Epigenetic approach of Prader-Willi syndrome diagnosis in Romanian population

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Abstract

This work aimed to compare the clinical validity of a newly developed assay for Prader-Willi syndrome diagnosis in Romanian population, as compared to an existing assay. This syndrome is a complex multisystem, genetic and epigenetic disorder, which arises from a defect in imprinted genes regulation and contribution. So far, a molecular cytogenetic method (fluorescence in situ hybridization) has been the most frequently used assay in primary diagnosis, but because it is targeted towards genetic factors it only covers deletional cases in this pathology. However, gene expression control is realized both through physical presence of the corresponding DNA sequence and through specific methylation patterns on parental alleles of the given gene: normal healthy state is defined by unmethylated (expressed) paternal allele, in the presence of methylated (repressed) maternal allele; by contrast, the pathological condition is defined by the presence of only methylated (repressed) maternal allele, thus the lack of unmethylated (contributing) paternal allele. This is the first report presenting a methylation mapping assay, based on methylation specific amplification, for Prader-Willi syndrome in Romanian population, suggesting it might be used as primary diagnosis tool based on its cost-efficiency and covering ability of almost entire Prader-Willi syndrome etiology (deletions, unimernal disomy, imprinting defect).

Keywords: fluorescence in situ hybridization (FISH); methylation-specific PCR (MS-PCR); Prader-Willi syndrome (PWS).

Introduction

Prader–Willi syndrome is a complex human disease first described as a genetic congenital condition that causes a neurodevelopmental disorder associated with life-threatening obesity [1, 2]. It is a rare disease affecting, according to recent epidemiological studies, 1:15000-1:25,000 births independent of race, sex, or ethnic group [3]. However its cause has been referred to as genetic; its non-Mendelian transmittance has challenged many genetic counseling activities and raised the question of epigenetic factors implications.

A detailed research of the critical chromosome 15q11-q13 region revealed the contribution of both genetic and epigenetic factors, which converge towards the main characteristics of Prader-Willi syndrome: defects in imprinting process associated with lack of paternal contribution affecting the expression of imprinted genes [4]. Several main chromosomal features in the critical PWS region were described, which explained the complex PWS etiology (deletions [5, 6, 7], uniparental disomy [6, 7, 8], rare imprinting defects [9, 10, 11, 12], rare chromosome 15 rearrangements and few atypical deletions [13].
One feature refers to the cluster of imprinted genes contained in about 8 million base pairs of the 15q11-q13 region, and the second one to low copy repeat DNA sequences that are clustered at the three common chromosome brake-points (centromere proximal BP1, BP2 and distal BP3) (figure 1) [14].

Deletional subtypes represent the most frequent PWS (ca 75%) and are explained by the genomic instability at BP loci. The typical PWS deletions consist of type I and II classes involving BP 1, BP 2, respectively BP 3; type I deletion is about 6.6 Mb and respectively type II of 5.3 Mb [16].

There are imprinted and nonimprinted genes located between the described BPs. Their contribution depends on their physical presence and their epigenetic control including allele-specific DNA methylation [17, 18]. While non-imprinted genes are expressed equally by the parental alleles, the imprinted ones are expressed only form one allele in a parent-of-origin manner.

DNA methylation status contributes to the gene expression control, and it is achieved through specific methylation patterns on parental alleles: normal healthy state is defined by unmethylated (expressed) paternal allele, in the presence of maternal methylated (repressed) allele. By contrast, the pathological condition is defined by the presence of only maternal repressed allele and thus the lack of the unmethylated (contributing) paternal allele [17, 18]. The clustered imprinted genes in 15q11-q13 region are regulated by the imprinting control region (ICR). The locus encoding the gene for small nuclear ribonucleoprotein polypeptide N (SNRPN) within ICR is also imprinted and expressed only from the paternal chromosom [19]. It is required for switching to and maintenance of, the paternal active epigenotype, not only for itself but for all the clustered imprinted genes in the critical PWS region. Therefore the Romanian Biotechnological Letters, Vol. 17, No. 6, 2012
epigenetic status of SNRPN has been considered so far the best biomarker in PWS condition [19]. Its presence and imprinting state defines the contribution of parental alleles, and it is essential for defining the health versus pathological states. Therefore, the methylation pattern in this locus is informative about its paternal contribution [20].

