2. Method of study

Materials

DNA Samples

DNA for MS-PCR optimization and control DNA used in standard methods were obtained from peripheral blood and amniotic fluid including DNA from known FXS patients and their relatives and DNA from patients with mental retardation or delayed development but negative for FXS tests.

In prospective study, DNA for MS-PCR and standard methods were obtained from peripheral blood of patients with mental retardation/ delayed development of unknown etiology. All peripheral blood samples were sent from Pediatric Clinic and Genetic Unit at Songklanagarind Hospital. The recruitment of patients was followed the clinical checklist, and inclusion/ exclusion criteria (see Appendix A). In addition, DNA from amniotic fluid that was sent for prenatal diagnosis in known FXS families was included.

The protocols in this study were approved by the Ethic Committee, Faculty of Medicine, Prince of Songkla University (SUB.EC 46/364-015)

Primers for PCR amplification

Table 1 shows the primers and sequences of primer for PCR amplification. The first set of primers was used for PCR across a CGG repeats region. The second to the fifth sets of primers were used for MS-PCR according to the method of Weinhäusel and Haas (2001). The sixth to the last sets of primers were used for MS-PCR according to the method of Zhoe and colleagues (2004). GenBank access number 29704 is the reference for the *FMR*1 gene sequence and U80460 for the *XIST* (*X-Inactive Specific Transcript*) gene sequence.

Table 1 Primers and their sequences for PCR amplification

Set	Primer name	Primer sequence
1	Primer 1	5’ GAC GGA GGC GCC GCT GCC AGG 3’
1	Primer 3	5’ GTG GGC TGC GGG CGC TCG AGG 3’
2	PMF	5’ GTT GCG GGT GTA AAT ATT GAA ATT ACG 3’
2	PMR	5’ ATT TAA TTT CCC ACG CCA CTA AAT ACA C 3’
3	PUF	5’ GTG TTT GAT TGA GGT TGA ATT TTT G 3’
3	PUR	5’ ATT TAA TTT CCC ACA CCA CTA AAT ACA C 3’
4	XMF	5’ AAT TAA AGT AGG TAT TCG CGG TTT CG 3’
4	XMR	5’ TTT TTC CTT AAC CCA TCG AAA TAT CG 3’
5	XUF	5’ AAA AGT GGT TGT TAT TTT AGA TTT GTT 3’
5	XUR	5’ CTA CCT CCC AAT ACA ACA ATC ACA C 3’
6	Met-F	5’-CCG CCT CTA AAC GAA CGA CGA ACC GAC G-3’
6	Met-R	5’-GGT TGC GGG CGT TCG AGG TTT AGT CGT C-3’
7	Non-met F	5’-CCG CCT CTA AAC GAA CGA CGA ACC GAC G-3’
7	Non-met R	5’-GGT TGC GGG CGT TCG AGG TTT AGT CGT C-3’
8	mTP-F	5’-GCC GCT ACC AAA AAA CGT ACG ACA ACG CG-3’
8	mTP-R	5’-TAC CGA TAC GCA TCC CAG TTT GTC AGC (TCG)7 TCG-3’
8	Tail-R	5’- TAC CGA TAC GCA TCC CAG TTT GTC AGC-3’

Chemicals and enzymes

PCR for CGG repeats

D1S80 allelic ladder with probe

Taq DNA Polymerase (5 u/µl, Invitrogen)

Chemiluminescent detection kit (Orchid Diagnostics)

- Quick light hybridization solution (Cat No. 120661)

- (CGC)n oligonucleotide/ alkaline phosphatase coupled probe DNA (Cat No. 140006)

- Lumiphos-480 Dioxetane (Cat No. 120171)

- Quick light wash component A and B (Cat No. 100040, Cat No. 100050)

-10X Quick light buffer (Cat No. 100030)

Southern blot analysis

Eco RI (New England Biolab)

Eag I (New England Biolab)

2-log DNA ladder (New England Biolab)

Hybridization buffer (Amersham Pharmacia Biotech)

Gene Images AlkPhos Direct labeling and detection system (Amersham Pharmacia Biotech; RPN 3680)

- Labeling reagent

- Cross linker solution

- Reaction buffer

- Blocking reagent

Chemiluminescent detection kit (Orchid Diagnostics)

Methylation specific PCR

Hydroquinone (Sigma)

Sodium bisulphite (Sigma)

DNA wizard clean up kit (Promega A7280)

Hot start Taq DNA Polymerase (5 u/µl Immolase, Bioline)

Hot start Taq DNA Polymerase (5 u/µl, Qiagen)

Equipments

PCR Thermal cycler (Perkin Elmer 480)

PCR Thermal cycler (MJ research PTC 200)

Semidry electroblotting (Biorad)

Power supply (Biorad)

Hybridization oven and bottles (Robin Scientific)

