Multiplex Methylation Specific PCR Analysis of Fragile X Syndrome: Experience in Songklanagarind Hospital

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Methylation specific PCR (MS-PCR) is a technology for a sensitive detection of methylation in the gene. This assay was developed for diagnosis of methylation-related diseases including fragile X syndrome (FXS), the most common X-linked mental retardation caused by a CGG trinucleotide repeat expansion. Affected individuals (full mutation, FM) have CGG greater than 200 repeats, while normal individuals and premutation (PM) carriers have 6-54 and 55-200 repeats, respectively. Only FM individuals are correlated with methylation of the gene. The authors tested this assay on known 35 DNA samples (15 normal, 2 PM and 18 FM) and a prospective study of 60 males referred for FXS screening in Songklanagarind hospital. In addition, the authors tested on 2 prenatal cases. All results were corresponded to PCR for CGG repeats and/or Southern blot analysis. The authors concluded that MS-PCR provides an accurate method for methylation detection of FXS.

Keywords: *Methylation specific PCR, Mental retardation, FMR1 gene*

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Fragile X syndrome (FXS) is the most common cause of mental retardation secondary to Down syndrome. In Thai boys, the frequency of FXS among mental retardation of unknown cause is approximately 7%[1]. The gene responsible for FXS, fragile X mental retardation 1 (*FMR1*) gene, contains an unstable repeat sequence of Cytosine-Guanine-Guanine (CGG). Among normal individuals, the number of CGG repeats ranges from 6 to 54 repeats. Individuals with CGG repeats between 55 and 200 repeats; premutation (PM), have a variable risk for producing an affected offspring. In affected individuals; full mutation (FM), the CGG repeats are massively expanded to over 200 repeats[2]. Other mutations, such as deletions or point mutations, of the *FMR1* gene have rarely been reported. Individuals with repeat number in normal and PM range, the *FMR1* gene is unmethylated. However, FM individuals almost always have expanded repeats with methylation of the promoter of *FMR1* gene resulting in lack of fragile X mental retardation protein (FMRP)[3,4]. Rarely, individuals with unmethylated FM and near normal FMRP level have been reported as having normal intelligence[5]. Thus, methylation of the *FMR1* promoter rather than the expanded repeat impairs gene function and influences the severity of the phenotype.

Standard methods for diagnosis of FXS have been developed. For instance, the Polymerase Chain Reaction (PCR) analysis is applied mainly to determine the length of the triplet repeats[6,7]. Although, it is highly sensitive for allele sizes in the normal and low PM ranges, it may fail to detect the allele sizes in the upper PM range and FM due to the high GC content of the repeat region. Individuals with PM/FM mosaicism reveal PCR results in the normal or premutation range, leading to a false negative diagnosis. Southern blot analysis is a definite diagnosis used for all suspicious cases. This procedure, genomic DNA is digested by using a pair of restriction enzymes, methylation sensitive and methylation insensitive, before hybridizeation with a specific probe[8]. The advantage of Southern blot analysis is that methylation status is obtained as well as the approximate repeats; however, the main

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disadvantages of this technique are that it is time consuming, requiring a large quantity of DNA and low sensitivity for distinguishing a low ratio of mosaicism. Other techniques, such as protein expression detection and mRNA level analysis are also available[9,10], but they are not appropriate for FXS screening.

In the past few years, methylation specific PCR (MS-PCR) has been developed for diagnosis of methylation-related diseases including Prader Willi syndrome, Angelman syndrome, FXS and some types of cancer. The advantages are requiring a small amount of DNA, inexpensive and less time consuming[11-14]. It is based on bisulphite modification of DNA, wherein unmethylated cytosine residues are converted to uracil, while methylated residues remain unconverted[15,16]. Subsequently, the sequence of *FMR1* promotor of FXS cases (methylated) and unaffected individuals (unmethylated) after bisulphite treatment can be distinguished by PCR. The authors tried this method in FXS genetic testing at Songklanagarind Hospital, and found that it is useful and can be an alternative method for FXS screening.

Material and Method

DNA samples

Peripheral blood was collected from pediatric patients who were referred to Songklanagarind Hospital due to mental retardation or developmental delay. History taking and physical examination were performed by pediatricians, following the FXS checklist[17]. For prenatal diagnosis, DNA was extracted from the amniotic fluid cell culture and fetal cord blood after birth. The DNA was extracted using a standard phenol-chloroform method and was divided for 2 assays; MS-PCR and PCR for CGG repeats. The PCR reactions were performed in parallel.