So far the molecular cytogenetic method (fluorescence in situ hybridization, FISH) has been extensively used in primary diagnosis, however it covers only deletional cases in this pathology. Abnormal methylation is commonly detected by methylation mapping methods (methylation specific PCR [21-23], methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; MRC-Holland, Amsterdam, The Netherlands [24-26]. This is the first report presenting a methylation mapping assay based on methylation specific amplification [21], for screening Prader-Willi syndrome in Romanian population. This study suggested MS-PCR assay first use in primary diagnosis based on its cost-efficiency and covering ability of almost entire Prader-Willi syndrome etiology (deletions, unimatalernal disomy, imprinting defect).

This paper aimed to compare the clinical validity of molecular cytogenetic method (FISH) and the molecular methylation test (methylation specific PCR - MSPCR) in order to explain our suggestion for MSPCR primary diagnosis on clinically PWS suspected cases.

Materials and Methods

Patients: 59 patients with clinical suspicion of PWS, and a control group of 30 normal individuals consisting of non-related individuals and parents of the patients, were recruited in this study after obtaining their informed consent, in accordance to The Declaration of Helsinki. This protocol was approved by the Bioethics Committee of the University of Medicine and Pharmacy Victor Babes, Timisoara. Induced lymphocyte cultures and extracted DNA from peripheral blood of patients and their normal relatives, (parents as controls) were used for FISH and MS-PCR assays performance.

FISH assay was performed according to standard protocols on metaphase spreads from cultivated blood lymphocytes using a chromosome 15 specific probe (CEP 15) (15q11.2) fluorescence-labeled in spectrum green and also locus specific DNA probes for SNRPN and PML (15q22) labeled in spectrum orange (all probes Abbott/Vysis). HYBrite (Vysis) denaturation and hybridization system, Axio Imager M1(Zeiss) fluorescence microscope and Isis (MetaSystems) fluorescence in situ imaging system were used to obtain the molecular cytogenetic results [27].

MS-PCR assay included DNA extraction, bisulfite conversion and amplification (PCR).

DNA extraction was performed from the collected peripheral blood of individuals. The DNA extraction was performed with Qiagen, DNeasy Blood & Tissue Kit, from whole blood according to the manufacturer protocol. Extracted DNA was quantified by a Nanodrop spectrophotometer, and optimal DNA concentration of 50-75 ng/μl was used in further steps.

Bisulfite conversion and cleanup of DNA was performed with EpiTect Bisulfite (Qiagen) kit according to the manufacturer protocol which consisted of 2 major steps: mutagenesis and purification.

Processed DNA amplification (in Corbett thermal cycler) was performed using the following PCR program: denaturation 95°C/10 min, 35 cycles - denaturation 95°C/15 sec, primer annealing 64°C/20, extention-72°C/30 sec, and a final step consisting of final extension 72°C/5 min. PCR reaction was performed in a total volume of 20 μl with: 3,5 μl DNA bisulfite-treated, PCR buffer 1x, MgCl₂ 1,5 mM, 225 μM of each dNTP, 1 μM of Maternal and Common primer, and 0,25 μM of Paternal primer and 1,5 U of Platinum Taq DNA Polymerase (Invitrogen).
The primer sequences were chosen after Zeschnigk et al. [21]:
Maternal: 5´-TATTGCGGTAAATAAGTACGTTTGCGCGGTC-3´
Paternal: 5´-GTGAGTTTGGTGTAGAGTGGAGTGGTTGTTG-3´
Common: 5´-CTCCAAAAACAAAAACTTTAAAAACCCAAATTCC-3´

The optimized amplification conditions after the Zeschnigk et al. [21] protocol consisted of raised alignment temperature, (64°C instead of 60°C) and the use of Hot Start type DNA polymerase.

The amplicons (aliquots of 10μl) were resolved by electrophoresis using 2% agarose gel and 0.5x TBE buffer, ethidium bromide-stained and observed under UV light. The bisulfite conversion and MS-PCR amplification was repeated twice for each sample to prove its reproducibility.

**Results**

Molecular cytogenetic and methylation assays were clinically validated on 59 PWS clinically suspected individuals (aged 7 months - 28 years), and the relevant results were illustrated (figures 2 and 3). Positive PWS cases are specifically marked by the signal from the SNRPN probe, proximal to CEP15 probe: absence of the signal suggests deletion of the paternal SNRPN allele (figure 2).

