ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2012, *57*(12), 1041-1047

PARENTAL ALLELE METHYLATION MAPPING METHOD FOR PRADER-WILLI SYNDROME PRIMARY DIAGNOSIS IN THE ROUMANIAN POPULATION

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Received September 12, 2012

We described a simple assay, methylation specific polymerase chain reaction, to simultaneously analyze the genetic and epigenetic factors involved in Prader-Willi syndrome. In this paper, its cost-effectiveness toward its counterpart, commonly used technique in our country for the primary diagnosis of this disease, based on a molecular cytogenetic method, is demonstrated. The described method relies on the epigenetic principle of gene expression control through DNA methylation. The primary issue in evaluating its clinical utility is the correct interpretation of the results and the awareness of the limitations imposed by polymerase reaction chemistry. Therefore, we described the basic issues of this methylation mapping technique explaining also the need for appropriate choice of reactives and reaction conditions. The analytical and clinical validation of this methylation specific amplification method on clinically suspected cases in the Roumanian population aims to suggest its implementation in genetic laboratories as first, primary diagnosis approach.

INTRODUCTION

The epigenetic principle underlying methylation specific PCR (MSPCR) method is referred to as biochemical control of gene expression or transcriptional state through methyl group tags distribution on DNA sequences (DNA methylation patterns).[1-2] DNA marking by methyl groups or DNA methylation represents a vital biochemical process (DNA methyltransferase enzyme catalyzed) that underlies the attachment of methyl groups on DNA strands, namely on its cytidine residues, where their pyrimidine rings of cytosine (C) bases are transformed into minor 5-methyl-cytosine (5meC) bases.[3] This epigenetic modification of DNA preserves the **genotype** or the nucleotide sequence and does not introduce mutations, but instead, independently of DNA sequence, it establishes a specific **epigenotype.** This is defined by specific distribution of 5meC and C residues on DNA sequence, which encode molecular **signals in gene regulatory regions (promoters and exon 1) for instructing the gene to function or to be silenced**.[4-6] Gene transcriptional state is regulated through an inverse relationship with its DNA methylation pattern: unmethylated form of a DNA sequence instructs the gene for expression, therefore for its contribution to the corresponding phenotype, while its methylated form remains suppressed and the lack of its contribution is encountered. Normally, healthy state needs proper DNA methyl tags

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distribution on specific genes in specific cell types during a specific developmental stage of an organism. Numerous sporadic or non-familial diseases that are transmitted in non-Mendelian manner involve altered epigenotypes associated with gene expression variation (such as imprinting diseases, cancer, Alzheimer's disease, metabolic syndrome). [6-9]

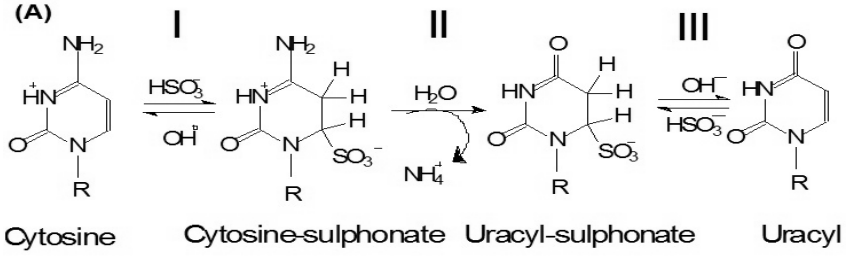
Prader-Willi syndrome (PWS) is caused by deletions or by defects in DNA marking by methyl groups in certain gene types, named imprinted genes, on 15 chromosome.[9-11] Contrary to the majority of genes in our genome that are biallelically expressed from their both parental gene copies (alleles), these genes are expressed only from one parental copy, whereas the other one is silenced. Proper parent-of-origin marking through methyl groups on parental alleles is essential for the healthy state. The critical gene selected for PWS diagnosis, small nuclear ribonucleoprotein N (*SNRPN*), is expressed only from its **paternal** allele, meaning that its regulatory region (promoter and exon 1) is normally unmethylated, while methylation of maternal allele assures its repression. However both have the same DNA sequence.[9-17] Methylation mapping on the critical gene *SNRPN* alleles may be a good biomarker for primary PWS diagnosis.[17, 18]

Cytogenetic methods (fluorescence in situ hybridization, FISH), performed on individual chromosomal preparations, can detect only deletional cases (representing, according to published reports, only cca 75% of PWS cases). [11, 19] We described an alternative molecular method at DNA level, methylation specific PCR (MS-PCR), which relies on epigenetic concept of gene expression control through DNA methylation, and gives information on both deletional and non-deletional cases, in an attempt to extend the PWS diagnosis coverage in Romanian population to almost 99% of cases, according to reports on other populations.[19] Mapping the DNA methyl tags on parental *SNRPN* alleles, represents a qualitative measure of DNA methylation, which is correlated with the function of each allele. The paternal allele activity or contribution is therefore central of the MSPCR method use in PWS diagnosis.[18, 20]