Sodium bisulphite treatment

Two g of DNA in a volume of 50 l was denatured by adding 5.5 l of 3 M NaOH and boiling at 98 C for 6 minutes. Thirty microlitre of 10 mM freshly prepared hydroquinone (Sigma) and 520 l of 3 M freshly prepared sodium bisulphite (Sigma) were added and incubated at 55 C under mineral oil layer, for 5 hours. The mixture was purified using the Wizard DNA purification resin (Promega) following the manufacturer's protocols. The modified DNA was desulphonated by adding 5.5 l of 3 M NaOH and incubated at room temperature for 10 minutes. The modified DNA was precipitated and then resuspended in 25 l TE (10 mM TRIS, 1 mM EDTA, pH 8.0) buffer. This modified DNA was used as a template for PCR or long term storage at -70 C.

Methylation specific PCR

Amplification was carried out in 25 l volumes comprising of 2 l of modified DNA, 1X buffer II (Immolase, Bioline), 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.24 M each, of PUF, PUR, PMF, and PMR primers, 0.06 M each, of XUF and XUR, 0.32 M each, of XMF and XMR, 1 unit Hot start Taq DNA polymerase (Immolase, Bioline). This PCR was simultaneously amplified multiple sequences in a single reaction: *FMR1* and *XIST* (X-Inactive Specific Transcript) genes. Primer sequences were according to a previous report by Weinh usel et al[14]. PCR reactions were performed in a thermal cycler (MJ research PTC 200 or Perkins Elmer 2400) at 95 C (10 min) for initial DNA denaturation, followed by 38 cycles of 95 C (30 sec), 62 C (30 sec) and 72 C (1 min), with a final extension at 72 C for 10 min. The PCR products were separated on 2.5% agarose gels, stained with ethidium bromide and visualized under a UV transilluminator. The PCR for CGG repeats and Southern blot analysis were performed, according to a previous report[17]. Data were analyzed for comparison of MS-PCR with standard methods for FXS diagnosis.

Results

First, the authors optimized for the best condition of MS-PCR in known 18 FM, 2 PM and 15 normal cases. In addition, a known prenatal FM case was retrospectively tested. The methylation patterns were correctly identified in all samples. Fig. 1 shows MS-PCR product patterns. The authors used MS-PCR to evaluate 60 prospective males of unknown FXS status. The results showed 7 FM males and 53 normal males. All of these MS-PCR results corresponded to the results from PCR for CGG repeats and/or Southern blot analysis. In addition, a prenatal case was prospectively tested and showed a normal male pattern corresponding to the result from the standard methods.

Discussion

Methylation of DNA plays an important role in the epigenetic regulation of gene expression. In human being, methylation is almost exclusively in a cytosine preceding a guanosine (CpG) in the DNA sequence. CpG is almost always found in the promoter of the genes and normally unmethylated (review in ref. 18). MS-PCR has been developed to rely on the

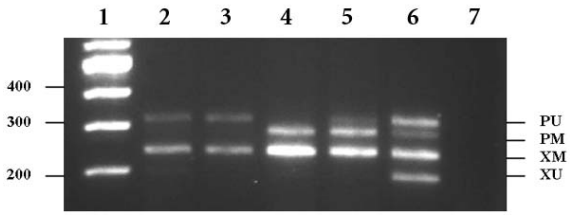

Fig. 1 Patterns of MS-PCR products. *FMR1* promoter unmethylated (PU); 318 bp, *FMR1* promoter methylated (PM); 288 bp, *XIST* promoter methylated (XM) 241 bp, and *XIST* promoter unmethylated (XU); 198 bp. Lane1 100 bp marker, Lane 2 normal male, Lane 3 premutation male, Lane 4 full mutation male, Lane 5 male with mosaic premutation and full mutation (presence of both PU and PM products), Lane 6 female, Lane 7 H_2O