![Figure 2](image)

**Figure 2. Chromosomal spreads and their fluorescence marks pattern obtained by FISH assay:** A) normal individual, B) PWS patient (white arrow indicates the locus in the PWS/AS region where the lack of fluorescent signal indicates deletion). The probes used are CEP 15 (D15Z1) 15p11.2-spectrum green, LSI SNRPN (D15S10) 15q11-q13-spectrum orange, LSI PML 15q22-spectrum orange.

![Figure 3](image)

**Figure 3. MS-PCR band patterns generated by agarose gel electrophoresis.** 1-PWS positive control, 2 – normal control, 3, 4 – PWS cases, 5 – normal, 6, 7 – PWS cases, 8 – H2O, L- Ladder, maternal amplicon-313bp, paternal amplicon-221bp.
Band pattern (Figure 3) indicating a PWS positive case consists only of one methylated amplicon (313 bp) which represents maternal SNRPN sequence; normal band pattern consists of both bands standing for methylated (313 bp) and unmethylated (221 bp) amplicons, the two amplicons representing maternal, respectively paternal SNRPN alleles. Positive control was considered from FISH confirmed PWS patient, and negative controls from normal/healthy individuals or parental DNA.

Discussions

The core principle of the PWS molecular diagnosis through DNA methylation mapping is that this rare disease is due to defects in imprinting on the chromosomal region 15q11-q13, which result in the absence of paternal allele contribution to the SNRPN phenotype [3, 4]. PWS is a complex disorder whose diagnosis may be difficult to establish due to its variable etiology [6, 7, 8, 9].

The primary issue in evaluation of MS-PCR clinical applicability is the result interpretation. The hallmark of PWS condition in MSPCR is the lack of unmethylated amplicon from the paternal allele [21]. Three scenarios may be associated with this pattern: (i) deletion of the unmethylated paternal SNRPN sequence, (ii) replacement of paternal SNRPN unmethylated sequence with maternal methylated one, (iii) presence of the paternal SNRPN sequence which is abnormally methylated. This is confirmed by the simple assay of MS-PCR [21]. A small amount of DNA is needed for MSP, and the results can be obtained in 2 days [28].

False positive results are extremely rare and imply a sequence polymorphism in the 3’ region where the primers aligns [22]. In this study the primer pairs for the methylated and unmethylated region, amplified the expected amplicon in all the 30 healthy control individuals.

The clinical validity of the MSPCR and FISH assays was studied on 59 samples of PWS clinically suspected cases: first validation group was FISH positive (19 deletional cases), the second validation group was FISH negative (40 cases among which 5 were MSPCR confirmed and 35 MSPCR nonconfirmed) (figure 4A). All the 19 FISH positive cases were confirmed by MSPCR method. These results showed a larger coverage, cca 46%, for the MSPCR assay as compared with only cca 32% for the FISH assay.

The deletional cause was encountered with a frequency of ~79.16 % among PWS cases, while non-deletional cause (maternal uniparental disomy or imprinting defect) estimated based on the correlation of the MS-PCR results and the FISH ones, was encountered with a frequency of ~20.84 % (Figure 4B).

Figure 4. Diagrams of efficiency of FISH and MS-PCR diagnosis assays (A) and of PWS subtype prevalence in Romanian population (B).
These results are in accordance with literature, where reports on FISH analysis indicated on a frequency of deletional subtype of ~70-75% of the PWS cases [3, 29, 30].

Conclusions

Methylation specific PCR can be used for the primary diagnosis of the PWS suspected individuals, as it can confirm 99% of the cases: it is not limited to the epigenetic factors causing only one PWS subtype or etiology; it is able to concomitantly inform about almost all PWS subtypes. However, MSPCR reaction is not informing about the specific cause of PWS condition.

Information about subtypes are only relevant for recurrence risk in parents of PWS patients, estimated by genetic counselors for couples willing to conceive again: very low (under 1%) risk of recurrence occurs in couples having a PWS child with a deletional and uniparental disomy (UPD) subtype and very high recurrence risk (cca 50%) occurs in couples having imprinting defect subtype PWS child, if the imprinting centre is affected by a microdeletion. Hence, only counseling may impose new etiological informations provided by approaching a secondary method (for example, FISH or MLPA), after the primary MSPCR diagnosis had been performed. Clinical data along with fluorescent in situ hybridization and MS-PCR can distinguish the deletional from the non-deletional cases of PWS.

The molecular tests make possible an early and precise diagnosis providing a clear etiology of the disease, giving the possibility of an adequate treatment, improving the quality of life of both parents and PWS patients.

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