frequency were detected paternal chromosomal translocations (less that 1% of cases). Other explanations of the lack of paternal contribution are grouped in the class of epigenetic defects that impaired the imprinting process (around 3% of cases). In Fig. 1 a schematic view of the human chromosomal region 15q11q13 the genes expressed from different alleles are differently colored; there is evidenced also the localization of two common deletions [del (15)(q11-q13)] (class or type I and class II deletions) and their localization. Such deletions include the entire imprinted domain plus certain non-imprinted (green boxes) genes. Different allelic localizations of such large deletions demonstrated the involvement of the parent of origin marking by epigenetic factors. In PWS, they are always on the paternal allele, whereas in AS, they occur on the maternal chromosome. The deletion regions are flanked by the three break-points (BP1, BP2 and BP3) (Fig.1). In certain rare cases even larger deletions were detected extending telomeric region including the more distal break-points, named BP4 and BP5 (Sahoo et al., 2007).

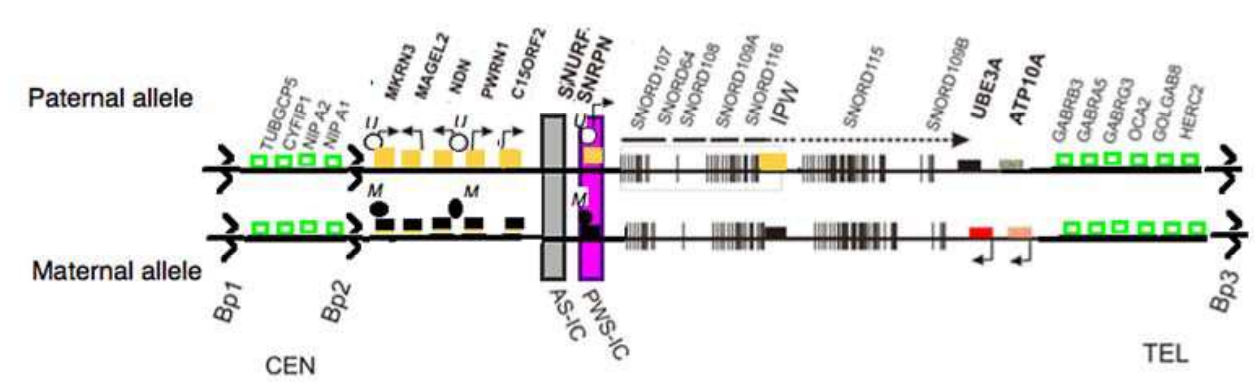


Fig. 1. A map of the 15q11-q13 critical region. Imprinted, paternally-expressed genes are represented by yellow boxes, while imprinted, maternally-expressed genes are represented by red boxes. Black boxes represent imprinted (silenced) genes and green boxes represent genes expressed biallelically. Dashed and dotted lines demarcate the snoRNA clusters. The PWS- and AS-ICs are represented by two differently colored domains. Black circles indicate methylated (M) CpG islands and the white circles the unmethylated (U) CpG islands. BP1, BP2, and BP3 are the common deletion breakpoints and are represented by zigzag lines. (Chamberlain and Lalande 2010)

The structure of these BPs consists of repetitive blocks of 250-400 kb, that explains their instability and hence their role in non-homologous recombination events during parental meiosis (Christian et al., 1998, Amos-Landgraf et al., 1999). It was demonstrated that the deletions can occur via cross-over interchromosomal (between the two homologous chromosomes) or intrachromosomal (between different regions of one chromosome 15) events (Carrozzo et al., 1997; Robinson et al., 1998). The causes of maternal uniparental disomy [upd(15)mat] occurrence was ascribed to maternal meiotic non-disjunction followed by mitotic loss of the paternal chromosome 15, during fertilization (Buiting, 2010).

PWS epigenetics investigates the causes of the imprinting defects and hence the occurrence of the lack of expression of the paternally inherited genes. In patients with PWS and an imprinting defect, the paternal chromosome carries a wrong (maternal) imprint in terms of the epigenetic marks on DNA and histones or noncoding RNA (wrong distribution of DNA methylated cytidine residues in the paternal and maternal genes concomitant with wrong histone modification and ncRNA expression). In PWS, these epigenetic modifications are repressive not only for the maternal allele, but also, in a wrong way for the paternally expressed genes in the 15q11q13 region.

Much of research has been performed in order to find the causes of such non-genetic defect. This epigenetic factor in PWS may be or may be not accompanied by deletions. The first situation was rarely observed (8-15%), but the second one is more frequent (85% in PWS cases).

The discovery of cases of critical DNA regions containing both small deletions and the imprinting defect led to the definition of a bipartite imprinting controlling (IC) region: its role is regulation in cis- the process of imprint resetting and imprint maintenance in the whole critical chromosomal region 15q11q13 (Sutcliffe et al., 1994; Buiting et al., 1995). Thus the paternal-only expression of MKRN3, NDN, and SNURF-SNRPN genes is regulated by the parent-of-origin epigenetic modification of the promoter regions of these genes. Another parental expressed gene, C15orf2 has a special feature of expression: it has been reported to be biallelically expressed in testis, however, in brain its expression is restricted to the paternal allele (Wawrzik et al., 2010). A recent review of the genetics and epigenetics in PWS revealed about 21 IC-deletions in patients with PWS (Buiting, 2010).

The most complex gene in the critical chromosome 15 region is linked with the IC region and is considered SNURF- SNRPN gene. IC contains the major transcriptional start site of SNURF- SNRPN gene. It consists of 10 exons which encodes in fact two different proteins: exons 1-3 encode SNURF (SNRPN upstream reading frame), a small polypeptide of unknown function (Gray et al., 1999), while exons 4-10 encodes a small nuclear (SmN) ribonucleoprotein named SNRPN, a spliceosomal protein involved in mRNA splicing in the brain (Ozcelik et al., 1992). SNURF gene, along with upstream noncoding exons, has been considered the major site of imprinting defects, because disruption of this gene leads to altered imprinting of SNRPN and many other 15q11-q13 imprinted genes. However, numerous 5' and 3' exons of SNURF- SNRPN gene identified up to now do not encode proteins and they occur in many splice forms of the primary transcript (Dittrich et al., 1996; Farber et al., 1999). Exon 1 and the promoter region of this complex genetic locus overlap with the IC. Also, as it has been mentioned earlier, the SNURF-SNRPN region also serves as a host for all snoRNA genes encoded within its introns. These genes lack a direct methylation imprint, but their imprinted expression is indirectly regulated by the same SNURF-SNRPN methylation (Horsthemke and Buiting, 2008; Buiting, 2010).

