Final Report

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การศึกษาระดับอณูของยีนในกลุ่มอาการโครโมโซมเอกซ์เปราะ (*FMR1*) ในคนไทยที่ถูกคัดเลือก

Molecular studies in Fragile X Syndrome gene (*FMR1*) among selected Thai subjects

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Abstract

Objectives:

1. To find a frequency of Fragile X Syndrome (FXS) in Thai children with developmental delay of unknown cause.

2. To find patterns and relationship of CGG repeat numbers, and haplotypes in selected normal Thai and FXS subjects.

Methodology:

1. We screened 293 boys and 69 girls with developmental delay of unknown cause (age $\leq$ 15 years). Four hundred and five non-FXS subjects were randomly selected as control normal DNA.

2. A six- item clinical checklist was used including family history (FH), long and narrow face (F), prominent and large ears (E), attention deficit/hyperactivity (AH), autistic-like behaviors (AT) and testicular volume (T). These were scored as 0 if absent, 1 if borderline, and 2 if present.

3. We used PCR and/or EcoRI/EagI double digestion and hybridized with StB12.3 probe, to screen the patients. The PCR was also used for typing microsatellites and single-nucleotide polymorphisms. The specific probes or restriction enzyme were applied depend upon types of microsatellties and single-nucleotide polymorphisms.

4. We used a logistic regression model from a computer program to analyze the clinical checklist data (Stata, version 5.0). Chi-square and t-tests were used to test significant difference between comparative groups at P =0.05 as a threshold.

Results:

1. We screened for FXS in 293 Thai boys with developmental delay (DD) of unknown cause. We found 21 (7.2%) to have FXS. We ascertained these 21 families and 2 previously known FXS families. We found 39 affected males, 7 affected females, 4 male carriers and 30 female carriers. Carriers had 60-125 CGG repeats while, affected individuals had > 200 CGG repeats.

2. We studied 179 unrelated non-FXS cases and 27 FXS cases from 18 families. We proposed a five-item clinical checklist for screening for FXS in DD Thai boys. We found that a five-item checklist, 2FH + F + 0.5E + 2AH + T = total score, was the best model. When we used this clinical checklist with a threshold of total score of 4, 78.7% (specificity) of the screened cases with total scores $\leq$ 4 could be eliminated as negative cases. In addition, all positive FXS cases had total scores > 4 (sensitivity = 100%).

3. To determine if FXS may have a specific haplotype association we analyzed 125 unrelated control subjects and 25 unrelated FXS patients. We used three markers and two single nucleotide polymorphisms. We found 54 and 14 haplotypes in control and FXS subjects, respectively. No significant of a specific haplotype association in either the controls or FXS group.

Discussion and Conclusions

We found approximately 7% of FXS in DD Thai boys. This is somewhat high compared to previous reports on other populations. This may due to our use of more strict screening criteria. We propose a cost-effective five-item clinical checklist for FXS screening in male pediatric population with developmental delay of unknown cause, particularly from Asian population settings. In addition, we found no specific haplotype association in either normal control nor FXS groups suggesting no founder effect. This result contrast with most other reports on FXS founder effects in different ethnic groups. Our studies might support the thought that FXS is common enough to screen in Thai DD patients. In addition, our experience will lead to the prevention of this disease using molecular testing for prenatal diagnosis.

Suggestions/further implication/implementation

1. Screened cases with normal *FMR1* gene need to be further investigated to find a possible cause of developmental delay, particularly a case with high checklist score or a case with DD family history. The FRAXE may be one of the etiology. Otherwise, screening the whole *FMR1* gene may need to be done to test whether this candidate case has another mutation.
2. The analysis of AGG interruption is an interesting project. This is to predict a risk of having an affected child and CGG repeat instability mechanism.
3. Protein expression is a good predictor for genotype-phenotype correlation, particularly in prenatal cases. However, we need to find a simple and liable protein expression method.
4. Our studies provide a cost effective clinical checklist for FXS screening. In addition, we show modified DNA testing techniques in FXS. These methods can apply for clinical service in prenatal and postnatal cases.

Keywords clinical checklist, fragile X, *FMR1* gene, haplotype, founder effect

บทคัดย่อ

วัตถุประสงค์

1. เพื่อหาความถี่และแบบประเมินลักษณะทางคลินิกของผู้ป่วยกลุ่มอาการโครโมโซมเอ็กซ์เปราะในผู้ปัญญาอ่อนและพัฒนาการช้าโดยไม่ทราบสาเหตุในเด็กไทย

2. เพื่อหาลักษณะและความสัมพันธ์ของจำนวนซ้ำของ CGG และ Haplotype ในผู้ป่วยกลุ่มอาการโครโมโซมเอกซ์เปราะและคนไทยที่มีจำนวน CGG ปรกติ

ระเบียบวิธีวิจัย

1. ผู้ป่วยเด็กชายไทย 293 คน ผู้ป่วยเด็กหญิง 69 คน ผู้ป่วยทุกคนอายุน้อยกว่าหรือเท่ากับ 15 ปี จากโรงพยาบาลสงขลานครินทร์ โรงพยาบาลรามาธิบดี และ โรงพยาบาลราชานุกูล ผู้วิจัยเลือกแบบสุ่มในคนที่ไม่ใช่กลุ่มอาการโครโมโซมเอกซ์เปราะจำนวน 405 คน เพื่อใช้เป็นกลุ่มคนที่ปกติ

