Chulalongkorn Medical Journal

Volume 45
Issue 3 *March 2001*

Article 5

3-1-2001

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## Recommended Citation

Sakdikul, S. and Mutirangura, A. (2001) "Diagnosis of Prader-Willi, Angelman and male fragile X syndromes at King Chulalongkorn Memorial Hospital by methylation-specific PCR.," *Chulalongkorn Medical Journal*: Vol. 45: Iss. 3, Article 5.
Available at: https://digital.car.chula.ac.th/clmjournal/vol45/iss3/5

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# Diagnosis of Prader-Willi, Angelman and male fragile X syndromes at King Chulalongkorn Memorial Hospital by methylation-specific PCR

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**Sakdikul S, Mutirangura A. Diagnosis of Prader-Willi, Angelman and male fragile X syndromes at King Chulalongkorn Memorial Hospital by methylation-specific PCR. Chula Med J 2001 Mar; 45(3): 223 - 32**

**Background** : *Definite diagnoses for patients suspected of having Prader-Willi, Angelman and fragile X syndromes are crucial. Among a number of available chromosomal and molecular tests, methylation-specific PCR is the most specific method and requires the lowest unit cost.*

**Objective** : *To establish methylation - specific PCR for these syndromes in Thailand.*

**Material and methods** : *DNA samples from known or suspected patients were treated in sodium bisulfide to alter unmethylated, but not methylated, cytosine to uracil. Then, following published protocols, PCR reactions and primers specific to methylated and unmethylated sequences on SNRPN, the imprinted gene on chromosome 15q11-13, and FMR1 were used to detect the modified DNA associated with Prader-Willi, Angelman, and male fragile X syndrome patients respectively.*

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**Result** : *The PCR products from Prader-Willi and male fragile X syndrome patients showed only methylated sequences and were distinguishable from normal controls.*

**Conclusion** : *Methylation-specific PCR was established to diagnose Prader-Willi, Angelman and male fragile X syndromes in Thailand.*

**Key words** : *Prader-Willi syndrome, Angelman syndrome, Fragile X syndrome, Methylation - specific PCR.*

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Received publication. November 8, 2000.

**สายรุ้ง ศักดิกุล, อภิวัฒน์ มุทิรางกูร. การวินิจฉัยกลุ่มอาการ Prader-Willi, Angelman และ fragile X ในโรงพยาบาลจุฬาลงกรณ์ด้วยวิธี methylation - specific PCR. จุฬาลงกรณ์เวชสาร 2544 มี.ค; 45(3): 223 – 32**

**เหตุผลของการวิจัย** : *การวินิจฉัยผู้ป่วยที่สงสัยว่าเป็นกลุ่มอาการ Prader-Willi, Angelman และ fragile X มีความสำคัญ ในวิธีการตรวจทางห้องปฏิบัติการทั้งในระดับโมเลกุลและโครโมโซม methylation-specific PCR นับเป็นวิธีที่จำเพาะและใช้ค่าใช้จ่ายน้อยที่สุด*

**เป้าหมาย** : *สร้างการตรวจ methylation-specific PCR สำหรับกลุ่มอาการเหล่านี้ในประเทศไทย*

**วิธีการทำวิจัย** : *ดีเอ็นเอจากผู้ป่วยที่พิสูจน์แล้วหรือสงสัยว่าป่วยด้วยกลุ่มอาการดังกล่าวถูกนำมาทำปฏิกริยากับสารโซเดี่ยมไบซัลไฟด์ทำให้เบสไซโตซีนที่ไม่มีเมททิเลชั่นกลายเป็นยูราซิลในขณะที่เมททิลไซโตซีนไม่เปลี่ยนแปลง หลังจากนั้นตรวจโดยใช้ปฏิกริยาพีซีอาร์ตามที่ตีพิมพ์ ไพรเมอร์ที่ใช้ตรวจกลุ่มอาการ Prader-Willi&Angelman คือลำดับเบสที่จำเพาะกับยีน SNRPN ที่ถูกและไม่ถูกเติมเมททิล ส่วนกลุ่มอาการ fragile X ชายก็ใช้ไพรเมอร์จำเพาะกับยีน FMR1*