The IC region investigations resulted in its definition by two critical elements that are named the smallest regions of overlap (SRO) that control the imprinting process in PWS and AS: the AS-SRO and the PWS-SRO (Buiting et al., 1995; Buiting, 2010). The 4.3 kb long PWS-SRO overlaps with the SNURF-SNRPN exon 1/promoter region (Ohta et al., 1996b). This IC element is required for the maintenance of paternal imprint during early embryonic developments (El-Maarri et al., 2001).

The cases of imprinting defects with no IC deletion are classified in a subgroup of cases defined by primary epimutations or epigenetic modification (Buiting et al., 2003; Horsthemke and Buiting, 2008).

3. Clinically PWS diagnosed cases in Romanian population

Diagnostic testing: approaching classical genetic and epigenetic methods, as well as advanced combined sequencing and epigenetic methods, in a study of a cohort of 17 clinically PWS diagnosed cases in Romanian population (Table 1).

Nr crt	Clinical diagnosis	Molecular tests confirmation			Classical cytogenetic confirmation
		MSPCR	FISH	MLPA	Karyotype
1	PWS	-	-	-	-
2	PWS	-	-	-	-
3	PWS	-	-	-	-
4	PWS	-	-	-	-
5	PWS	-	-	-	-
6	PWS	-	-	-	-
7	PWS	-	-	-	-
8	PWS	+	+	del NDN, SNRPN, UBE3A	-
9	PWS	-	-	-	-
10	PWS	+	+	-	-
11	PWS	-	-	-	-
12	PWS	+	+	-	-
13	PWS	-	-	-	-
14	PWS	+	-	Nondeleted NDN, SNRPN, UBE3A	-
15	PWS	+	+	-	-
16	PWS	+	-	Nondeleted NDN, SNRPN, UBE3A	-
17	PWS	-	-	-	-
18	PWS	-	-	-	-
19	PWS	-	-	-	-

Nr crt	Clinical diagnosis	Molecular tests confirmation			Classical cytogenetic confirmation
		MSPCR	FISH	MLPA	Karyotype
20	PWS	-	-	-	-
21	PWS	+	+	-	-
22	PWS	+	+	-	-
23	PWS	-	-	-	-
24	PWS	+	+	-	-
25	PWS	-	-	-	-
26	PWS	-	-	-	-
27	PWS	+	+	del NDN, SNRPN, UBE3A, TUBGCP5, MKRN3, APBA2	-
28	PWS	+	-	-	-
29	PWS	+	+	-	-
30	PWS	-	-	-	-

Table 1. Patient Sample characterstics: clinical, karyotipical and molecular aspects

3.1 Clinical methods

The most obvious features observed were obesity and mental retardation and the rest of criteria being variable. Our survey in Romanian population resulted in a DNA bank designed to characterize the PWS features. Initial surveys contributed to gathering 13 females and 7 males, between 6 month and 28 years old. Positive clinical diagnosis of PWS was based on major and minor criteria, the minimum diagnostic score being 3 - up to age 3 and 5 – for patients older than 3 (up to the adult age) (Gunay-Aygun M et al., 2001, Holm VA et al., 1993, P. Goldstone et al., 2008, Suzanne B Cassidy and Daniel J Driscoll, 2008). Consequently, clinical diagnosis of PWS has been established based on characteristic clinical features that differ with age (Ledbetter DH and Engel E, 1995, Ohta T et al., 1999). In the newborn infant, the suggestive feature remained hypotonia; it resulted from the history in our study (patients aged 6 month to 7 years – 6 cases and more than 14 years – 11 cases) (Table 2 in Annexes). Obesity, moderate mental retardation, behavioral disturbances related to food and learning difficulties are present in all studied cases. Facial features of PWS (periorbital fullness, almond-shaped and down-slanting palpebral fissures, malar hypoplasia, down-turned mouth corners and thin upper lip) are also present (Figs.2, 3, 4).

3.2 Classical genetic approach

Karyotyping- for the monitoring of the variations in chromosomal morphology as a result of DNA sequence modifications (deletions, translocations) is the classical genetic approach. Initially, the gold standard was thought to be the karyotype. But this classical chromosome investigation commonly did not reveal any of the complex genetic and epigenetic defects lately described for PWS patients (Fig. 3). Also, only very rare paternal translocations and even more rare deletions have been detected in the restricted HBII-85 region, more recently.