2. ใช้แบบประเมินลักษณะทางคลินิก 6 ข้อ คือ ประวัติครอบครัว (FH), ใบหน้ายาวแคบ (F),หูกางและ/หรือใหญ่ (E), สมาธิสั้นและ/ หรือซนอยู่ไม่นิ่ง (AH), พฤติกรรมแบบออทิสติก (AT) และขนาดของอัณฑะ (T) การให้คะแนนมี 3 ระดับคือ 0 คะแนนเมื่อไม่พบ, 1 คะแนนเมื่อไม่ชัดเจนหรือพบลักษณะแบบใดแบบหนึ่งในข้อประเมินนั้น และ 2 คะแนนเมื่อชัดเจน

3. ตรวจดีเอ็นเอของยีนในกลุ่มอาการโครโมโซมเอกซ์เปราะ (*FMR1*) โดยการทำพีซีอาร์ และ/หรือการตัดด้วยเอนไซม์ตัดจำเพาะ EcoRI/EagI แล้วตรวจจับด้วยโพรบ StB12.3 ส่วนไมโครแซทเทลไลต์และความแปรผันของนิวคลิโอไทด์เดียวใช้การทำพีซีอาร์ แล้วนำไปตรวจจับด้วยโพรบจำเพาะหรือตัดด้วยเอนไซม์ตัดจำเพาะ

4. วิเคราะห์ผลการศึกษาจากข้อมูลผู้ป่วยใช้ Logistic regression จากโปรแกรมคอมพิวเตอร์ Stata version 5.0 และทดสอบค่าความแตกต่างด้วยไคว์สแควร์ หรือ t-test

ผลการศึกษา

1. การศึกษาในกลุ่มเด็กชายไทยที่มีพัฒนาการช้าโดยไม่ทราบสาเหตุจำนวน 293 รายโดยการตรวจดีเอ็นเอด้วยวิธี PCR และ Southern blot พบ FXS 21 ราย คิดเป็นร้อยละ 7.2 เมื่อศึกษาต่อไปในครอบครัวทั้ง 21 รายนี้และอีก 2 ครอบครัวที่เคยตรวจพบแล้วว่าเป็น FXS พบผู้ป่วยชาย 39 ราย ผู้ป่วยหญิง 7 ราย ผู้ชายที่เป็นพาหะ 4 ราย และผู้หญิงที่เป็นพาหะ 30 ราย ผู้ที่เป็นพาหะมีจำนวนซ้ำของ CGG 60-125 ซ้ำ แต่ผู้ป่วยมีจำนวนซ้ำของ CGG มากกว่า 200 ซ้ำ

2. ผู้วิจัยศึกษาเด็กชายที่มีพัฒนาการช้าโดยไม่ทราบสาเหตุที่ไม่ใช่กลุ่มอาการโครโมโซมเอกซ์เปราะจำนวน 179 คนและผู้ป่วยกลุ่มอาการโครโมโซมเอกซ์เปราะจำนวน 27 คนจาก 18 ครอบครัว ผลการวิเคราะห์ได้แบบประมินที่สำคัญ 5 ข้อคือ 2FH + F + 0.5E + 2AH + T = total score เป็นโมเดลที่ดีที่สุด ค่าของคะแนนรวมที่เท่ากับ 4 เป็นจุดวิกฤตของการตัดสินใจว่าจะส่งตรวจดีเอ็นเอในกลุ่มอาการโครโมโซมเอกซ์เปราะหรือไม่ ในกลุ่มที่ไม่ใช่กลุ่มอาการโครโมโซมเอกซ์เปราะมีคะแนนน้อยกว่าหรือเท่ากับ 4 คือมีความจำเพาะ (specificity) ร้อยละ 78.7 แต่กลุ่มอาการโครโมโซมเอกซ์เปราะทุกรายมีคะแนนรวมมากกว่า 4 คือมีความไว (sensitivity) ร้อยละ 100

3. ศึกษาในกลุ่มคนปกติ 125 ราย และผู้ป่วย FXS 25 ราย ที่ไม่เป็นญาติกันจากประวัติครอบครัว การศึกษาใช้ marker 5 ตัวที่ใกล้กับยีน CGG-*FMR1* พบว่าไม่มีความแตกต่างของ haplotype ในกลุ่มทั้งสอง