**ผลการศึกษา** : *ผลผลิตของเทคนิคพีซีอาร์ของผู้ป่วยกลุ่มอาการ Prader-Willi และ fragile X ชายพบเฉพาะสายที่มีเมททิเลชั่นและต่างจากตัวอย่างปกติที่ใช้เปรียบเทียบ*

**สรุป** : *วิธี methylation-specific PCR ถูกนำมาใช้เพื่อวินิจฉัยกลุ่มอาการ Prader-Willi, Angelman และ fragile X ในประเทศไทย*

Laboratory investigation in medical genetics is crucial for making a proper clinical judgment. General indications include confirmatory, presymtomatic and prenatal diagnosis as well as carrier detection. The methylation-specific polymerase chain reaction (MSPCR) is a molecular technique for diagnosing diseases whose causes are related to DNA methylation.[1] These causes include genomic imprinting, triplet repeat expansion, and promoter hypermethylation, as seen in, for example, Prader-Willi (PWS) & Angelman (AS) syndromes, the fragile X syndrome, and the inactivation of some tumor suppresser genes in sporadic tumors, respectively.[1-3] This study reports the establishment of the tests for PWS, AS and fragile X syndrome in Thailand.

PWS is characterized by severe muscular hypotonia and feeding difficulties in infancy, hypogonadism with undescended testes in males, early onset of childhood hyperphagia and consequent obesity, short stature, small hands and feet, almond-shaped eyes, and hypopigmentation.[4] AS is characterized by mental retardation of greater severity, microcephaly, seizures, lack of speech development, inappropriate bouts of laughter, ataxic gait, puppet-like upper-limb movements, large mandible, an open mouth with protruding tongue, and hypopigmentation.[5] Although these two disorders share almost no clinical features, they are both caused by abnormalities involving the same chromosomal region. Almost all PWS and majority of AS are caused by deletion of chromosome 15q11-13, uniparental disomy (UPD) and imprinting center mutation. Whereas absence of the paternal copy or mutation of the paternal imprinting center of 15q11-13 leads to PWS, AS results from absence of the maternal copy, alteration of the maternal imprinting center of 15q11-13 or maternal mutation of the AS gene, *UBE3A*.[6] Since *SNRPN* is methylated only on the maternal chromosome 15q11-13 according to genomic imprinting control, analysis of DNA methylation at the imprinted gene shows a distinct pattern among PWS, AS, (except in *UBE3A* mutation cases) and normal people.[6]

Fragile X syndrome is the most common cause of inherited mental retardation. It is found more commonly in males with a frequency of approximately 1 in 2500. Patients are characterized by facial abnormalities (including a narrow face with a prominent forehead jaw and ears), macroorchidism in 90 % of postpubertal affected males, mild connective tissue abnormalities, autism and moderate to severe mental retardation.[7] The disorder is X-linked and predominantly caused by a large expansion of a CGG trinucleotide repeat present in the 5' region of the *FMR1* gene.[8] The CGG trinucleotide repeat is highly polymorphic in the normal population, ranging from 6 to 50 repeats. An intermediate expansion of 50 - 200 repeats is seen in normal transmitting males and unaffected carrier female. Patients with fragile X syndrome have an expansion to the full mutation, which is greater than 200 repeats. A full mutation of the trinucleotide repeat correlates with methylation of the gene and silencing of gene transcription which is believed to result in the fragile X phenotype.[9] Consequently, detection of *FMR1* DNA methylation can be used for diagnosing males with the full CGG repeat mutation.