PCR amplification reactions underlie numerous techniques currently used for DNA methylation mapping. However, methylation analysis is not possible with classical, straightforward PCR reaction. It should detect and amplify on DNA template both genetic (DNA nucleotide sequence) and epigenetic (methyl tags distribution on the same DNA sequences of parental alleles) information.[18] However, (i) DNA polymerase is not able to distinguish methylated versus unmethylated C residues in template DNA, and (ii) DNA polymerase is able to incorporate only unmethylated C mononucleotides during PCR amplification. Proper DNA template processing through specific treatment with sodium bisulfite may enable the discrimination of both genetic and epigenetic information that subsequently may be amplified during PCR reaction.[18]

Such **mutagenesis based DNA processing for MS-PCR reaction** was reported in early 70s [21] and it has been extensively used in epigenetics since late 90s.[22-25] It is based on different sensitivity to bisulfite treatment of methylated versus unmethylated C residues in DNA sequences: DNA incubated in the presence of bisulfite at acid pH, would contain unmethylated C residues deaminated to uracil (U), while for methylated C residues, the reaction is extremely slow and conversion process may be stopped before transformation of methylated C to thyminidine (T) residues can occur (Fig. 1A).[21] Therefore, **bisulfite converts DNA to a new template for PCR**, having **new genetic information** or nucleotide sequence, where U stands for initial unmethylated CpG site, while C stands for initial methylated CpG site in former DNA template.

Choice of proper primer sets is the next critical step of any MS-PCR reaction as they must discriminate between former methylated and unmethylated molecules in bisulfite-treated DNA.



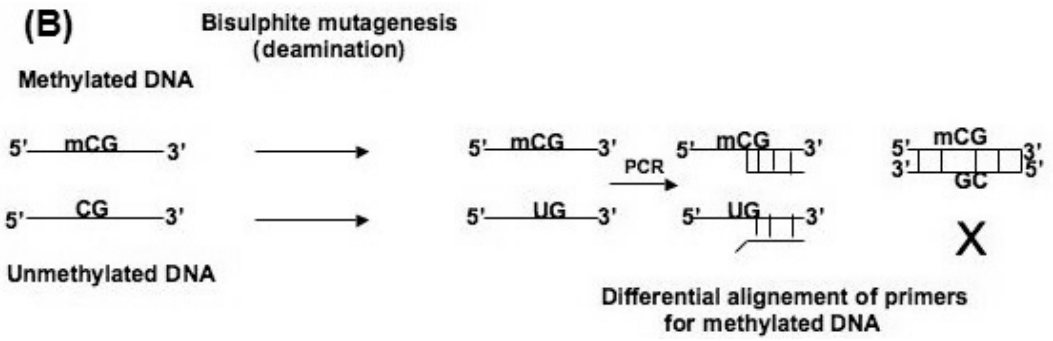


Fig. 1 – Core principle of MSPCR method: A. Chemical reaction equation showing the three steps of cytosine deamination by bisulfite: sulphonation (addition of bisulphate to the 5-6 double bond of cytosine), hydrolytic deamination of the resulting derivative (cytosine-bisulfite) to uracyl-bisulfite derivative and removal of sulphonate group by alkali treatment, that results in uracyl. B. Primer alignment at the primer binding sites on converted DNA. Primer sequences are essential for differential alignment and further amplification reactions.