วิจารณ์และสรุป

การศึกษากลุ่มอาการโครโมโซมเอกซ์เปราะนี้แสดงให้เห็นว่ามีโรคนี้ในเด็กชายไทยที่มีพัฒนาการช้าที่ไม่ทราบสาเหตุค่อนข้างสูงเมื่อเปรียบเทียบกับรายงานอื่นๆ อาจจะเป็นเพราะการคัดเลือกที่มีแบบประเมินอย่างชัดเจน ผู้รายงานเสนอว่าควรนำแบบประเมินลักษณะทางคลินิก 5 ข้อที่ศึกษานี้ไปใช้ในการตรวจกรองกลุ่มอาการโครโมโซมเอกซ์เปราะในผู้ป่วยเด็กชายโดยเฉพาะจากคนเอเชีย นอกจากนี้ผู้วิจัยยังพบว่าการถ่ายทอดยีนที่ผิดปกติในคนไทย ไม่มี haplotype ที่จำเพาะ บ่งชี้ว่าไม่น่าจะมีบรรพบุรุษร่วมกัน ซึ่งต่างกับการศึกษาส่วนใหญ่ในคนเชื้อชาติอื่นๆ การศึกษาทั้งหมดนี้แสดงให้เห็นว่า FXS พบได้บ่อยพอที่จะมีการตรวจกรองในผู้ป่วยเด็กไทยที่มีพัฒนาการช้า และสามารถป้องกันโรคได้โดยการตรวจวินิจฉัยก่อนคลอดโดยการตรวจดีเอ็นเอที่ยีนโดยตรงหรือการตรวจ haplotype

คำแนะนำ/ การศึกษาเพิ่มเติม/การนำไปใช้ประโยชน์

1. ควรมีการศึกษาผู้ป่วยในรายที่ไม่ใช่กลุ่มอาการโครโมโซมเอกซ์เปราะต่อไปว่าเกิดจากสาเหตุอะไร โดยเฉพาะรายที่มีคะแนนรวมจากแบบประเมินลักษณะทางคลินิกสูง หรือมีประวัติครอบครัว โดยการตรวจกรองกลุ่มอาการโครโมโซมเอกซ์เปราะตำแหน่ง E หรือ ตรวจกรองหาความผิดปกติของยีน *FMR1* ทั้งหมดเพื่อหาความผิดปกติแบบอื่น
2. การศึกษาหาลักษณะ AGG interruption ในส่วนลำดับการซ้ำของ CGG เพื่อนำมาใช้ประโยชน์ในการให้คำแนะนำปรึกษาทางพันธุกรรมเกี่ยวกับความเสี่ยงของการมีลูกเป็นโรคมากหรือน้อยและกลไกการเกิดความไม่เสถียรในการซ้ำของ CGG
3. การพัฒนาการตรวจการแสดงผลของโปรตีน (protein expression) ให้ทำได้ง่ายและเชื่อถือได้ เพื่อนำมาใช้ทำนายลักษณะทางคลินิก โดยเฉพาะการตรวจก่อนคลอด
4. การศึกษานี้ได้พัฒนาแบบประเมินทางคลินิกและเทคนิคการตรวจดีเอ็นเอในกลุ่มอาการโครโมโซมเอกซ์เปราะให้เหมาะสมกับคนไทย โดยสามารถนำแบบประมินไปใช้ตรวจกรองผู้ป่วยก่อนที่จะมีการส่งตรวจดีเอ็นเอเพื่อลดค่าใช้จ่าย และนำเทคนิคการตรวจดีเอ็นเอไปใช้ตรวจวินิจฉัยทั้งก่อนและหลังคลอดเพื่อป้องกันการเกิดโรคซ้ำในครอบครัว

คำสำคัญ: แบบประเมินลักษณะทางคลินิก, โครโมโซมเอกซ์เปราะ, ภาวะปัญญาอ่อน, ยีน *FMR1* แฮปโลไทป์, บรรพบุรุษร่วม

Chapter I Review of literatures

Fragile X Syndrome (FXS) is the most common cause of inherited mental retardation. FXS was first described as a X-linked mental retardation by Martin and Bell in 1943. Therefore, the disease was previously known as Martin-Bell Syndrome. Lubs (1969) had shown that FXS was associated with the marker X chromosome with a gap at the nearby tip of the long arm. Its name derived from the cytogenetic fragile site observed at Xq. Sutherland (1977) described the method of expressive fragile X site using folate deficiency culture medium. Later, other methods were added such as excess deoxythymidine, metrotrexate addition and 5'fluoro-deoxyuridine (reviewed by Jacky et al., 1996). Based on molecular analysis, the incidence of disease in Europe and North America is about 1:3000-4000 in males and 1:6000-8000 in females (Turner et al., 1996; Morton et al., 1997, de Vries et al., 1997). Although this disease distribute worldwide, it is rarely reported in Asian populations. The typical clinical findings in an affected male include long and narrow face, prominent and large ears, mild to moderate mental retardation, enlarged testicles, hyperactivity, and autistic-like behaviors. An affected female has milder clinical features with no significant appearance. However, some cases exhibit problems in social adjustment and borderline mental retardation (Hagerman 1996). The syndrome is remarkable because of its unusual X-linked dominant pattern known as Sherman Paradox (Sherman 1984, 1985). Males with fragile X site have abnormal phenotype ~80% while females with fragile X site have abnormal phenotype ~35%. Risk of having an affected child depend on genotype of the parents. A daughter of male carrier has a lower risk than the daughter of a female carrier. The higher risk increases in successive generation (anticipation). Thus the number of individuals with FXS families increases with each generation.