Providing definite diagnosis for patients suspected of having PWS, AS or male fragile X syndrome is of great clinical importance. These three syndromes are difficult to be diagnosed by

clinical examination alone and some of their clinical characteristics are commonly found in other conditions. These non-specific phenotypes include mental retardation or autism in childhood males with fragile X syndrome, hypotonia in infancy or obesity in childhood with PWS and seizure with severe mental retardation for AS. In addition, fragile X syndrome and a small proportion of PWS & AS can be transmitted in a Mendelian fashion. Definite diagnosis is necessary for informative genetic counseling, proper clinical management and further investigation aimed for disease prevention such as carrier testing or prenatal diagnosis.

## Methods

### Samples

Three to 5 ml of blood in anticoagulant EDTA (CBC tube) was sent to the Genetics Laboratory, Department of Anatomy, Faculty of Medicine, Chulalongkorn University for to prove the PWS, AS or fragile X syndrome diagnosis.

### DNA preparation

DNA (1 μg) in a volume of 50 μl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5, both freshly prepared, were added and mixed, and samples were incubated under mineral oil at 50°C for 16 hr. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer]s instructions (Promega) and diluted into 50 μl of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at -20 °C.

### MSPCR

MSPCR for both PWS&AS and fragile X syndrome was performed essentially as previously described.[2,3] The sequences of PCR primers are listed in table 1. PCR reactions were carried out in a

Table 1. PCR primers used for methylation-specific PCR.

| Syndrome | Primer set | Primer sequence, 5'-3' | Size |
|---|---|---|---|
| Prader-Willi | SNRPN-M for | TAAATAAGTACGTTTGCGCGGTC | 174 |
| & | SNRPN-M rev | AACCTTACCCGCTCCATCGCG | |
| Angelman | SNRPN-P for | GTAGGTTGGTGTGTATGTTTAGGT | 100 |
| | SNRPN-P rev | ACATCAAACATCTCCAACAACCA | |
| Fragile X | FX-BS-for | ACCGATTCCCAACAACGCGCATA | 255 |
| | FX-BS-rev | TTTCGTTATCGTCGTCGTTCGC | |
| | NM-BS-for | ACACACATACACACACTCCCAAA | 163 |
| | NM-BS-rev | TTGAAATGGAGTTGAGTGTTTGAT | |

30 μl volume containing 1xPCR buffer (Perkin-Elmer), 200 μM dNTP, 2.0 or 4.5 mM MgCl2 for PWS & AS or fragile X respectively, 0.4 μM primers, SNRPN-M or SNRPN-P, (in the duplex PCR 1.2 μM SNRPN-M primers and 0.4 μM SNRPN-P primers) for PWS & AS or 0.5 μM NM-BS primers, 1.0 μM FX-BS primers for fragile X, respectively, 0.6 U of AmpliTaq Gold (Perkin-Elmer) and 30 ng of bisulphite-modified DNA. The reaction was incubated at 95 °C min. DNA was amplified for 35 cycles at 94°C for 30 s, 62°C or 64°C for 30 s for PWS & AS or fragile X respectively and 72°C for 30 s, followed by a final extension at 72°C for 10 min. A negative control consisting of the reaction mixture without the DNA template was included for each set of reactions. PCR products were separated on a 3 % agarose gel, stained with ethidium bromide, and visualized under UV illumination.

## Result

MSPCR entails chemical modification of cytosine to uracil by sodium bisulfite, in which methylated cytosines in the CpG dinucleotides are

