

Prader–Willi Syndrome and diagnostic protocols: a preliminary study in Romania

Sindromul Prader–Willi si protocole de diagnostic: studiu preliminar in Romania

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Abstract

Prader Willi Syndrome (PWS) is a neurometabolic genetic disorder affecting 1/12.000-1/15.000 newborns. Molecular mechanisms that could lead to this disorder include chromosomal deletions, uniparental disomy (UPD), intragenic mutations, and epigenetic modifications in the process of imprinting and rarely reciprocal translocations. A common defect is noticed in all cases: loss of parental contribution for the functioning of specific genes in normal conditions, due to genetic instability of the critical region 15q11-q13. Objectives of the study concerned the implementation of molecular genetic/ epigenetic methods of investigation and development of an interdisciplinary clinical investigation algorithm specific for the disease (geneticist, pediatrician, endocrinologist, psychiatrist, neurologist, psychologist, orthopedist, pneumologist, nutritionist) aiming for early recognition of the clinical features, resulting in early diagnosis and early intervention. Materials and methods: a multicentric study started in 2008, being included in a research project (CNMP / Partnerships, 2008-2011), and coordinated by UMF Timisoara. So far, 9 suspected cases of PWS have been investigated and in 3 cases family members have been tested (brother, sister, parents, grandparents). Results: The investigation protocol applied, including multidisciplinary clinical evaluation, laboratory investigations, and specific genetic and epigenetic tests relevant for the subtypes of PWS, allowed accurate diagnosis of patients. This approach was applied for the first time in our country. Conclusions: Early recognition and diagnosis is essential in PWS, as complex treatment applied in due time leads to prevention of obesity installation and other redoubtable complications. This is possible by interdisciplinary approach and detection of molecular mechanisms involved, allowing an appropriate genetic counseling.

Keywords: Prader Willi Syndrome (PWS), genetic, epigenetic.

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Rezumat

Sindromul Prader Willi (PWS) este o boală genetică neurometabolică, având o frecvență de 1/12.000-1/15.000 nou născuți. Mecanismele moleculare responsabile de producerea bolii implică deleții cromozomiale, disomia uniparentală (DUP), mutații intragenice, modificări epigenetice în procesul de amprentare și rar, translocații reciproce. În toate cazurile se remarcă un numitor comun: pierderea contribuției parentale specifice pentru funcționarea unei gene în condiții normale, datorate instabilității genetice a regiunii critice, 15q.11-q13. Obiectivele studiului au vizat implementarea metodelor moleculare de investigare genetică/epigenetică și dezvoltarea unui algoritm de investigație clinică interdisciplinară specifică bolii (genetician, pediatru, endocrinolog, psihiatru, neurolog, psiholog, ortoped, pneumolog, nutriționist), care să permită suspectarea și diagnosticarea precoce a bolii, având ca rezultat diagnosticul precoce și intervenția timpurie. Material și metodă: studiul multicentric a demarat în anul 2008 și se realizează în cadrul unui proiect de cercetare (CNMP/Parteneriate, 2008-2011), coordonat de UMF Timișoara. Până în prezent au fost investigate 9 cazuri suspecte de PWS și la 3 cazuri au fost analizați și membri ai familiei (frate, soră, părinți, bunici). Rezultate: protocolul de investigație aplicat, incluzând consult clinic multidisciplinar, investigații paraclinice specifice, teste genetice și epigenetice relevante pentru subtipurile SPW a permis o încadrare diagnostică corectă a pacienților, această abordare fiind posibilă pentru prima dată în România. Concluzii: Recunoașterea și diagnosticul precoce sunt importante pentru stabilirea unui protocol terapeutic care poate evita sau temporiza instalarea obezității și a altor complicații redutabile. El este posibil în cadrul colaborării interdisciplinare, iar stabilirea mecanismului molecular implicat permite un sfat genetic adecvat.