The Fragile X Mental Retardation 1 gene, *FMR1*, was cloned in 1991 (Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991). Affected individuals with full mutation, have expanded CGG trinucleotide repeats (>200 copies) in the first exon of the *FMR1* gene. The normal chromosome contains 6-54 copies and is faithfully transmitted from parent to offsprings. Phenotypically normal carriers with 52-200 copies are called "premutation" (Fu et al., 1991). However, there is no well-defined CGG repeats between normal and premutation state. The term "borderline" is used to describe this region of repeat numbers (~40-60 repeats). The expansion of CGG repeats is accompanied by hypermethylation at the CpG island adjacent proximal to the gene, resulting in an absence of the FMR1 protein (Pieretti et al. 1991). FXS does not behave like mendelian pattern and is now known as a dynamic mutation that is

heritably unstable in premutation and full mutation. In addition, a small change of CGG repeats transmitting to offspring was observed in individuals within the borderline CGG repeats (Murray et al., 1996). Most female carriers transmit an increased size of CGG repeats to their offspring, therefore they are at a higher risk of having affected offsprings (Fu et al., 1991 Snow et al., 1993; Fisch et al. 1995; Nolin et al., 1996). In contrast, male carriers, known as normal transmitting males (NTM), always have phenotypically normal daughters. This might come from the observation that the full mutation male always carry premutation size of CGG repeats in sperm (Reyniers et al., 1993). Likewise, the studies of Malter et al., (1997) showed that the contraction of CGG repeat occur at 17 week gestational age in testes of the full mutation male fetus but not in the ovaries of the full mutation female fetus.

Fragile site of the FXS is located on the long arm of chromosome X (Xq27.3) designated as FRAXA locus. Previously, the diagnosis of FXS was based on cytogenetic expression of FRAXA in a proportion of cultures cells. However, there were a number of technical problems with the method. First, cytogenetics cannot reliably distinguish FRAXA from the other three neighboring fragile sites which are FRAXD (Xq27.2), FRAXE (Xq28), FRAXF (Xq28) (Sutherland and Baker 1990,1992; Hirst et al., 1993a). Secondly, there are differences in the proportion of affected cells regarded as diagnostic, although guidelines have been published recommending 4% as the lower limit (Jacky et al., 1991). In addition, it is time consuming and tedious work since it requires analysis at a minimum of 100 cells in a male and 150 cells in a female (normally 15-25 cells). A rapid and inexpensive method is to amplify the DNA using the Polymerase Chain Reaction (PCR). However, its ability to detect large premutation or full mutation is limited because of failure to amplify (Fu et al., 1991). The modified PCR reaction using 7-deaza GTP replacement instead of normal GTP and Dimethysulfoxide (DMSO) addition can improve sizes resolution of large premutation and some full mutation (Erster et al., 1992; Brown et al., 1993). The best protocol is to perform a PCR on all samples and, if there is a possible failure or ambiguous result, then carry out a Southern blot using EcoRI and EagI double digestion and hybridization with StB12.3 (Rousseau et al., 1991). Normal males demonstrate a single band (2.8 kb), whereas a second band is seen in females (5.2 kb) representing the methylated inactive X chromosome. A four banded pattern is produced by the female with a premutation because she will have the premutation and a normal X chromosome in both inactive and active states. A larger band or smear band (> 5.2 kb) is a methylated inactive chromosome in full mutation (reviewed by Murray et al., 1997).

Haplotypes studies using polymorphic markers near the CGG repeats have demonstrated linkage disequilibrium between the unstable expanded alleles and normal alleles

of adjacent markers in different populations (Richards et al., 1992, 1994; Oudet et al., 1993; Hirst et al., 1993b; Buyle et al., 1993; Zhong et al., 1996; Chiurazzi et al., 1996a, 1996b; Eichler et al., 1996; Syrrou et al., 1996). Furthermore, the haplotypes mostly found on fragile X chromosomes are associated with a few patterns. This has been interpreted as indicating that a few number of founder chromosomes are responsible for the majority of abnormal chromosomes in different populations. In addition, the associated haplotype might originate from upper normal range predisposing to large expansion according to Morton and Macpherson (1992). The CGG repeat in *FMR1* is occasionally interrupted by AGG trinucleotides (Kunst and Warren 1994; Hirst et al., 1994; Snow et al., 1994; Eichler et al., 1994, 1995 1996; Zhong et al.,1995). Most CGG repeat possess two interspersed AGGs at position 9-10 and 19-20 [$(CGG)_{9-10}AGG(CGG)_{19-20}(CGG)_n$]. (CGG)n is defined as a pure CGG repeat at 3' end of the CGG sequence. The majority of premutations were shown to have lost one or both of their AGG interruptions in contrast to normal stable alleles. In addition, a comparison of stable and unstable alleles of similar size in the general population (< 55 CGG repeats) showed that all unstable alleles had lost one or both of their AGG interruptions. Significant instability was revealed to initiate at a threshold of 35 pure CGG repeats (Eichler et al., 1994). This observation led to the suggestion that the loss of the AGG interruptions provide instability to the repeat. Zhong et al., (1995) showed that the chromosomes with > 14 pure CGG repeats were associated with the longest alleles of two nearby microsatellites, *FRAXAC1* and *DXS548*, and with increased microsatellites heterozygosity. They suggested that the association of long pure CGG regions with the longer and more heterozygous microsatellites may be related. Falik-Zaccai et al., (1997) reported an association between founder haplotype and loss of AGG interruption in the Tunisian Jews.