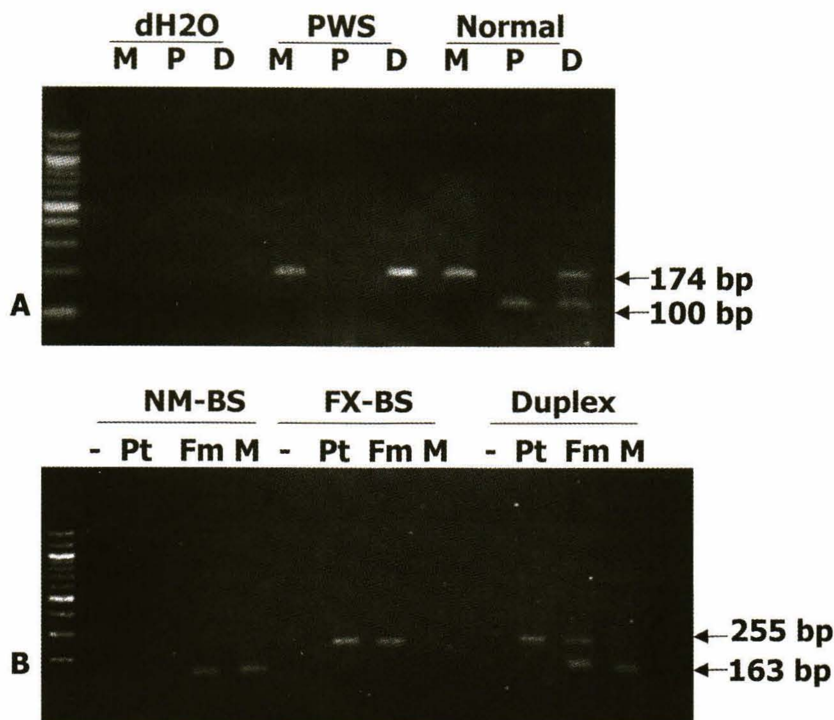


Figure 1. Demonstrate methylation-specific PCR products for Prader-Willi and fragile X syndromes respectively. A) PCR products of SNRPN primer set. dH2O, PWS and normal are PCR products from water, PWS DNA and normal control DNA respectively. M, P and D are PCR products from SNRPN-M, SNRPN-P and duplex primer sets respectively. B) PCR products of FMR1 primer set. -, Pt, Fm and M are water, known fragile X male, normal female, and normal male respectively. The bisulfided DNAs were tested with NM-BS, FX-BS and duplex PCR of both primers respectively.

resistant to the modification. Subsequently PCR amplification with primer pairs specific for either the methylated or the unmethylated alleles is performed. We applied this technique to test for PWS & AS and male fragile X syndrome by using published primer pairs specific for methylated alleles, SNRPN-M and FX-BS, and unmethylated alleles, SNRPN-P and NM-BS, for analyzing DNA methylation status on the *SNRPN* and *FMR1* genes respectively.

An example of MSPCR for PWS is shown in fig 1A. Normal human DNA demonstrated a positive PCR result from both from SNRPN-M and SNRPN-P showing 174 and 100 bp respectively. Duplex PCR showed both PCR products. Nevertheless using PWS DNA, previously confirmed to have a paternal deletion of chromosome 15q11-13 or maternal UPD chromosome 15 by microsatellite analysis, [10] MSPCR was positive only from the SNRPN-M but not from the SNRPN-P substrate from both simplex and duplex PCR. Thirty-six samples suspected to have PWS were tested and the diagnosis was confirmed for 12 cases. Only one typical AS case was analyzed but showed a normal methylation pattern.

Fig 1B shows the MSPCR result for fragile X syndrome testing in normal male, female and typical fragile X male DNA samples. NM-BS amplified unmethylated DNA sequence was positive in the normal male and female but not in the fragile X male showing a 163 bp PCR product On the contrary FX-BS, specific for methylated sequence, amplified product from the normal female and the fragile X male but not the normal male. This confirmed methylation at FMR1 in the fragile X male. In addition, the normal female showed both methylated and unmethylated alleles because of normal DNA methylation by the random X-inactivation process. Thus this test gave definite diagnosis when testing male patients. 16 questionable samples were tested to detect the male fragile X syndrome and 2 were positive.