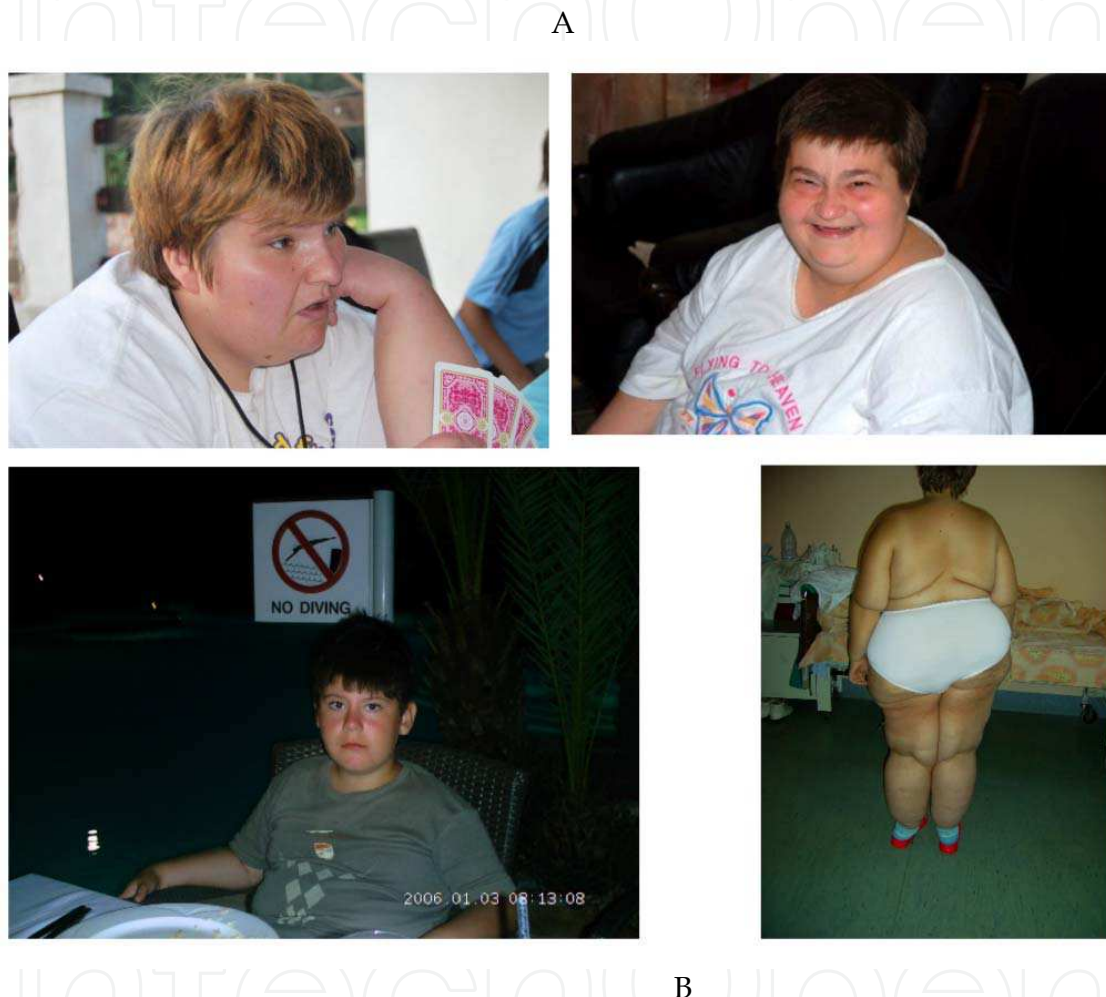


Fig. 2. A,B. Typical features with PWS cases. A. Facial features of the Prader Willi syndrome (periorbital fullness, almond-shaped palpebral fissures, malar hypoplasia, down-turned mouth corners and thin upper lip). FISH test identified 15q deletion in these patients with typical clinical features. B. Short stature and typical feet and hands dimensions. Obesity is another typical feature with all represented cases

3.3 Molecular tests

Confirmation of the clinical phenotypes has been realized by approaching molecular methods. Molecular cytogenetic approach (FISH fluorescence in situ hybridization technique) brought numerous PWS cases that presented deletions as detected by fluorescent probes in FISH. Further investigations by this method revealed an interesting, non-mendelian transmittance aspect with PWS, that required another molecular approach, based

on the epigenetic, methylation method. The need for discussing the recurrence risk with any of these situations determined a new, more precise approach, by sequencing methods (MLPA or even MS-MLPA) for the detection of the microdeletions as causes of the impaired IC role in imprinting control concomitant with the estimation of the methylation status on paternal allele.

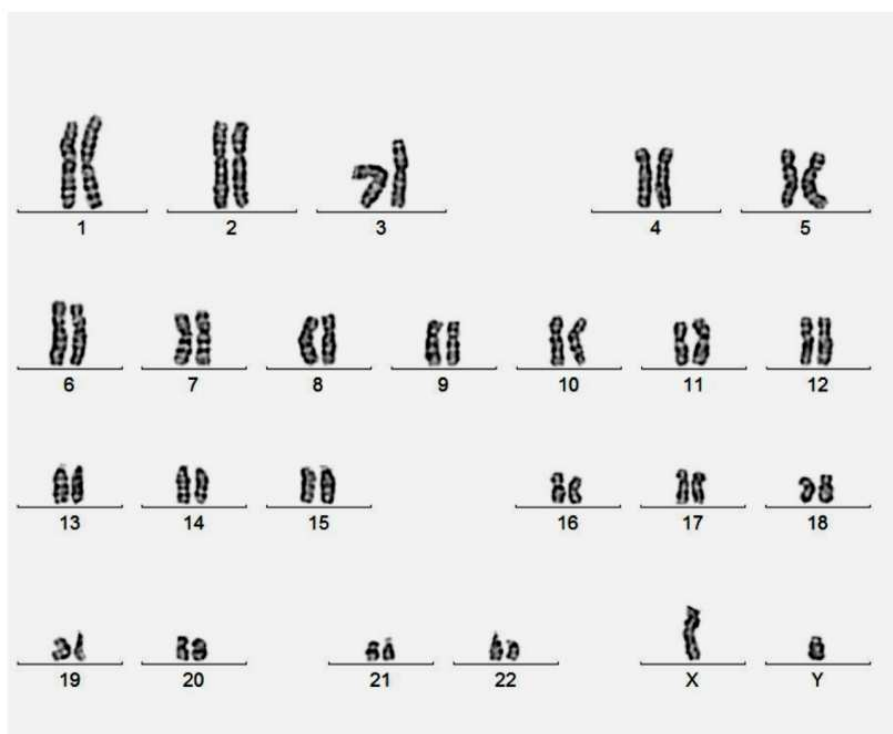


Fig. 3. A normal karyotype frequently obtained for the PWS patients

A more efficient and correct scheme of molecular testing have been suggested in the recent literature that included the following sequence of techniques in order to reveal not only the causes of genetic and epigenetic defects, but meantime to decipher the molecular mechanism involved in the establishment of the birth defect:

- Methylation analysis for the general diagnosis (about 99% cases confirmed);
- FISH detection of deletions for the establishment of the disease mechanism
- Sequencing methods that were imposed in the cases when FISH was negative and suspicion for UPD had to be solved- MLPA and MS-MLPA.