Cuvinte cheie: *Sindrom Prader Willi (SPW), genetică, epigenetică.*

Introduction

Prader Willi Syndrome (PWS) is a contiguous genes syndrome, affects 1/12.000-1/15.000 people and its clinical features are consecutive to the lack of expression of the paternal alleles in the 15q11.2-q13 region (1-3). 70% of patients have a paternal chromosome deletion involving 15q11.2-q13 region that can be identified by molecular cytogenetic analysis - FISH test (4). In 1% of patients the karyotyping may detect a deletion of the critical region (1, 5). Less than 1% of patients experience a balanced chromosomal rearrangement with break points within 15q11.2-q13, that can be detected by cytogenetic and FISH analysis. 25% of cases are caused by native 15 uniparental disomy. There are some genes identified in the region, some of which are imprinted, but the precise cause of the syndrome is still not completely elucidated (5). In most of the instances, due to the particular phenotype, careful history and clinical examination allows detailed clinical diagnosis. Positive clinical diagnosis must be

followed by karyotype analysis to identify chromosomal deletions or rearrangements involving the critical region of chromosome 15. In the context of a normal karyotype, molecular genetic testing is indicated (6). It is essential to define the clinical criteria that indicate genetic testing for each age group. Molecular evaluation of a patient clinically diagnosed with PWS is done in stages, adapted to the mechanism that produces the syndrome (7).

Materials and methods

The study started in 2008, in a multicentric collaboration, bringing together the efforts of several research teams from medical centers Timisoara, Iasi, Bucharest, Cluj Napoca and the Romanian Prader Willi Association.

1. Clinical methods. The lot included 9 cases, with the most obvious features being obesity and mental retardation and the rest being variable. The patients were 8 females and one male, aged between 4 and 22 years. Positive clinical diagnosis of PWS was based on ma-

major and minor criteria, the minimum diagnostic score being 3 - up to age 3 years and 5 - for patients older than 3 (up to the adult age) (1-4) .

2. Blood samples were taken from clinically diagnosed patients who presented at the "L. Turcanu" Clinical Hospital in Timisoara, "Sf. Maria" Children's Hospital in Iasi and "IC Parhon" Endocrinology Institute in Bucharest. The patients were enrolled in the list of the Romanian PWS Association (APWR / Zalau). Blood samples were used for molecular cytogenetic tests (sodium heparin tubes) and molecular investigations (EDTA tubes). Blood samples collected on EDTA were either processed immediately or stored at -80 C until processing.

3. Fluorescence *in situ* hybridization (FISH) Analysis. Patients with clinical features suggestive of Prader-Willi Syndrome were tested for deletions of 15q11-q13 region.

FISH was performed using the following probes from Vysis: Probe 1: LSI D15S10 (spectrum orange), LSI PML control of orange fluorescent hybridization, CEP 15 (spectrum green-hybridizes to the centromere of chromosome 15), ish(del15)(q11.2q11.2)(D15S10-) and Probe 2: LSI SNRPN (spectrum orange),

LSI PML (spectrum orange) control of orange fluorescent hybridization, CEP 15 (spectrum green-hybridizes to the centromere of chromosome 15), ish(del15)(q11.2q11.2)(SRNPN-).

The amount of blood taken in sodium heparin (green-top) tubes was: minimum 0.5 -2.0 mL for newborn infants (< 3 months) and minimum 3.0 -5.0 mL for children and adults. Initially, FISH analysis was performed in European laboratories for some cases. Currently, patients have to undergo this analysis in cytogenetics laboratory operating in the University of Medicine and Pharmacy, Timisoara, Department of Medical Genetics.

4. Genomic DNA extraction was performed from 0.7 - 1 ml EDTA-blood, using the Invitex - Invisorb Spin DNA Extraction Kit.

5. Sodium bisulfite treatment. Before methylation-specific polymerase chain reaction (MSPCR), a sodium bisulfite treatment was performed, converting unmethylated cytosine to uracil and leaving methylated cytosine unchanged. The DNA samples were first diluted and then the appropriate amount of DNA was used for the conversion accordingly to the manufacturer indications (Qiagen - EpiTect Bisulfite Kit).