**Discussion**

It is necessary for a reference hospital in Thailand to develop a DNA diagnostic laboratory service. This is required for better counseling and management for families suffering genetic diseases. Compared to infectious diseases, most Mendelian medical disorders and chromosomal abnormalities are rare, and molecular genetic investigation requires specialized techniques. We previously established PCR based methods to test several genetic diseases such as Duchenne muscular dystrophy, [11,12] spinal muscular atrophy, [13] and PCR *SRY* gene to identify testis-determining factor for Turner's syndrome or patients with ambiguous sexual organs. At the moment, either fresh blood in EDTA or DNA is required for these tests. However, since all tests require only a little amount of DNA, more convenient sample collection methods such as Guthrie's test filter or a drop of blood on a piece of paper, will be feasible in the future. [14]

There are several laboratory methods applicable to test PWS & AS including chromosome analysis and fluorescence in situ hybridization (FISH), microsatellite analysis and methylation analysis by Southern blot or PCR.[6] Studying chromosome abnormalities using karyotyping and FISH detects only patients with deletions (approximately 70 % of cases). Microsatellite analysis demonstrates deletion and UPD (approximately > 97 % of PWS and 80 % of AS). Nevertheless, this method requires parent DNA to observe the pattern of 15q11-13 segregation. Thus

methylation analysis is the most efficient test since parent DNA is not needed and it will show a positive result in almost all PWS and a majority of AS. [2] In addition, by using this improved PCR based technique, less cells or DNA are needed, giving potential for a better referring system and a lowered cost of investigation. In cases of AS with *UBE3A* mutation, clinical evaluation and a typical EEG pattern will be useful for definite diagnosis. MSPCR alone will not provide information regarding the exact mechanism causing this disease. This may be significant in rare inheritable cases such as balanced chromosomal translocation or an imprinting center mutation of PWS & AS and the *UBE3A* mutation of AS. In these cases classification of the disease cause and carrier detection by FISH or microsatellite analysis may be performed. However, if these techniques are not feasible, MSPCR can still be offerred for prenatal diagnosis.[15]

There are also several tests for fragile X.[8,16] These include chromosome analysis, PCR across the CGG repeats, Southern blot to observe methylation status and length of the trinucleotide expansions and MSPCR. Unlike PWS & AS, diagnosis for fragile X syndrome is aimed not only to confirm diagnosis but also to detect the allele in other family members. These include affected males with the full mutation, carrier or affected females with the full mutation and males with the permutation (normal transmitting males). Experienced cytogeneticists can use a special cell culture technique to induce the fragile site in cells from affected patients. PCR across the repeats is them used to analyze triplet repeat length, especially of the premutation from a normal transmitting male. Southern blot and hybridization is the best method to diagnose a full mutation from both males and females. MSPCR is applicable for only males with the full mutation. Due to normal DNA methylation in X inactivation, using MSPCR for to diagnose female fragile X requires quantitative comparison giving a higher possibility for false positive or negative results. Thus, unlike PWS, MSPCR for fragile X is limited to use in fragile X males. Nevertheless, MSPCR is a more practical diagnostic and screening test because of the lower unit cost and because radioactive isotopes are not necessary.

In conclusion, we have developed MSPCR diagnostic techniques for PWS & AS and fragile X syndrome in Chulalongkorn University Hospital. This technique is useful not only to confirm a diagnosis but also for prenatal testing. In addition, patients showing some significant clinical phenotypes, such as hypotonia in infancy, childhood obesity, severe mental retardation with seizure, male mental retardation, and childhood autism can be differentially diagnosed and these diseases confirmed or excluded. Finally, this technique requires only a little amount of DNA and has a lower unit cost, giving the potential to analyze referred DNA samples from the rest of Thailand.

## Acknowledgment

We express our gratitude to all pediatricians and geneticists who sent patients' blood samples for DNA diagnosis. This study was supported by the Molecular Biology Project, Faculty of Medicine, Chulalongkorn University, and the Thailand Research Fund.

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