There has been a large number of documented FXS cases in several medical practices world-wide but none in Thailand before 1991. The first Thai case was reported using the cytogenetic method (Wasant et al.,1994). Eigel et al., (1995) investigated 644 unrelated individuals (213 males, 431 females) unselected for mental retardation or FXS from Chiang Mai province. Based on PCR and Southern blot analysis, only three borderline CGG repeats (52,54,54) were found out of 1075 chromosomes analyzed. Uraiwan et al.,(1996) studied on unknown cause of mental retardation or delayed developmental patients at Songklanagarind Hospital during 1991-1996 using cytogentic and molecular analysis (most cases were from the lower part of southern Thailand). They found 7 index cases from 260 cases (2.7%).

Chapter II : Molecular screening for Fragile X syndrome in Thailand

Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental retardation with a prevalence of approximately 1: 4,000 males in Caucasians based on molecular analysis (Turner et al 1996; Morton et al, 1997; de Vries et al, 1997). Although this disease is distributed worldwide, there are few reports in Asian populations. The syndrome is remarkable because of its unusual X-linked dominant pattern (Sherman 1984, 1985). The typical clinical characteristics in affected males include variable degrees of mental retardation, narrow and long face, large and prominent ears, enlarged testicles, hyperactivity, attention deficit, and autistic-like behaviors. An affected female has milder clinical features with no significant appearance. Some female patients exhibit problems in social adjustment and learning difficulties (Hagerman, 1996).

The Fragile X Mental Retardation 1 gene, *FMR1*, was cloned in 1991 (Oberle et al, 1992; Verkerk et al, 1991; Yu et al, 1991). The *FMR1* contains a polymorphic CGG repeat at the 5' untranslated region. Most patients, called full mutation, have expansion of the CGG repeats (>200 repeats) accompanied by methylation of the adjacent CpG island causing absence of the FMR1 protein. (Fu et al, 1991; Pieretti et al, 1991). The normal chromosome contains 5-55 repeats. Phenotypically normal carriers with repeat sizes of 56 to approximately 200 are called premutation which are at higher risk to give birth to affected children. The terms "borderline" or "grey zone" are used to describe individuals with 35-52 CGG repeats and have a low risk to transmit unstable CGG repeat to the next generation (Brown, 1996a).

The first Thai FXS case was reported by Wasant et al (1992). Later, Jinorose et al (1997) reported that the frequency of FXS at Songklanagarind Hospital was 2.7% in selected males and females based on the cytogenetic method and in part confirmed by molecular methods. The first molecular diagnosis for FXS in Thailand has been established at Songklanagarind Hospital since 1997. Our study was carried out to find a frequency of FXS and ascertained FXS families using molecular screening in Thailand. This study provides an estimated frequency of FXS. Also, it points the way toward the means of the prevention of mental retardation by genetic counseling and prenatal diagnosis.

Materials and Methods

Subjects

The 293 Thai males (age $\leq$ 15 years) were primarily collected from May 1991-December 1999 at Songklanagarind Hospital (southern Thailand) and from June 1997-December 1999 at Ramathibodi Hospital (central Thailand). Of 293 cases, 32 patients were referred from Nawabutra Women and Children Medical Center, Chulalongkorn and Rachanukul Hospitals. These three medical centers are in Bangkok designated as central Thailand. Most subjects had unknown causes of mental retardation and delayed development without specific syndrome nor abnormal karyotyping. The others were diagnosed as FXS and autism. Figure 2.1 showed the schematic screening. The 405 randomly selected males were used for studying the distribution of normal CGG repeat size.

Molecular methods

DNA was extracted from whole blood with the phenol/chloroform method. The modified non-radioactive PCR method (Primer 1 & 3) was used to amplify the CGG repeat region of *FMR1* gene (Brown et al, 1993). (Primer 1: 5'-GAC GGA GGC GCC GCT GCC AGG-3', Primer 3: 5'-GTG GGC TGC GGG CGC TCG AGG-3'). The PCR reaction is shown in Table 2.1. One third of PCR reation was mixed with stop buffer (formamide with 0.05% bromphenol blue and 0.05% xylene cyanol) and loaded in 6% polyacrylamide gel (16 X16 Cm plate) at constant 9 watt for 2 hours and 20 minutes. The PCR product was transferred to positive charged nylon membrane by using semi-dry electrobloting (Biorad). The nylon membrane was dried at 80 ^{o}C for 30 minutes. (CGC)n alkaline phosphatase labelling probe was used for hybridization. The probe was supplied from the Lifecode Company (Stnaford, CN, USA) including hybridization buffer and washing solution. The membrane was pre-hybridized with wash I in hybridization oven (Robbin Scientific) at 55^{o}C for 10 minutes. The membrane was hybridized with (CGC)n probe (1 μl probe in 10 ml hybridization buffer) at 55 ^{o}C for 20 minutes. The membrane was washed in wash I and wash II, respectively,at 55 ^{o}C for 20 mintues. The membrane was rinsed in 1X Quicklight buffer (Lifecode) 3 minutes twice. The membrane was placed on plastic bag and sprayed with Lumiphos 480 (Lifecode) or CDP^{Tm} star (Amersham). The plastic bag was sealed by electrical sealing mechine. The membrane was exposed to X-ray film at 37 ^{o}C for 1 hour. Repeat sizes were estimated with known size markers.