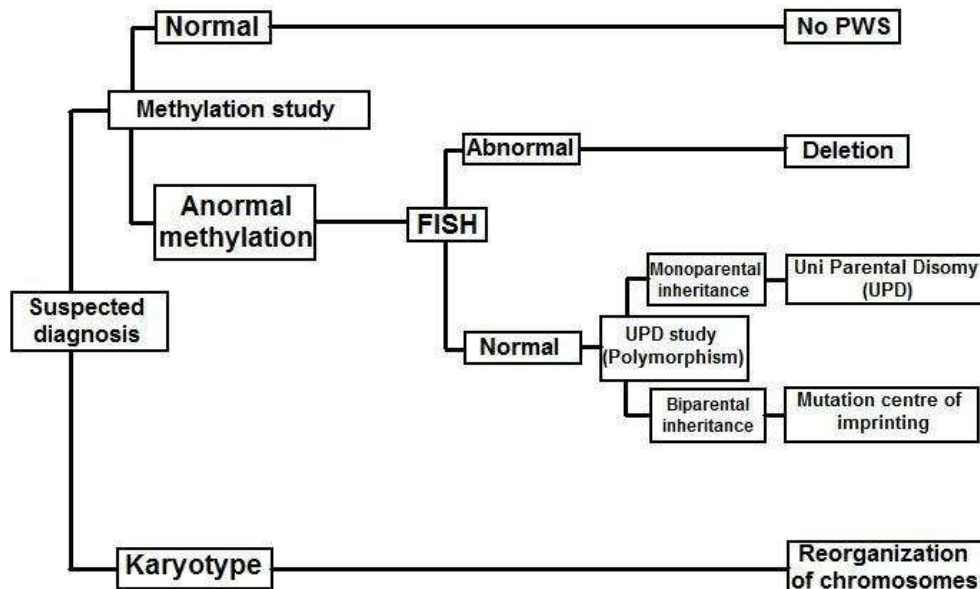


Figure 1. Diagnostic algorithm in subjects suspected of Prader Willi syndrome

6. Methylation specific PCR (MSPCR).

A methylation-specific polymerase chain reaction (MSPCR) was performed on bisulfite converted DNA. Primers for SNPRN exon 1 were used in order to establish the differentially methylated state on parental alleles (8-10).

Molecular investigations are currently conducted in the Epigenetics Laboratory, University of Bucharest and National Institute of Virology, Bucharest.

This approach will confirm a diagnosis, but will provide no further information regarding the mechanism that produced the disorder, necessitating follow up studies (FISH and/or microsatellite analysis).

Results

Diagnostic protocol applied with this group included: physical evaluation, cytogenetic investigation (karyotype and FISH) and methylation analysis (*Figure 1*) (11, 12).

Clinical diagnosis of the 9 cases was based on the major and minor criteria of the diagnostic score shown in *Table 1*.

FISH analysis and methylation PCR results are shown in *Table 2*. FISH analysis is positive in 4 cases and methylation specific analysis is positive in 6 cases (*Figure 2*).

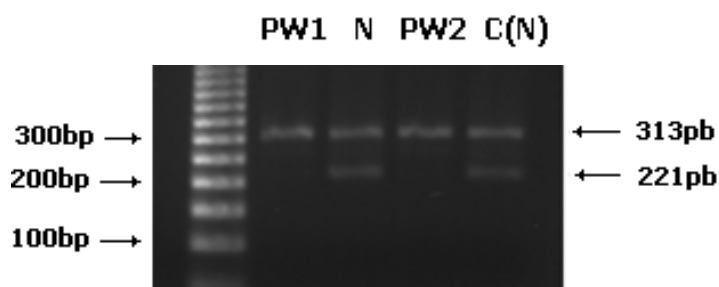


Figure 2. Methylation specific PCR (MSPCR) analysis:

“PW” – individuals that have a gel electrophoresis pattern specific for PWS - maternal 313bp MSPCR product present only; “N” – individuals suspected of PWS based on clinical diagnosis, both MSPCR products are present - the maternal allele product (313bp) and the paternal allele product (221bp); “C(N)”- normal individuals used as control, both MSPCR products are present.