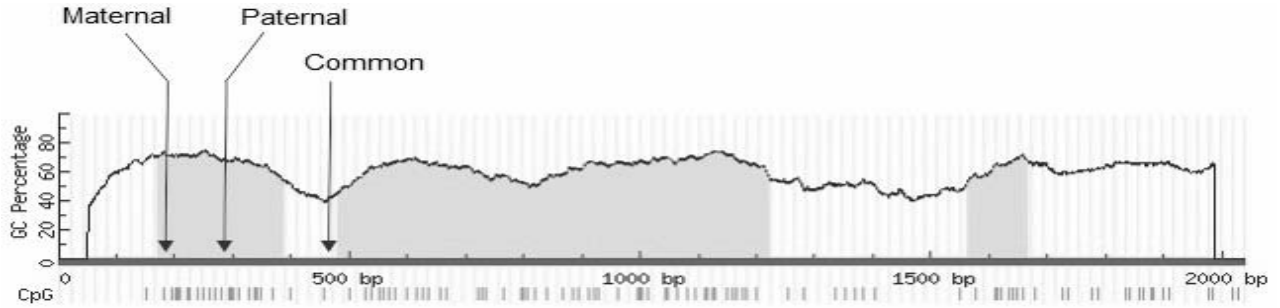


Fig. 2 – **MethPrime diagram generated for CpG dinucleotide repeats density variation** in *SNRPN* (promotor/exon1) gene region comprising 2037 bp. It is grouped in three CpG dinucleotide rich regions according to their density (dashed marked domains): island 1 of 216 bp, island 2 of 741 bp and island 3 of 101 bp. Maternal/Paternal and Common primer sequences that were chosen according to Zeschnigk *et al.* protocol[18] were validated by the Meth Prime sofware according to the standard MS-PCR rules: two primers, corresponding to maternal and paternal alleles were provided from the highest density region, while the common primer, from the zero density region. This diagram was generated by the MethPrime software.[26]

Zeschnigk group suggested an ingenious rule for optimal discrimination, based on targeting two different sequence regions by different primers: (i) one region, that is **methylation prone** in healthy or pathological state, with two primers and (ii) the other region, that is never methylated in either state, with one primer.[18] Presently, these two sequence regions on template DNA may be discriminated by targeting them with primer sequences that are recommended by the recently developed MethPrime software: its gold standard rule in primers sequence choice is referred to **CpG dinucleotide density**.[26]

PCR amplification per se results further in products named amplicons and their electrophoregram is interpreted based on the presence or lack of the amplicon band corresponding to the normal contribution of paternal, unmethylated allele versus maternal, normally methylated allele.[27, 28]

The reason for which specialized genetic laboratories have avoided so far the MSPCR approach for molecular confirmation of PWS cases in Roumanian population is the lack of basic epigenetic principles knowledge and of the complex chemistry of the MS-PCR technique. This paper aims to describe basic issues and critical steps of this molecular method in order to use it routinely as primary PWS diagnosis assay. In this paper, its cost-effectiveness toward its counterpart, commonly used technique in our country for the primary diagnosis of this disease, based on a molecular cytogenetic method, is demonstrated.

RESULTS

Based on the outlined assay principles, MS-PCR reactions were performed as indicated by literature[18] and our optimized conditions. MS-PCR assay was used first in attempts to analytically validate DNA methylation mapping capacity on already confirmed clinically suspected PWS cases, by FISH method. Secondly, the optimized conditions were used for confirmation of more

clinically suspected cases, which were double checked by FISH method, in Roumanian population that were assigned to our laboratory by Roumanian Prader-Willi Association and hospital partners during the period of 2009-2011 (a total of 59 clinically suspected cases). As controls, normal relatives, the parents of probands, and non-related individuals were considered.

We demonstrated that MS-PCR protocol can be set in user-defined conditions. First optimization is referred to as designing of proper primer sequences. Our setting designed three primers from Zeshnigk protocol[18] that were verified by the gold-standard principle used by the MethPrime software (Fig. 2). Further optimizations regarded annealing temperature, concentration of primers and the DNA Taq Polymerase type, which are essential for the configuration of PCR chemistry in order to achieve accuracy and avoid false results. Alignment temperature has been raised to 64ºC instead of 60ºC (as initially suggested by Zeshnigk *et al.*, (1997);[18] also a Hot Start type Taq DNA Polymerase has been used instead of common Taq polymerase, and the paternal primer concentration was raised from 0.25 μM[18] to 0.35 μM. These optimizations aimed to avoid amplification of mismatched partially complemented sequences at low temperature that may result in obtaining unspecific amplicons.

Two different amplicon eletrophoresis patterns were obtained for normal individuals respectively PWS cases, obtained with optimiyed and unoptimiyed conditions.

As shown in Fig. 3, **healthy individuals** were represented by **two bands** corresponding to two normal alleles of **imprinted *SNRPN* gene.** This normal state of *SNRPN* gene is defined by two DNA amplicons representing both parental alleles with the same DNA sequence or genetic information, however having different mass according to different methylation marks or epigenotypes on them: the heavier, 313 bp band, corresponding to methylated *SNRPN* maternal allele and the second, lighter 221 bp band, corresponding to unmethylated paternal allele DNA sequence. The **lighter band** is critical in methylation assay, as its presence indicates the normal state, while its lack, the pathological condition linked with lack of the functional, active, unmethylated paternal allele. The **PWS patient**, presented **only the** 313 bp **methylated DNA band**. These results were obtained according to literature.[18]

The clinical validity of the MSPCR assay was studied further 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). This results showed a larger coverage, cca 46%, for the MSPCR assay as compared with only cca 32% for the FISH assay.