[Wash I (500 ml): 20 ml Wash component A + 25 ml Wash component B + water 455 ml Wash II (500 ml): 2 ml Wash component A + 25 ml Wash component B + water 473 ml]

EcoRI/EagI double digestion and southern blot analysis (Rousseau et al, 1991, Brown 1994) was tested in all affected FXS individuals and suspected PCR results. The DNA was digested with 100 Units EcoRI and 50 units EagI in the final concentration of 100 mM Tris HCl pH 8.0, 100 mM NaCl, 20 mM $MgCl_2$ and 0.001 M Dithiothreitol at 37 ^{o}C, for 14-16 hours. The digested DNA was run on 0.8% agarose gel in 1X TAE at 30 volts for 16 hours. Then transfer (southern blot) the DNA to positive charged nylon membrane using 500 ml 0.4 N NaOH overnight. The membrane was neutralized with excess 2X SSC 10 minutes. The membrane was dried at 80 ^{o}C for 30 minutes. The StB12.3 probe was labeled with fluorescein and detected using the Gene Images CDP-Star protocol from Amersham. The membrane was hybridized at 60 ^{o}C in hybridization oven for 14-16 hours and washed in 0.1% SDS and 0.5 X SSC at 65 ^{o}C, for 15 minutes twice. StB12.3 probe was supported from Dr. W Ted Brown with Dr. L. Mandel's permission. Figure 2.2 show the diagram of FMR1gene, primer 1 & 3, StB12.3 probe and restriction map.

Results

We found 21 FXS index cases from 293 screened boys. Therefore, the frequency of FXS was 7.2% in Thai boys with developmental delay of unknown cause. We divided the samples into two groups based on the location of collected samples. The frequency of FXS at Songklanagarind Hospital in southern Thailand was 6.8% (9/132). Similarly, the frequency of FXS in central Thailand was 7.5% (12/161).

We did DNA testing in members from 21 these screened FXS families and 2 additional known FXS families comprising of at least 39 affected males and 7 affected females. Of 7 affected females, 2 individuals were mothers. In addition, we found at least 30 premutation carrier females and 4 premutation males (non transmitting males). The expanded CGG repeats (>200 repeats) were found in all affected individuals. However, mosaic premutation/full mutation patterns were observed in 9 from 39 affected full mutation males (23.1%). The premutation individuals had CGG sizes ranging from 60 to about 125 repeats. Figure 2.3 and 2.4 show PCR result and southern blot in a FXS family. The normal range of CGG repeats was 19-50 repeats from 405 randomly selected normal males (26 alleles, heterozygosity of 68.6%). The two most common alleles were 29 (44.4%) and 30 CGG repeats (32.6%)

followed by 36 CGG repeats (9.4%). Most normal individuals had chromosome with less than 41 CGG repeats (98.3%). Figure 2.5 shows the CGG distribution in normal Thai subjects.

Discussion

Since the identification of the FMR1 mutation, two reliable molecular methods have replaced cytogenetic methods. These are polymerase chain reaction (PCR) and southern blot methods. We used these methods to screen FXS in our study. The study of Jinorose et al (1997) was the first report on FXS screening in Thai population. However, the study was done in a medical center and most cases was based on cytogenetic methods. Our study expanded a FXS screening project to a few medical centers in two major parts of Thailand. We had a FXS frequency of 7.2% in Thai boys with developmental delay of unknown cause. In comparison with a frequency of 3.5% FXS in males reported by Jinorose et al (1997), the higher frequency in our study might be due to our use of more strict screening criteria and possible false negative by their cytogenetic methods (Jinorose, personal communication). However, frequencies of FXS were not much different between central and southern Thailand (6.8% vs 7.5%). This suggest that a frequency of FXS is about 7% in Thai boys with developmental delay of unknown cause.

Some molecular FXS screening studied in Asian populations were previously reported from Japan (Hofstee et al, 1994), Singapore (Yoon et al, 1997), India (Baskaram et al, 1998), Chinese-Hong Kong (Pang et al, 1999) and Indonesia (Faradz et al,1999). We compared our study to these reports in Table 2.2. Frequencies of FXS in selected Asians varied from 0.4% to 7.1%. These findings was not surprising since we knew that FXS frequencies in selected Caucasians varied from 0.5% to 11.0 % depending on criteria used when screening cases (Perroni et al, 1994, Murray et al, 1996; Arvivo et al, 1997; de Vries et al, 1997; Gerard et al 1997; Marini et al, 1997; Mila et al, 1997; Mazzocco et al, 1997, 1998).

The normal distribution of CGG repeat in Thai population was similar to the reports in Chinese population with 29 and 30 CGG repeats as the two most common alleles followed by 36 CGG repeats (Zhong et al 1994; Chen et al, 1997). In Caucasians, 29 and 30 CGG repeats were also common alleles but 30 CGG repeats was the most common instead of 29 CGG repeats as seen in Thai and Chinese (Fu et al, 1993; Brown et al, 1996b; Kunst et al, 1996; Murray et al 1996). However, these findings were different from the Japanese which had 28 and 29 CGG repeats as common alleles (Arinami et al, 1993; Holsfee et al, 1994).

Normal chromosomes with 41-50 CGG repeats were very rare (1.7%) in our study. Likewise, Eigel et al (1995) found 3 chromosomes (52/54/54 CGG repeats) from 1,075 Thai chromosomes.