X-Ray film 8 x 10 inches and X-Ray cassette

Vertical gel electrophresis (Protean II Xi cell, 20 x 20 cm or 16 x 20 cm, Biorad)

Transverse gel electrophoresis (C.B.S. Scientific Co)

Positive charge nylon membrane (Hybond-N)

Methods

2.1 DNA extraction

Genomic DNA was extracted from peripheral blood, collected in EDTA anti-coagulant. Peripheral blood was washed with TE buffer to collect the white blood cell pellet. The collected cell pellet was lysed with solution A and 10% SDS, and then digested with Proteinase K at 37ºC overnight. The DNA was extracted using a combination of phenol and chloroform and precipitated with cold absolute ethanol. Finally, the DNA was resuspended in TE buffer and stored at 4ºC.

2.2 PCR for CGG repeats

This method, according to Brown and colleagues (1993) with some modification, was used for all patients to determine repeat sizes.

2.2.1 Primers design

Primers 1 and 3 reported by Brown and colleagues (1993), amplified across CGG repeats (Fig. 2).

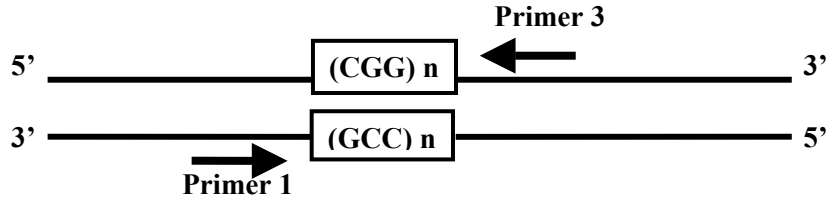

Fig. 2 Diagram showing an amplification site of PCR for CGG repeats

2.2.2 PCR amplification and detection

PCR was set up in 10 µl reaction volumes, with 50-100 µg of DNA, 1X buffer (Invitrogen), 0.75 mM $MgCl_2$, 1.25 µM each, of primer 1 and primer 3, 0.2 mM dNTP with substitute of dGTP by 7-deaza dGTP (Boehringer Mannheim), 10% DMSO (Sigma), 0.25 unit Taq DNA Polymerase (Invitrogen). PCR reaction was performed in a thermal cycler (Perkins Elmer 480) at 95°C (4 min) for initial DNA denaturation, followed by 30 cycles of 95°C (1 min), 65°C (1 min) and 72°C (2 min), with a final extension at 72°C for 10 min. The PCR products were separated on 6% denaturing polyacrylamide gel electrophoresis using 1X TBE buffer at 8 watts for 2.5 hours or until xylene cyanol FF dye was ran out of the gel. The PCR products were electroblot transferred to a nylon membrane at 400 mA for 45 min, followed by denatureing in 0.4 N NaOH, then neutralized in 2X SSC and baked at 80°C for 30-60 min. The membrane was hybridized with a (CGC)n probe at 56°C for 20 min after prehybridization with wash A for 10 min. The membrane was washed at 56°C with 30 ml of wash A for 20 min and 30 ml of wash B for 20 min. The results were visualized by chemiluminescent detection using 1X Quick Light buffer, Lumiphos-480 (Quick

Light genome mapping probe kit; Orchid Diagnostics) and autoradiography for 1.5-2 hours (see Appendix B).

2.3 Southern blot analysis

This method, following the method by Rousseau and colleagues (1991) with some modification, was used for all affected individuals and in case of suspect PCR results. The result showed both methylation status and approximate size of CGG repeats.

Eight to ten microgram of DNA was digested with restriction enzymes: *Eco*RI (methylation insensitive) and *Eag*I (methylation sensitive) at 37°C for 16-18 hours. The digested DNA was separated on 0.8% agarose gel electrophoresis at 30 volts for 17 hours. DNA was transferred to a nylon membrane using 500 ml of 0.4 N freshly prepared NaOH for 16-18 hours. The membrane was baked at 80°C for 30-60 min, then prehybridized with primary wash buffer for 45-60 min and hybridized with an StB12.3 probe (kindly supply by Prof. W. Ted Brown, NYS Institute for Basis Research in Developmental Disabilities, New York with official permission from Dr. Jeans Louise Mandel, Institut de Genetique et Biologie Moleculaire et Cellulaire, Strasbourg, France) for 16-18 hours. This probe was labeled using Gene Images AlkPhos direct labeling and detection system (Amersham Pharmacia Biotech; RPN 3680). The membrane was washed 2 times with a primary wash buffer at 56°C for 10 min and 2 times with a secondary wash buffer at room temperature for 5 min. The results were visualized using chemiluminescent detecting, 1X Quick Light buffer and Lumiphos-480 (Quick Light genome mapping probe kit; Orchid Diagnostics) and autoradiography for 1.5-2 hours (see Appendix B). Fig. 3 shows the recognition sites of the restriction enzymes, restriction fragment sizes and location of the StB12.3 probe on the *FMR*1 gene.