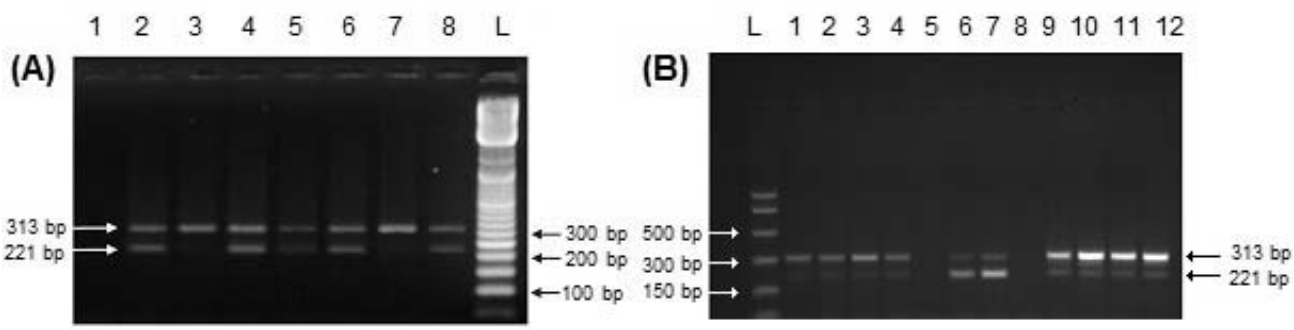


Fig. 3 – **Electrophoretic band patterns generated with optimized (A) and unoptimized (B) conditions. A.** 1-H_20; 2- **normal**, parental, control, with **two bands**: normal unmethylated (paternal-221 bp) and normal methylated (maternal-313bp) ones; 3-**PWS-positive case**, comprising only **one**, **methylated band -313 bp**; 4, 5, 6, 8- normal, two bands pattern, 7- positive PWS case with one band – 313 bp (methylated), **B.** 1-4 and 9-12- PWS positive case that should had one methylated band shows **false-normal,** two bands pattern due to **faint unmethylated bands** as unspecific amplicons; 6, 7- normal, parental control – two bands; 5, 8- H_20. L- Marker Ladder; 313 bp maternal band, methylated; 221 bp paternal band, unmethylated.

DISCUSSIONS

The core principle of the PWS molecular diagnosis through DNA methylation mapping is that this rare disease is due to defects in imprinting processes on the critical chromosomal region 15q11-q13, which results in the lack of paternal *SNRPN* allele contribution.[17,18] PWS is a complex disorder whose diagnoses may be difficult to establish due to its variable etiology: deletional, nondeletional (15 maternal disomy, and imprinting defect), and rarely translocations and mosaicism.[29]

The primary issue in evaluation of MS-PCR clinical applicability is the results interpretation. The hallmark of PWS condition in MS-PCR is the **lack of unmethylated amplicon of the paternal allele**.[17,18] 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. All these scenarious are confirmed by the simple assay of MS-PCR. MSPCR greater coverage is explained thus by its ability to detect both genetic (deletional) causes and epigenetic (nondeletional) ones, thus covering almost all PWS etiologies.

The clinical criteria of PWS diagnosis established in 2001[30] are considered presently consensus criteria for all medical centers worldwide. However, they require confirmation by molecular genetic testing, which was not widely available when the criteria were developed. The first approached genetic test, based on fluorescent in situ hybridization (FISH) method, targets the genetic factors only, represented by paternal chromosome (allele) deletion through specific fluorophores carrying probes on critical 15 chromosome.[31] Its routine use in genetic laboratories proved its limitation regarding its informative power that refers only on deletional PWS subtype.[29] When epigenetic factors were recognized in this pathology, MS-PCR method gained attention due to its power to detect not only the imprinting methylation defects, but based on its results interpretation, both major PWS subtypes, deletional and nondeletional. Its use may cover thus almost all PWS etiologies, representing around 99% of cases.[19,29]

However, widely use of MS-PCR technique in foreign specialized laboratories since its first publication in late 90s[17, 18] proved difficulties in avoiding false negative results due to complex chemistry of its processing steps.[28] Moreover, in Roumanian genetic laboratories, epigenetic specific methods able to map DNA methylation patterns, have to be correctly interpreted through knowledge of epigenetic fundamentals in order to be correlated with molecular pathological mechanisms. In an attempt to persuade laboratory specialists to approach MS-PCR method as accurate routine, rapid, facile and cost-effective approach we described the principle of this method and the limiting conditions of its chemical reactions in order to suggest criteria for choosing proper commercial kits and to design optimal reaction conditions. MS-PCR needs less and low cost reactives and inexpensive equipment and may be performed in almost 2 days, which demonstrates its cost-efficiency as compared with FISH method, a time- and effort-consuming (requiring almost one week) and more expensive technique.