Although the FXS test has been strongly recommended for both males and females with unexplained mental retardation (Curray et al, 1997), this might not apply for many Asian countries with limited facilities. We screened for FXS in 69 unknown causes of mental retarded females, we did not find any positive case (data not shown). We suggest that an initial FXS screening should select male index cases. In addition, the clinical checklists should be considered to eliminate most negative cases. In our experience, we used a 5-item clinical checklist modified from the report of Giangreco et al, (1996). Using this checklist, we could diagnose all positive FXS cases and could eliminate 78.7 % of negative FXS cases. (Limprasert et al, chapter 3). Our study was an initial step to provide a prevention of mental retardation in Thailand by genetic counseling and prenatal diagnosis . In addition this finding might support the thought that FXS is common enough to screen in other Asian populations.

Table 2.1 The PCR reaction for amplifying CGG repeat tract at the *FMR1* gene

Reagent	**Stock**	**Vol (μl)**	**Final**
dd H_2O	-	2.4	-
Buffer II *	10X	1	1X
Magnesium	25 mM	0.3	0.75 mM
dNTPs**	2.5 mM	0.8	200 μM
Primer 1	10 pM	1.25	1.25 pM
Primer 3	10 pM	1.25	1.25 pM
DMSO	-	1	10%
Taq	5 unit/ml	0.05	0.25 unit
DNA	50-100 ng/μl	2	100-200 ng
Total	-	~10	-

Note: * 100 mM KCl, 100 mM Tris-Cl, pH 8.3

** replace dGTP with 7- deaza GTP (100%)

PCR cycles

Initial step: denaturation at 95 °C 4 min.

30 cycles 1 min at 95 °C (denaturation)

1 min at 65 °C (annealing)

2 min at 72 °C (elongation)

Final extension 72 °C 10 min

Table 2.2 Frequencies of FXS in Asian subjects using molecular screening

Authors	Populations	Subjects (males)	Frequencies (%)
Hofstee *et al* (1994)	Japanese	Selected MR	7/296 (2.3)
Yoon *et al* (1997)	Singaporean	Learning difficulties	8/339 (2.4)
Baskaran *et al* (1998)	Indian	MR	7/98 (7.1)
Pang et al (1999)	Chinese	MR	1/243 (0.4)*
Faradz et al (1999)	Indonesian	Special school	5/206 (2.4)
This study	Thai	DD of unknown cause	21/293 (7.2)