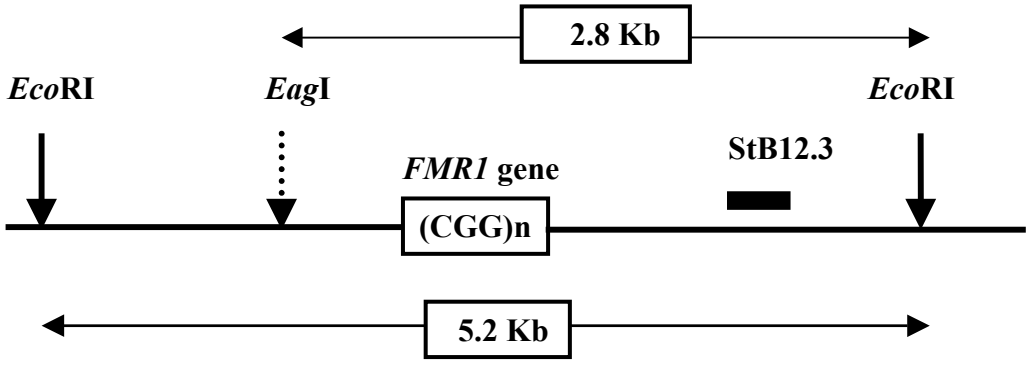

Fig. 3 Diagram showing recognition sites of restriction enzymes, restriction fragment sizes and location of StB12.3 probe on the *FMR*1 gene. Digestion with *Eco*RI produces a 5.2 Kb fragment. Digestion with *Eag*I produces a 2.8 Kb fragment. *Eag*I cuts unmethylated DNA but leaves methylated DNA uncut.

2.4 Methylation specific PCR

2.4.1 Sodium bisulfite modification

Two microgram of DNA in a volume of 50 µl was denatured by adding 5.5 µl of 3 M NaOH and boiling at 98°C for 6 min. Thirty microlitre of 10 mM freshly prepared hydroquinone (Sigma) and 520 µl of 3 M freshly prepared sodium bisulphite (Sigma) were added and incubated at 55°C under a 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 µl of 3 M NaOH and incubated at room temperature for 10 min. The modified DNA was precipitated and then resuspended in 25 µl of TE buffer. This modified DNA was used as a template for PCR or placed in long term storage at -70°C.

2.4.2 MS-PCR for male

2.4.2.1 Primers design

Two sets of primers, following the publication by Weinhäusel and Haas (2001) were used. The first set was comprised of 2 pairs of primers (PUF, PUR and PMF, PMR) that amplified the promoter region of the *FMR*1 gene on both unmethylated and methylated sequences (Fig. 4). The second set was comprised of 2 pairs of primers (XUF, XUR and XMF, XMR) that amplified the promoter region of the *XIST* gene on both unmethylated and methylated sequences. Also, the latter set served as an internal standard control.

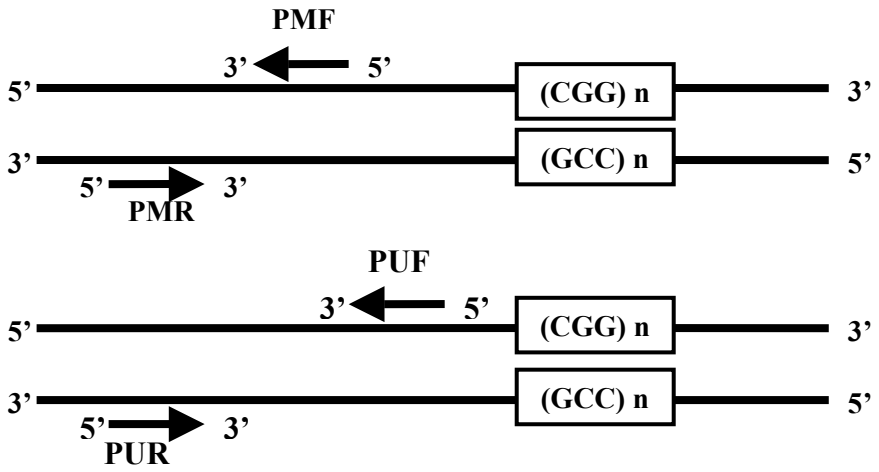

Fig. 4 Diagram showing an amplification site of unmethylated and methylated promoter regions of the *FMR*1 gene.

2.4.2.2 PCR amplification and detection

This PCR was simultaneously amplified through the *FMR1* and the *XIST* sequences in a single reaction. Amplification was carried out in 25 µl volumes comprising of 2 µl of the modified DNA, 1X buffer (Immolase, Bioline), 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.24 µM each, of PUF, PUR, PMF and PMR primers, 0.06 µM each of XUF and XUR, 0.32 µM each of XMF and XMR, 1 unit of Hot start Taq DNA Polymerase (Immolase, Bioline). PCR reactions were performed in a thermal

cycler (MJ research PTC 200) 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.