MSPCR method is not limited to the epigenetic factors causing only one PWS subtype or etiology. Providing its results are correctly interpreted it is able to concomitantly inform about almost all subtypes which include both genetic and epigenetic causes and therefore, it is suggested its use as primary diagnosis or molecular confirmation of clinically suspected cases.[29] However, MSPCR reaction is not informative on the specific etiology 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, as this risk depend on the type of etiology.[29] Hence, only counseling may impose new etiological informations provided by approaching secondary method (for example, FISH), after the primary MS-PCR diagnosis had been performed.[29]

EXPERIMENTAL

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 "Victor Babeş" University of Medicine and Pharmacy, Timişoara, where the FISH technique was performed as diagnosis approach. Extracted DNA from peripheral blood of patients and their normal controls were used for MS-PCR assays performance. Clinical diagnosis was based on consensus clinical diagnostic criteria for PWS developed in 1993[32] and proven to be accurate later in 2001.[30] This was applied by hospitals coordinated by the above-mentioned university in a common research project, which had as partner the University of Bucharest, where MSPCR technique was performed.

DNeasy Blood *&* Tissue Kit (Qiagen) for DNA extraction from whole blood was used. After Nanodrop quantification, DNA conversion with EpiTect Bisulfite (Qiagen) kit was performed. PCR conditions used (Corbett thermal cycler): primary denaturation 95ºC/10 min; 35 cycles program: denaturation - 95ºC/15 sec, primer annealing - 64ºC/20, extention -72ºC/30 sec, final extention 72ºC/5 min; PCR reaction mixture (20µl): 3.5 µl DNA, 1x, MgCl2 1.5 mM PCR buffer, 225 µM of each dNTP, 1 µM of each Maternal and Common primer, and 0.35 µM Paternal primer and 1.5 U of Platinum Taq DNA Polymerase (Invitrogen). Ethidium bromide marked PCR reaction products (amplicons) were

resolved on 1.7 % agarose gels and UV visualized. Selected maternal, paternal and respectively, common allele primer sequences have the following (5'- 3') sequences: TATTGCGGTAAATAAGTACGTTTGCGCGGTC, GTGAGTTTGGTGTAGAGTGGAGTGGTTGTTG, and CTCCAAAACAAAAAACTTTAAAACCCAAATTCC.

CONCLUSIONS

We proposed MSPCR as part of an algorithm for molecular confirmation of PWS clinically suspected cases. First approach should imply the MSPCR diagnosis, able to cover almost all etiologies. Starting with FISH method, may result an underscored number of real PWS cases in Roumanian population, as this technique is not able to cover nondeletional etiology. Moreover, this confirmation approach may delay the treatment intervention and meantime increase the test price by imposing the second, methylation test. However, when genetic counseling for parents is needed, as MS-PCR is not able to discriminate the PWS subtype, further FISH analysis is imposed for risk assessment.

DNA methylation mapping by MS-PCR assay is a qualitative estimation of DNA methylation status. This paper describes a methylation mapping protocol that may be used for diagnosis of any other imprinting defect disease providing particular optimizations are considered, such as primer sequences choosing criteria, increased alignment temperature and using a Hot Start DNA polymerase type. Considering the simple and cost-effective experimental model (which needs inexpensive thermocycle and electrophoresis line as compared with molecular microscopy technique) and its analytical characteristics of reliability, precision, reproducibility, and especially the informative quality regarding coverage of almost all PWS etiologies, MS-PCR technique may be considered as a better primary diagnosis method, instead of the cytogenetic FISH method and thus as a proper approach for a more accurate estimation of PWS incidence in the Roumanian population.

Acknowledgements: Cosmin Arsene PhD programe work was financed by the National Research Project entitled "Doctoral studies in the domain of life and earth sciences" cofinanced from the European Social Fund through the POSDRU/88/1.5/S/61150, 2007-2013, and coordinated by O.Z. The research work was also sustained from PN II project entitled "Correlation of clinical, genetic and epigenetic aspects in understanding the etiology of genomic diseases Prader-Willi and Angelman: a model of multidisciplinary approach of rare diseases in Roumania" (PWA- Clin-Epi-Gen), Programe 4 – Health – Partenerships, Contract CNMP: 42-113, 2008-2011, coordinated by NC at Partener 1: university of Bucharest, of the project consortiu, conducted by M.P..

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