3.3.1 Methylation analysis

The detection of methylation status solely at the SNRPN locus may be performed by using methylation specific PCR (MS-PCR) . This approach confirms a diagnosis but provides no further information regarding the disease mechanism requiring follow up studies (FISH and/or microsatellite analysis).

The(PCR)-based assay MS-PCR allows rapid diagnosis of PWS and AS. Methylated cytosines in the CpG dinucleotide are resistant to chemical modification by sodium bisulfite. In contrast, bisulfite treatment converts all unmethylated cytosines to uracil. Based on this differential effect, the bisulfite modified DNA sequence of a methylated allele was

successfully distinguished from that of an unmethylated allele using 2 sets of allele-specific primer pairs: a methylated allele-specific primer pair (M) and an unmethylated allele-specific primer pair (U). Bisulfite-modified DNA from patients with PWS amplified only with the M pair while modified DNA from patients with AS amplified only with the U pair (Jones et al. 1997) (Fig. 4). The results of MSPCR tests performed on clinically diagnosed individuals are represented in Figures 5 a,b.

This method has the following significant advantages over conventional analysis using methylation-sensitive enzymes and Southern blotting: (1) MSPCR can be completed in 2 days. Rapid turnaround of the test result may be especially useful when evaluating hypotonic newborn infants among whom the incidence of PWS is high [Gillesen-Kaesbach et al., 1995]; (2) Testing can be performed with as little as 50 ng of genomic DNA. Thus, in addition to whole blood, other potential sources of genomic DNA for analysis include dried blood spots and oral cell smears; (3) MSPCR does not require use of radioactivity.

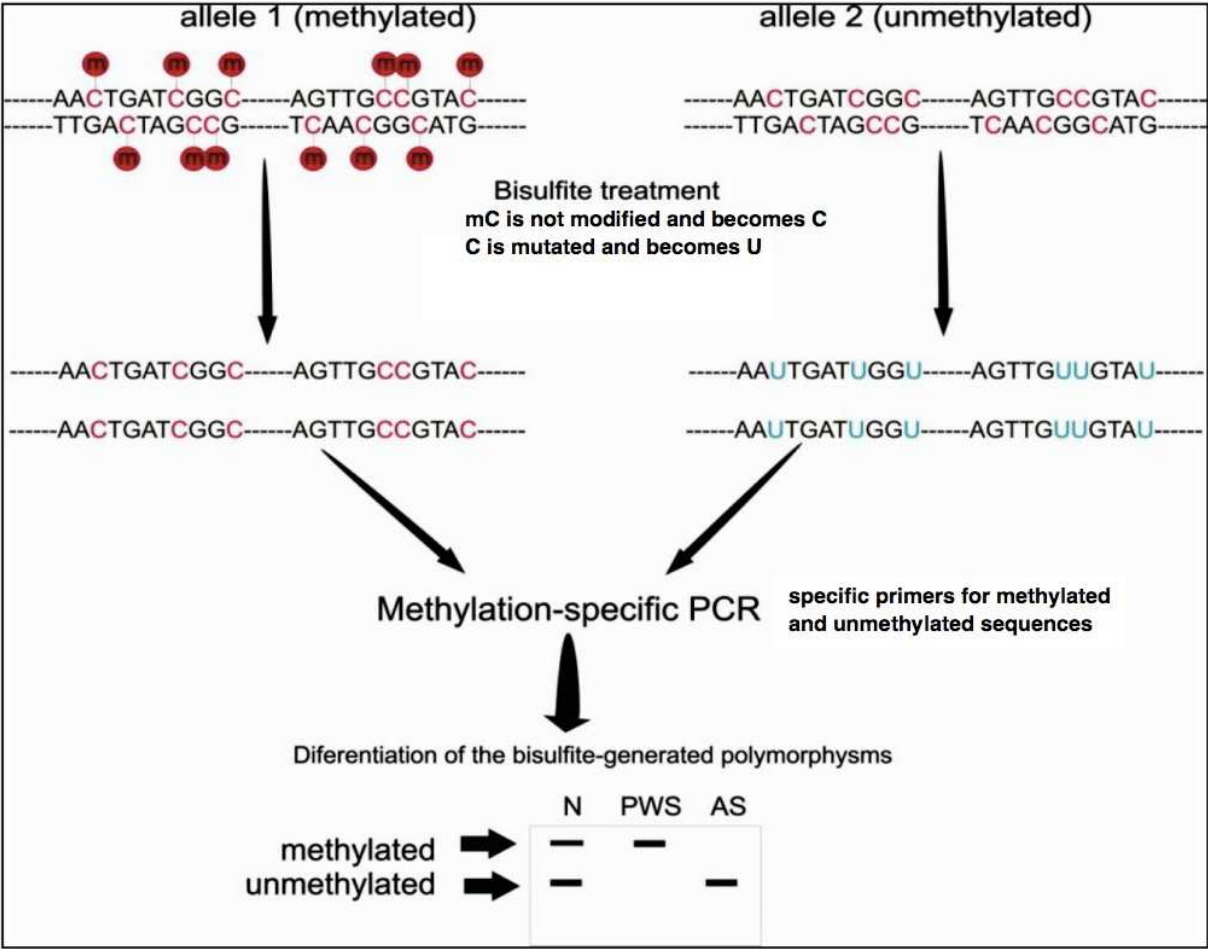


Fig. 4. The scheme of the steps in the methylation specific PCR protocol. It includes the mutagenesis by bisulfite conversion treatment, followed by the converted DNA purification and the PCR amplification of the methylated/unmethylated DNA fragment. The amplicon resulted from the amplification of the fragment flanked by the U primers stands for the fragment that was unmethylated. It is detected after its electrophoresis and its visualization in agarose gels by ethidium bromide in uv light