Discussion

This study is part of a research introduced for PWS and Angelman Syndrome (AS) patients. Multidisciplinary physical examination (geneticist, pediatrician, endocrinologist, orthopedist, neuropsychiatrist, pneumologist etc) allows for the correct establishing of the clinical score (1-3).

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 methylation pattern is parental specific in this region (13, 14) and detects patients with deletions, UPD and imprinting defects, which represent 99% of PWS cases.

MSPCR is the best approach to describe what is noticed, meaning the absence or presence of the relevant parental band, and not to simply say that a typical PWS methylation pattern is present. The lab specialists have to mention that this investigation cannot assess the molecular cause of the disorder (the proper mechanism) and they should ask for parental blood specimens for microsatellite analysis (in order to assess the mutation mechanism and recurrence risk).

Classic cytogenetic techniques have a low sensitivity in microdeletion detection, even with high resolution banding. Differences in the condensing degree of 15q12 region in homologous chromosomes renders the detection of deletion at this level difficult: in our study, only one of the 9 patients (11.1%) has a deletion detected using this method.

FISH technique, using specific probes for the 15q11-q13 region, is considered as the most efficient cytogenetic diagnostic method for PWS, identifying the deletion in approximately 80% of patients (2,3).

Table 1. Major criteria, minor criteria and supportive findings in patients from the lot

| | | Case number | | | | | | | | |
|--|--|-------------|-----|---|-----|------|------|------|-----|-----|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Major Criteria (1 point each) | CNS - central hypotonic infant | + | + | + | + | + | + | + | + | + |
| | Gastrointestinal - infant feeding difficulties and / or lack of growth | + | - | + | + | + | + | + | + | + |
| | Nutrition - excessive weight gain after age 1-6 years | + | - | + | + | + | + | + | + | + |
| | Craniofacial - narrow forehead, almond-shaped palpebral fissure, narrow nasal pyramid, oral commissure descended | + | + | + | + | + | +/- | + | + | + |
| | Endocrine - hypogonadism | + | +/- | - | +/- | + | + | + | + | + |
| | Developmental delay and / or mental retardation | + | + | + | + | + | + | + | + | + |
| Minor criteria (1 / 2 point each) | Neurology - poor fetal movements and / or lethargy in infant | - | - | + | +/- | + | ? | + | +/- | + |
| | Lung - sleeping and / or sleep apnea | + | +/- | + | + | + | ? | ? | ? | + |
| | Endocrine – short stature for age (teens) | - | + | | | + | + | +/- | + | + |
| | Dermatologic - hypopigmentation | +/- | - | - | - | - | - | ? | ? | - |
| | Orthopedics - small hands and feet | + | + | + | + | + | +/- | + | +/- | + |
| | Ophthalmology – esotropia and / or myopia | + | + | - | ? | ? | ? | ? | ? | - |
| | Dental - viscous saliva | + | - | + | + | + | + | + | ? | + |
| | Otolaryngology - defect in articulation of speech | + | + | + | + | + | + | + | + | + |
| | Psychiatry - pinching the skin | + | +/- | + | + | +/- | + | + | + | + |
| Criteria for support (no points) | Neurology - increased threshold of pain, normal results of neuromuscular assessment for hypotonia | + | + | + | + | + | + | + | + | + |
| | Gastroenterology – reduced vomiting | + | + | + | + | + | + | + | + | + |
| | Endocrinology: ineffective thermoregulation, early adrenarha and / or osteoporosis | + | + | - | - | - | + | ? | ? | + |
| | Orthopedics - scoliosis or kyphosis | - | - | - | - | + | + | + | + | + |
| | Psychological - high efficiency in a game of puzzle | + | - | - | ? | + | ? | + | + | + |
| TOTAL | | 9+/- | 6 | 8 | 8 | 9+/- | 7+/- | 9+/- | 8 | 9.5 |