2.4.3 MS-PCR for female

2.4.3.1 Primers design

Three sets of primers, following the publication by Zhoe and colleagues (2004) were used. All primers amplified across the CGG repeats region. The first set of primers (Non-met F and Non-met R) was used to amplify an unmethylated sequence of the *FMR*1 gene. The second set (Met-F and Met-R) and the third set of primers (mTP-F and mTP-R with tail-R) were used to amplify a methylated sequence of the *FMR*1 gene (Fig. 5).

2.4.3.2 Non-met PCR amplification

Amplification was carried out in 15 µl volumes comprised of 2 µl of the modified DNA, 1X buffer containing 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTP, 0.2 µ M each of Non-met F and Non-met R primers, 0.5X Q solution (Qiagen) and 1.5 unit Hot start Taq DNA polymerase (Qiagen). PCR reactions were performed in a thermal cycler (MJ research PTC 200) at 95°C (15 min) for initial DNA denaturation, followed by 40 cycles of 98°C (1 min), 62°C (1 min) and 72°C (2 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.

2.4.3.3 Met-PCR amplification

Amplification was carried out in 15 µl volumes comprised of 2 µl of the

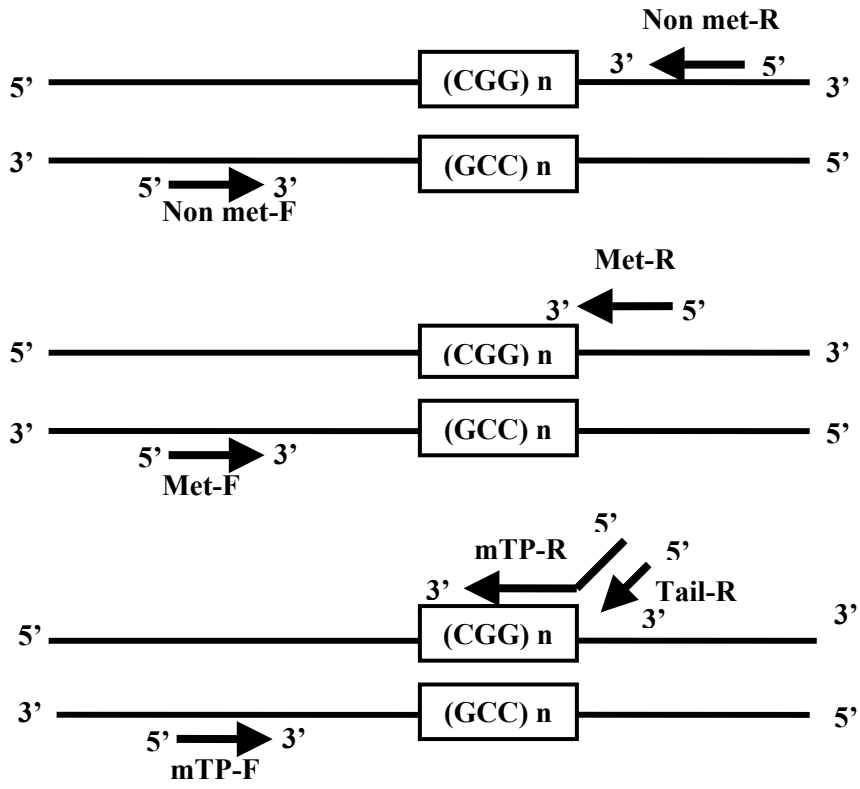

Fig. 5 Diagram showing an amplification site of unmethylated and methylated promoter regions of the *FMR*1 gene

modified DNA, 1X buffer containing 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTP, 0.2 μ M each of Met-F and Met-R primers, 1.5X Q solution (Qiagen) and 1.5 unit Hot start Taq DNA polymerase (Qiagen). PCR reactions were performed in a thermal cycler (MJ research PTC 200) at 95°C (15 min) for initial DNA denaturation, followed by 40 cycles of 98°C (1 min), 62°C (1 min) and 72°C (2 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.

2.4.3.4 mTP-PCR amplification

Amplification was carried out in 15 μl volumes comprised of 2 μl of modified DNA, 1X buffer containing 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM dNTP, 0.2 μM each of

mTP-F and Tail-R primers, 0.02 μM of mTP-R primer, 1.5X Q solution (Qiagen) and 1.5 unit Hot start Taq DNA polymerase (Qiagen). PCR reactions were performed in a thermal cycler (MJ research PTC 200) at 95°C (15 min) for initial DNA denaturation, followed by 40 cycles of 98°C (1 min), 62°C (1 min) and 72°C (2 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