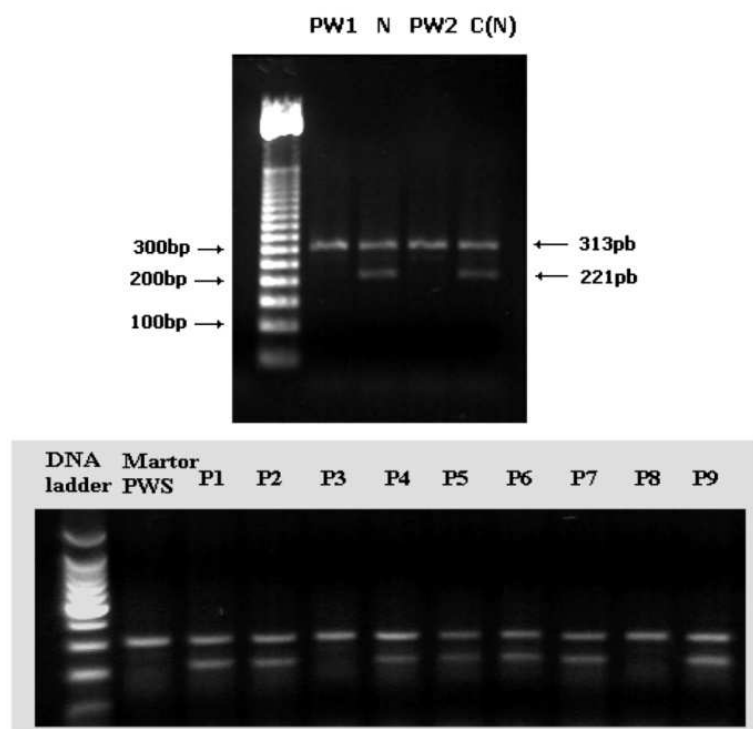


Fig. 5. a,b MSPCR analysis based on the distribution of the two MSPCR products (amplicons) of 313 and 221bp corresponding to the methylated (imprinted) maternal allele and, respectively, the unmethylated paternal allele. “PW” – individuals have an electrophoresis pattern specific for PWS - maternal only 313bp MSPCR product; “N” – individuals suspected of PWS based on clinical diagnosis, but not confirmed by methylation test- both MSPCR products are present - “C(N)” - normal individuals used as control, both MSPCR products are present

In Table 1 there are represented the results of our methylation analysis for the confirmation of the clinically diagnosed cases. From 30 clinically diagnosed cases, only 12 have been confirmed by methylation analysis. And among these latter ones, only 9 presented deletions, as confirmed by FISH method.

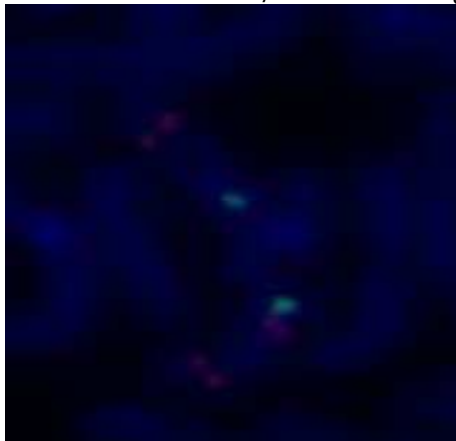
3.3.2 FISH method

Fluorescence in situ hybridization technique offers the possibility to use fluorochromes in order to specifically mark individual chromosomes over their entire length or defined chromosome regions in meta- and interphase preparations (Chevret et al. 2000); their presence is proved by using fluorescence microscopy. The first step in FISH procedures is the procurement of cells. Unlike many other chromosomal visualization techniques, FISH can be conducted on currently dividing or terminally differentiated cells. Cells are grown to a specific culture density and fixed with formaldehyde and placed on a functionalized glass slide. This slide is then allowed to dry, dehydrated with ethanol, and then treated with the hybridization buffer. DNA probes are then added and the slide is allowed to incubate. This gives the DNA probes enough time to hybridize with their complementary sequences.

Following hybridization, the slides are allowed to air dry and then are examined under microscope (Langedijk et al. 1995).

Many PWS/AS deletions are not detectable by G-banding. Even at higher band levels, variable G-banding quality, differences in homologue condensation/splitting of band 15q12, and possible presence of extra clinically benign G-bands in this region made interpretation difficult [Hoo et al., 1990; Ludowese et al., 1991]. Unreliability of G-banding for deletion detection in the region 15q11-q13 in comparison with FISH has been well documented [Delach et al., 1994; Butler, 1995; Bettio et al., 1995; Smith et al., 1995]. The development of molecular probes for the Prader-Willi syndrome and Angelman's syndrome region of 15(q11-13) enabled alternate or complementary means for the detection of deletions in patients (Chan et al., 1993; Butler, 1990; Knoll et al., 1989).

A. Deletion in PWS/AS critical region



B. Normal PWS/AS critical region

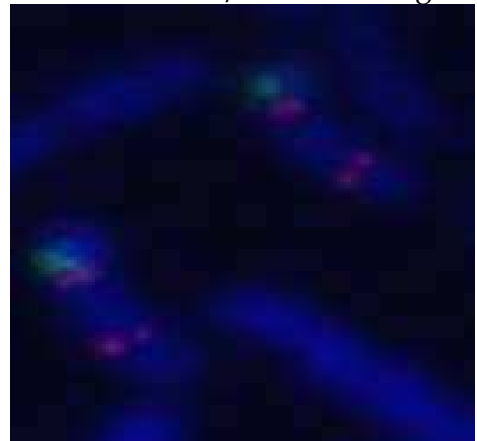


Fig. 6. FISH (fluorescent in situ hybridization for the region 15q11-q13) results for A-PWS case and B- normal case. The probes used: LSI for the common deletion D15S10 (spectrum orange/PML) and spectrum orange/CEP15 spectrum green for the common chromosome 15 mark (centromere) (Vysis, Abbott inc.)