Table 2. Clinical criteria, Fish Analysis and DNA Metylation in studied patients

| | Case number | | | | | | | | |
|-----------------|-------------|---|---|---|------|------|------|---|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Clinical score | 9+/- | 6 | 8 | 8 | 9+/- | 7+/- | 9+/- | 8 | 9.5 |
| FISH Analysis | + | + | - | - | - | - | - | + | + |
| DNA Methylation | + | + | + | + | ? | - | - | + | + |

In our study, only 4 of the 9 patients (44.4%) had a positive FISH test. Because FISH does not detect UPD and mutations of the imprinting center, the rest of the patients need a DNA investigation using microsatellite markers inside and outside PWS region. This investigation method is included in our study and will be available shortly.

The main disadvantage of this method is that it needs blood samples from the parents and, even if these are available, sometimes results may not provide extra information.

Thus, an efficient strategy for the routine diagnosis of PWS patients includes: a) methyla-

tion analysis, that allows diagnosis of 99% of PWS patients and does not 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 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 (15).

The results obtained in the study group, even if its size does not allow important statistic conclusions, differ from those reported in the specialized literature, both in the proportion of PWS cases confirmed by methylation analysis (66,6% compared to 99% in the literature) (4, 5), and that of cases confirmed by FISH analysis (44,4% compared to 70% in the literature) (5).

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 extra molecular investigations are indicated.

PWS patients in whom the FISH test has identified deletions present a classical clinical picture of the disorder (*Figure 3*), whereas patients in which FISH analysis is negative are characterized by the absence of a particular facies, a higher IQ and moderate behavioral problems.

Clinical diagnosis of PWS has been established based on characteristic clinical features that differ with age (16, 17). In the newborn infant, the suggestive feature is hypotonia, feature that resulted from the history in our study (patients aged 4 to 7 years – 2 cases and more than 14 years – 7 cases).

Obesity, moderate mental retardation, be-



Figure 3. Facial features of a Prader Willi syndrome patient.

Periorbital fullness, almond-shaped palpebral fissures, malar hypoplasia, down-turned mouth corners and thin upper lip. FISH test identified 15q deletion in this patient with typical clinical features (image reproduced with permission).

Table 3. Molecular genetic testing used for Prader-Willi Syndrome

| Test Method | Detected Mutation | Detection Frequency by Test |
|--------------------------|--------------------------|-----------------------------|
| Methylation analysis | Methylation abnormality | 99% |
| FISH/Quantitative PCR | Deletion of PWCR | 70%-75% |
| Uniparental disomy (UPD) | Studies UPD of PWCR | 25%-29% |
| Sequence analysis | Imprinting center defect | <1% |

After Suzanne B Cassidy, Stuart Schwartz, GeneReviews: Prader-Willi Syndrome, 2008, www.genetests.org

havioral 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 (*Figure 3*).

Because the genetics of PWS is complicated (paternal deletion — 70-75% of all cases of PWS, maternal uniparental disomy (UPD) — about 25% of cases, imprinting defect — less than 5% of cases), it usually takes more than one test to ascertain whether someone has PWS and what form of it they have (18, 19). Which genetic tests are used, and in what order, will depend on a number of considerations for each individual case (*Table 3*). Genetic testing usually requires a blood sample from the child and possibly from the parents as well (20, 21).

Diagnostic methods used in our study allowed PWS diagnosis confirmation in 6 out of the 9 cases. The 3 cases left will be analyzed with specific molecular tests to identify possible mutations of the imprinting center.

MLPA is a recently introduced method that can be used in the case of PWS either for deletion/microdeletion detection or for methylation defects. The relatively low price of the investigation makes it a competitive technique that should be considered for the final PWS investigation protocol.

Conclusions

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 (1, 2).

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 (22, 23).

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.

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Abbreviations

DNA: deoxyribonucleic acid
 EDTA: ethylenediaminetetraacetic acid
 FISH: Fluorescence in Situ Hybridization
 PCR: Polymerase Chain Reaction
 PWCR: Prader-Willi critical region
 PWS: Prader Willi Syndrome
 UPD: uniparental disomy

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