differential modification of methylated and unmethylated genomic DNA by sodium bisulphite and subsequent PCR amplification. Because it is PCR-based, this method has a higher sensitivity than the Southern blot analysis, which makes it more useful for methylation detection in small amount of DNA samples such as for prenatal diagnosis and in case of mosaicisms (PM/FM or Normal/PM). However, the MS-PCR can not be used for females; because of random X-inactivation, this method is unable to distinguish PM or FM females from normal females who have the same pattern (Fig. 1). However, the majority of FXS cases are males, thus this limitation is not a major problem for FXS screening. The authors compared MS-PCR with standard methods in Table 1. A previous study by Weinh usel et al[14] suggested that diagnosis in females might be obtained through the relative ratios of the intensity of *FMR1* methylated (288 bp) and *FMR1* unmethylated PCR products (318 bp), however, in the authors' experience this was difficult because of random X inactivation in most females and skewed X inactivation in some females. In addition, the intensity of PCR product in MS-PCR is not quantitative since the final PCR product is from amplification as an exponential curve.

MS-PCR does not identify the number of CGG repeats because the PCR does not amplified across this region. Therefore, it can not distinguish normal males from PM males. The number of CGG repeats is essential for genetic counseling in PM females, because knowing this information can allow a physician to discuss the risk of having an affected offspring. Zhou et al have developed a simple MS-PCR method for diagnosis in both males and females that simultaneously estimates the number of CGG repeats[19], which may provide a new method of screening in females. However, Southern blot analysis is still a gold standard method.

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Table 1. Comparison of MS-PCR with standard methods for FXS diagnosis

Comparison items	MS-PCR	PCR for CGG	Southern blot
Amount of DNA (g)	0.5-2	0.1-0.5	8-10
Time required (days)	2	2	5
Cost	Inexpensive	Inexpensive	Expensive
Repeat size	No	Yes (normal, PM*)	Yes (FM*)
		No (FM)	No (normal, PM)
Methylation status	Yes (only male)	No	Yes (male, female)

PM: Premutation, FM: full mutation
* Approximate size

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การใช้เทคนิค multiplex methylation specific PCR ในการตรวจวินิจฉัยกลุ่มอาการ โครโมโซมเอกซ์เปราะ: ประสบการณ์ของโรงพยาบาลสงขลานครินทร์

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Methylation specific PCR (MS-PCR) เป็นเทคนิคที่ใช้ในการตรวจภาวะเมทิลเลชั่น (methylation) ของจีนโดยไม่ต้องใช้เอนไซม์ตัดจำเพาะ แต่ใช้การเปลี่ยนแปลงลักษณะโครงสร้างของนิวคลิโอไทด์โดยโซเดียมไบซัลไฟต์แล้วใช้ไพรเมอร์ที่จำเพาะต่อลำดับนิวคลิโอไทด์เพื่อเพิ่มจำนวนด้วยปฏิกิริยาพีซีอาร์ เทคนิคนี้ใช้ในการตรวจวินิจฉัยโรคที่เกี่ยวข้องกับเมทิลเลชั่นหลาย ๆ โรค เช่น กลุ่มอาการโครโมโซมเอกซ์เปราะ (Fragile X syndrome) ซึ่งเป็นโรคปัญญาอ่อนแบบเอกซลิงค์ที่พบบ่อยที่สุด โรคนี้เกิดจากการเพิ่มจำนวนซ้ำของ CGG เกิน 200 ซ้ำ เมื่อใช้เทคนิคนี้ในการตรวจแบบย้อนหลังในคนปรกติ 15 ราย คนที่เป็นพาหะ 2 ราย และคนที่เป็นโรค 18 ราย และตรวจในผู้ป่วยปัญญาอ่อนเพศชายที่สงสัยกลุ่มอาการโครโมโซมเอกซ์เปราะ โดยที่ไม่ทราบผลการตรวจมาก่อน 60 ราย พบว่าเป็นกลุ่มอาการโครโมโซมเอกซ์เปราะ 7 ราย ผลที่ได้ทั้งหมดสอดคล้องกับผลการตรวจยืนยันโดยวิธีมาตรฐานเดิม นอกจากนี้ได้นำเทคนิคนี้มาใช้ในการตรวจวินิจฉัยก่อนคลอด 2 ราย ให้ผลเป็นที่น่าพอใจ ดังนั้นเทคนิค MS-PCR จึงเป็นวิธีที่ใช้ในการตรวจภาวะ เมทิลเลชั่นในผู้ป่วยกลุ่มอาการโครโมโซมเอกซ์เปราะได้อย่างแม่นยำ