For FISH analysis, probes for loci D15S11, SNRPN, D15S10, and GABRB3 within 15q11-13, and for identification of the chromosome 15 homologues an internal control probe for locus PML at 15q22, were co-hybridized to chromosomes following protocols provided by the manufacturer (Oncor, Gaithersburg, MD). Although it's an expensive and time-consuming, this method confirms the diagnosis of ~ 70% PWS and AS, and reveals the mosaic and translocation cases.

Patients with clinical features suggestive of Prader-Willi Syndrome and confirmed by MSPCR were tested for deletions of 15q11-q13 region and the results are illustrated in Figures 6 A and B.

3.3.3 Sequencing methods

The detection of microdeletions or duplications (CNVs) in critical 15 chromosomal region may be performed by classical MLPA method. MLPA is a technique based on semiquantitative genetic molecular method, initially developed by Schouten group (Schouten et al., 2002) while attempting to target 40 genomic loci containing variable copy numbers (deletions, duplications, triplications) by using a pair of probes for each target. Each probe contains a universal primer sequence and a sequence that is complementary to the target

DNA, named hybridization sequence. Both probes are hybridized adjacent one for the other avoiding the gap formation. When the probes are correctly hybridized to the target sequence, they are ligated by a thermostable enzyme (named ligase). Later the PCR primers contribute to the amplification of the linked probes during the exponential process that leads to a unique molecule. One of the primers is fluorescent labeled releasing a specific color, and therefore its amplification products to be visualized and thus detected and registered. Capillary electrophoresis enable after this the analysis of the PCR products and their comparison based on their dimensions.

In our first approach the MRC-Holland kit SALSA MLPA P245-A2 was used on the Applied Biosystem sequencer. It contained probes for the genes coding for: UBE3A, Necdin (NDN) and (2 probes) SNRPN (for two different regions a,b). The reactions were performed according to the producer’s instructions and the DNA quantity was 150 g genomic DNA in sufficient concentration, optimum 75-100ng/ml) in a volume of 5 l water. The identification of the altered peak height is basically the principle for the estimation of deletions. This is done by comparing the MLPA profiles obtained from the patients and the controls (parents). Softgenetics, LLC, State College, PA USA software was used in order to realize this comparisons.

In Figures 7 and 8 there are represented the normalized profiles of two individuals. Each peak represents the amplicon signal obtained from the corresponding exon, that is designed on X axis. The Y axis indicates the fluorescent intensity and the arrows indicate the positions of the four genomic targets on chromosome 15, analyzed by the kit.

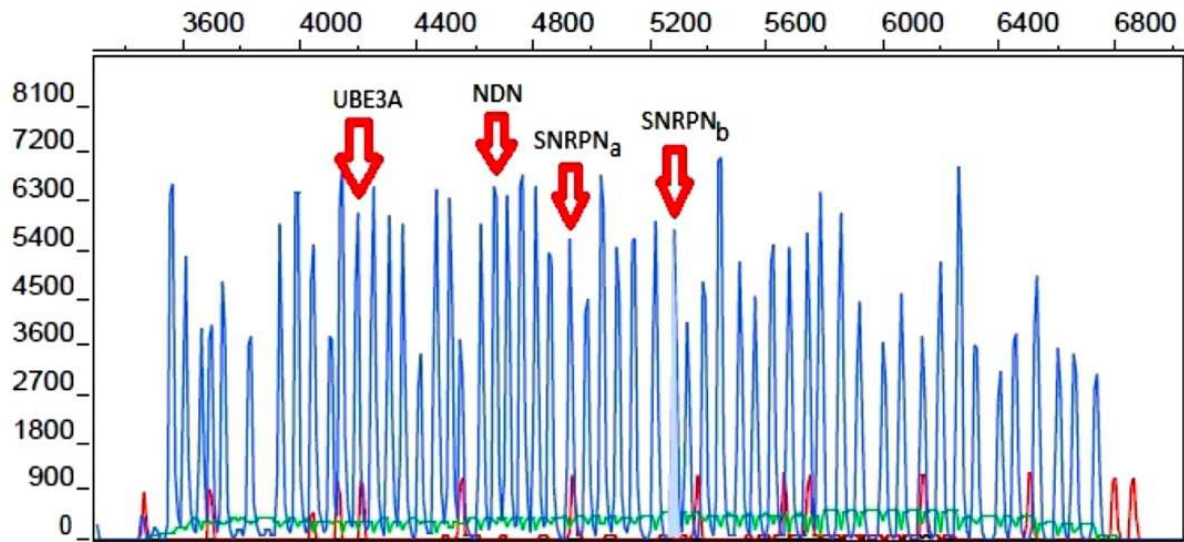


Fig. 7. The MLPA diagram obtained for a PWS patient, clinically diagnosed and confirmed by the molecular methylation test – the normal peak height does not suggest a deletion: UBE3A(15q12) – 6103, NDN (15q11.2) - 6593, SNRPN a (15q12) - 5653, SNRPN b (15q12) - 5802

The MLPA test is essentially a PCR technique. The characteristics that make it distinct from the other common techniques are as follows: 1. The amplification is dependent on the

ligation process, as the unligated oligomers are not amplified; 2. High ligation temperatures assure the specificity; 3. Amplification is performed in multiplex systems, thus enabling the analysis of up to 50 genomic targets, by a single test. This results in an effective low cost of the test, similarly to a mean high-throughput (HT) test. This type of analysis differs from the RT-PCR as the primers are in excess to the template, and the amplification is performed on a linear domain; therefore the number of generated amplicons is proportional with the template (the ligation products).

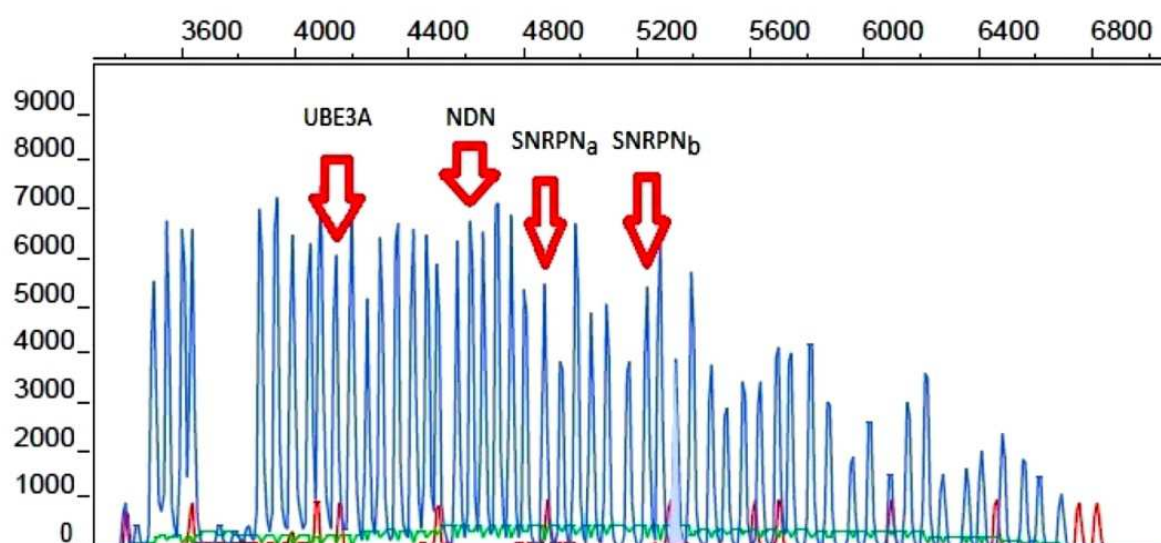


Fig. 8. The MLPA diagram obtained for a parent (normal control-mother); the normal height of the peak does not suggest a deletion: UBE3A(15q12) - 7530, NDN (15q11.2) - 6790, SNRPN a (15q12) - 5464, SNRPN b (15q12) - 5455

Due to its low cost and excellent sensibility and to the easy steps, the MLPA test is actually becoming a frequent tool approached in research and routine diagnosis. One negative aspect is however linked with the fact that it is not enough informative regarding the localization of the duplicated sequences as compared with the original copy, nor regarding their orientation.

The analysis of the genomic instability on chromosome 15 by this method resulted in the following conclusions: the chosen samples corresponded to a patient that was confirmed clinically and by molecular MSPCR and to his mother (control). The lack of the deletion in SNRPN region suggested an imprinting defect with no deletion, thus it is suggested a primary imprinting defect.

A more complete molecular approach is presently running in our investigations and would further involve the following test for a correct conclusion regarding the mechanism that led to the imprinting defect, a conclusion valuable for the genetic counselling: the confirmation or exclusion of UPD by microsatellite detection testing and the use of other, more informative MS-MLPA kits, in order to target the unique HBII-85 sequence, presently considered as characteristic for the PWS phenotypes. The simultaneous assessment of methylation status and genomic dosage at numerous sites across the 15q11-q13 region may be performed by the use of methylation sensitive multiplex ligation-dependent probe

amplification (MS-MLPA). This approach will confirm the diagnosis and further identify the presence of a causative deletion. However, in the absence of a deletion follow up studies (microsatellite analysis) are required to distinguish between UPD and an imprinting defect (Ramsden et al. 2010).

Trans factors influencing the imprinting process through the maintenance of proper cell methylome, such as the polymorphism in the critical gene *methfr* (a gene encoding methylenetetrahydrofolate reductase) are also reported in numerous literature. Our analysis on three families with one PWS proband reveals only one affected family, where the mother was a carrier of a homozygous C677T mutation; the other two families had a polymorphic profile with only one father being heterozygous for the same mutation. Hence this approach need a larger individual group for the *methfr* SNP test and perhaps the inclusion of other SNPs that may be relevant for the genome instability during gestational period or for the genome instability during the perinatal periods. The more cases will be accessible for providing the parental state of this gene, the more informative will be this algorithm for the detection of trans-factors influencing the mechanism of the imprinting process.

3.3.4 Interpretation of the analysis and further molecular approaches

For the laboratory diagnostic of the PWS one should start with the methylation analysis because it is the most sensitive method, confirming over 99% of the cases. If we start the diagnostic test with the MS-PCR (which will confirm a diagnosis but will provide no further information regarding the disease mechanism – i.e. about deletion, UPD, ID) and the result is positive, for genetic counseling, the next step is to perform a FISH or MLPA analysis (asking for deletion) and microsatellites analysis (asking for UPD) to determine the genetic mechanism and recurrence risk.

In the case the methylation test is negative, this result excludes paternal deletion, uniparental disomy and an imprinting defect result makes a diagnosis of PWS highly unlikely.

The other approach for the diagnosis of PWS is to start with the MS-MLPA assay. This test shows the methylation status and the dosage at 15q11-q13; it is more precise than MS-PCR as it investigates methylation status at several loci, thereby reducing the risk of a false positive or false negative result due to SNPs, and can also identify micro deletions in the IC as it uses many probes in the same reaction (up to 40). A positive result may indicate either a) the molecular cause of PWS is due to 15q11-q13 deletion, or b) the molecular cause of PWS may be due to maternal UPD or an imprinting defect. Laboratories should recommend in the later case microsatellite studies to confirm or exclude UPD.

In the case the methylation test is negative (in either MS-PCR and MS-MLPA), the result will exclude paternal deletion, uniparental disomy and an imprinting defect, but to be more accurate, FISH analysis is still recommended in order to confirm a translocation or a mosaic form of the syndrome.

Our work presents the simple MLPA method that used only few kits for the detection of deletions in critical 15 chromosome region. MS-MLPA assay may confer further both information about the methylation status and the dosage at 15q11-q13, and as described earlier it has some characteristics which makes it more reliable than MS-PCR. A deletion and methylation positive result suggests the following mechanisms: a) deletion confirms a

genetic cause; b) aberrant methylation suggests either maternal UPD or an imprinting defect.

PWS deletion patients present the classical clinical picture of the disorder (fig. 2), whereas negative FISH patients are characterized by absence of the particular facies, higher IQ and moderate behavioral problems.

Because the genetics of PWS is complicated it usually takes more than one test to ascertain the diagnosis and the form of disease (Roberts SE and Thomas NS. , 2003, Roof E et al., 2000). The genetic tests used and the order depend on a number of considerations for each individual case. Genetic testing usually requires a blood sample from the child and possibly from the parents as well (Simon C Ramsden et al., 2010, Gillessen-Kaesbach G et al., 1995).

The diagnostic methods used in our study allowed PWS diagnosis confirmation in 14 out of the 17 cases. The 3 cases left will be analyzed with specific molecular tests to identify possible mutations of the imprinting center.

4. Differential diagnosis

For PWS, there were described several disorders with a phenotype that can strongly resemble PWS consisting in neonatal hypotonia and later onset obesity. Their associated mechanisms implied: (i) upd(14)mat, which can be caused by uniparental disomy 14 and imprinting defects or deletions affecting the DLK1/GTL2 locus in the chromosomal region 14q32 (Temple et al., 2007; Buiting et al. 2008); (ii) a number of other conditions associated with obesity and developmental disability including Cohen syndrome, Bardet- Biedl syndrome, Alstrom syndrome, and (iii) the 1p36 microdeletion which characterize a specific syndrome (Goldstone and Beales, 2008).

5. Conclusions

The establishment of a practical set of molecular genetic testing guidelines for PWS and AS has been succeeded through numerous experiences linked with the technical performance, the complexity of the imprinting diseases and the basic concepts linked with the hereditary transmittance.

This study is part of a research programme for PWS and Angelman Syndrome (AS) patients. The diagnostic protocol applied with this group included: physical examination, cytogenetic investigations (karyotype and FISH) and methylation analysis (after a model of Glenn CC et al., 1996, Kubota T et al., 1997).

Multidisciplinary physical examination (geneticist, pediatrician, endocrinologist, orthopedist, neuropsychiatrist, pneumologist etc) allows for the correct establishing of the clinical score (Gunay-Aygun M et al., 2001, Holm VA et al., 1993, P. Goldstone et al., 2008).

The strategy we propose for the confirmation of the clinical PWS diagnosis includes initially a methylation analysis (MSPCR). This test is used as a diagnostic instrument for PWS, because the methylation pattern is parental specific in this region (Butler MG, 19990, Carrel AL et al., 2002) and detects patients with deletions, UPD and imprinting defects that represent 99% of PWS cases.

Thus, an efficient strategy for the routine diagnosis of PWS patients includes: a) methylation analysis which allows diagnosis for 99% of PWS patients and doesn't need parental samples; b) analysis of the microsatellite genotype of the family (child, mother and father), in order to identify deletions, UPD and mutations of the imprinting center; c) in noninformative cases

or if the parental samples are not available FISH technique is indicated because it can identify deletions (~ 75% of PWS patients). Cytogenetic studies using G banding should be routinely used in all patients in whom the clinical score highly suggests the PWS diagnosis, as approximately 5% of the PWS patients reported in the literature have a chromosomal rearrangement (Cassidy SB, et al., 1994).

Even if this study group size does not allow important statistic conclusions, the results obtained differ from those reported in the specialized literature both in the proportion of PWS cases confirmed by methylation analysis (82.35% compared to 99% in the literature) (Suzanne B Cassidy, 2008, Mellissa R. W. Mann, 1999), and in the number of cases confirmed by FISH analysis (41,1% compared to 70% in the literature) (Mellissa R. W. Mann , 1999).

The explanations could be related to a particular molecular profile of PWS patients in Romania. Such studies do not exist for the moment in our country and the confirmation will be possible by investigating a larger number of patients. In patients with a normal methylation pattern and without chromosomal abnormalities, we propose a clinical reevaluation in order to establish if further molecular investigations are indicated.

Due to the variability of expression and the importance of early diagnosis awareness is growing, and looking for evocative signs increases detection rate of patients with PWS (Gunay-Aygun M, et al., 2001, Holm VA, et al., 1993).

The study showed the relative correlation between clinical score and cytogenetic and molecular confirmation of PWS. The presence of short fingers seems likely to confirm the diagnosis. The triad brachydactyly - obesity - mental retardation is easy to follow by your practitioner, for the correct guidance of suspected cases to the specialist. The differential diagnosis of PWS, Fragile X and Prader-Willi-like syndrome has to be considered, especially when laboratory workup for PWS is negative.

Clinical behavioral pattern can be of assistance in guiding the investigations and final diagnosis. Further study and experience gathered by the project team will allow a refinement of techniques and an accurate diagnosis. Knowledge of the so called "open windows" of vulnerability of the genome during the crucial stages of development and their interaction with the environment would be beneficial for the activities of deciphering the cis and trans-acting factors in the altered imprinting mechanism that may lead to establishment of optimal diagnosis and therapeutic or preventive schemes.

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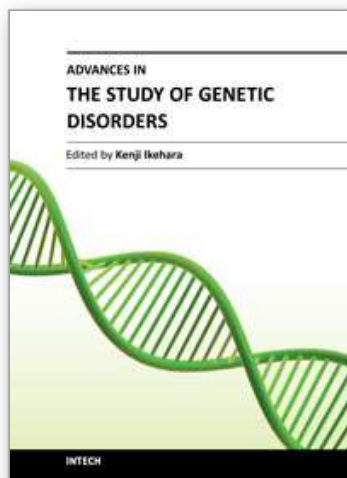
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The studies on genetic disorders have been rapidly advancing in recent years as to be able to understand the reasons why genetic disorders are caused. The first Section of this volume provides readers with background and several methodologies for understanding genetic disorders. Genetic defects, diagnoses and treatments of the respective unifactorial and multifactorial genetic disorders are reviewed in the second and third Sections. Certainly, it is quite difficult or almost impossible to cure a genetic disorder fundamentally at the present time. However, our knowledge of genetic functions has rapidly accumulated since the double-stranded structure of DNA was discovered by Watson and Crick in 1956. Therefore, nowadays it is possible to understand the reasons why genetic disorders are caused. It is probable that the knowledge of genetic disorders described in this book will lead to the discovery of an epoch of new medical treatment and relieve human beings from the genetic disorders of the future.

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