DD = Delayed development, MR = Mental retardation

* 2/324 = 0.6% in screened males and females

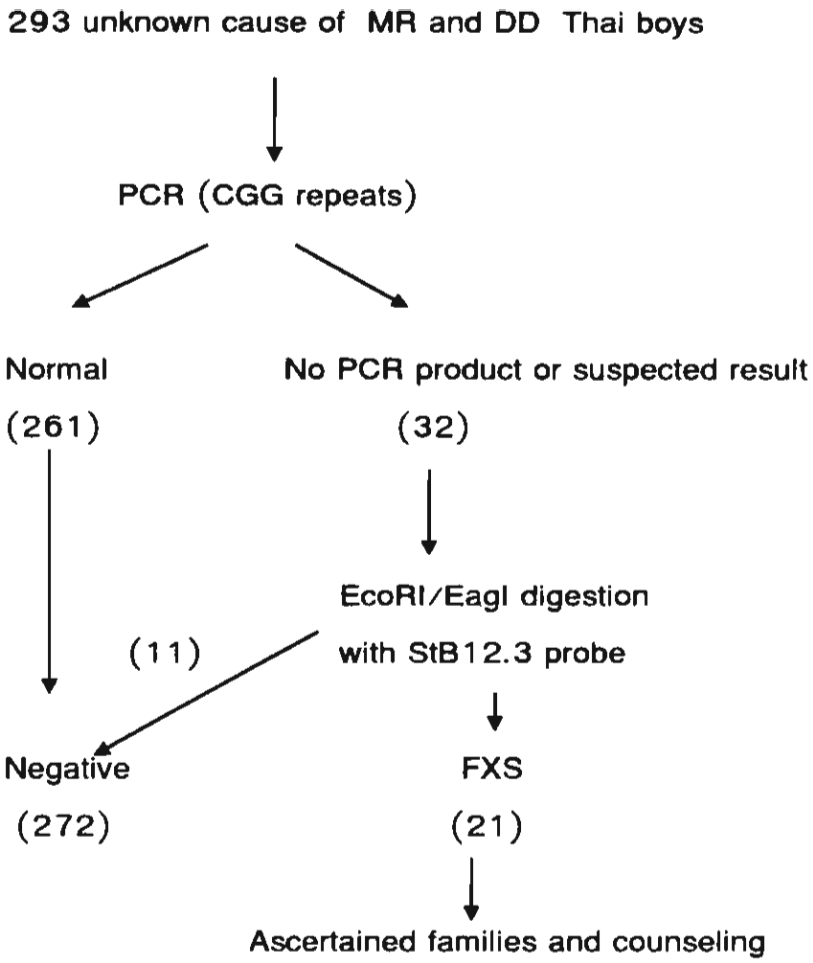


Figure 2.1 Schematic of FXS screening

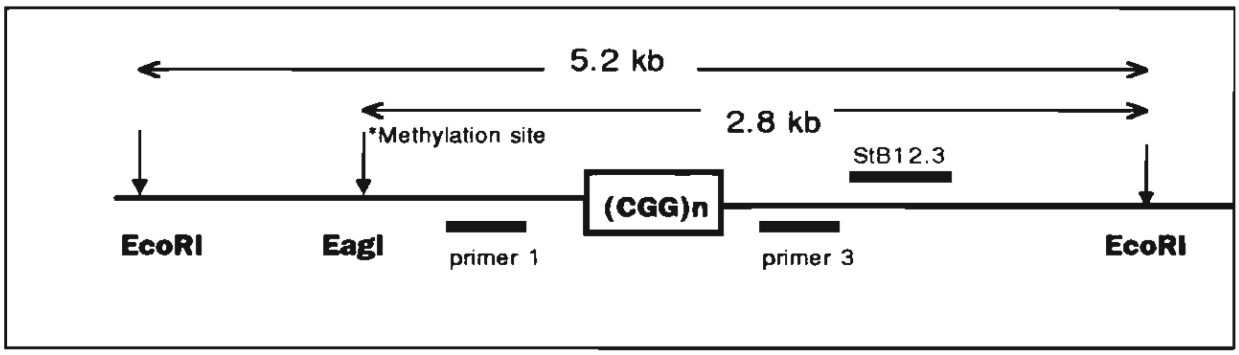


Figure 2.2 The FMR1 gene contains CGG repeats. The diagram shows primer 1 and 3 used for PCR. Three restriction sites, EcoRI and EagI, and StB12.3 probe were also shown. EagI is a methylation sensitive enzyme. Therefore, it will not cut this site when there is methylation. The southern blot patterns are: normal male (2.8 kb), affected male (> 5.2 kb), normal female (2.8 and 5.2 kb) and affected female (2.8 and > 5.2 kb).

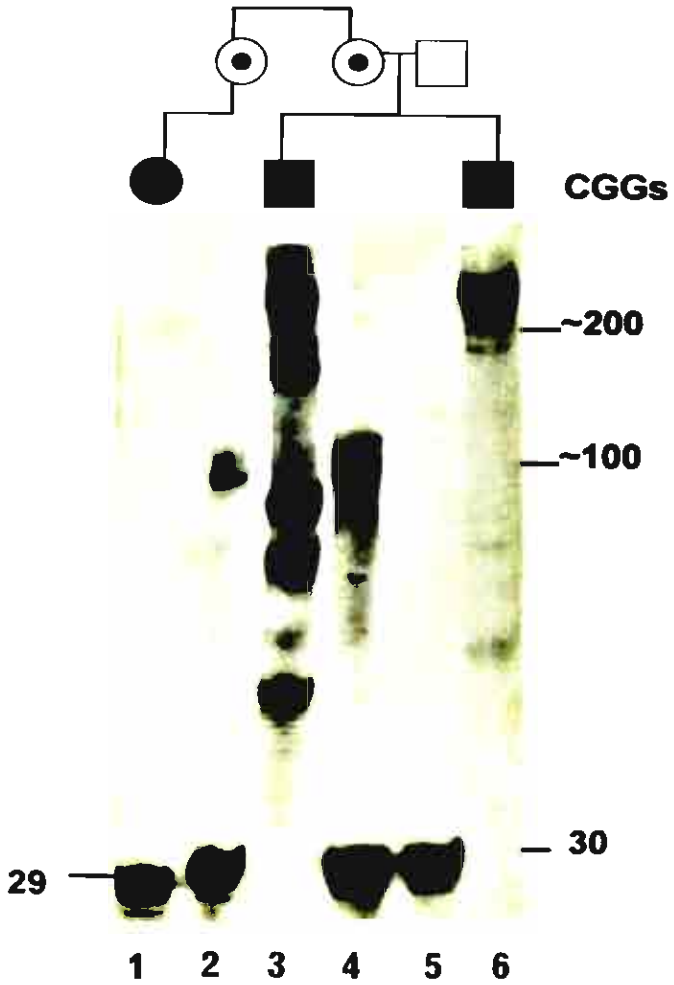
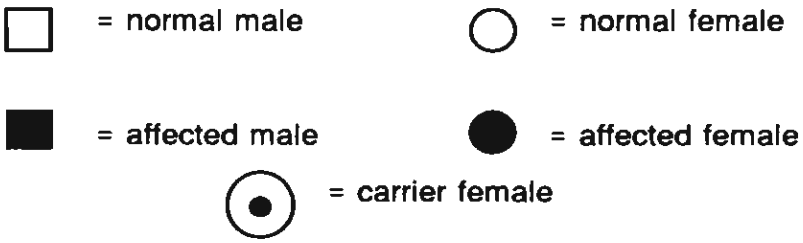


Figure 2.3 The PCR product is shown in a FXS family. The numbers of CGG repeat are indicated as 29, 30, ~100 and ~200. Lane 1 is an affected female (29, not amplified). Lane 2 and 4 are carrier females (30, ~100). Lane 3 is an affected male with different bands suggesting different sizes of CGG repeats or incomplete PCR amplification. Lane 5 is a normal male. Lane 6 is an affected male (> 200).



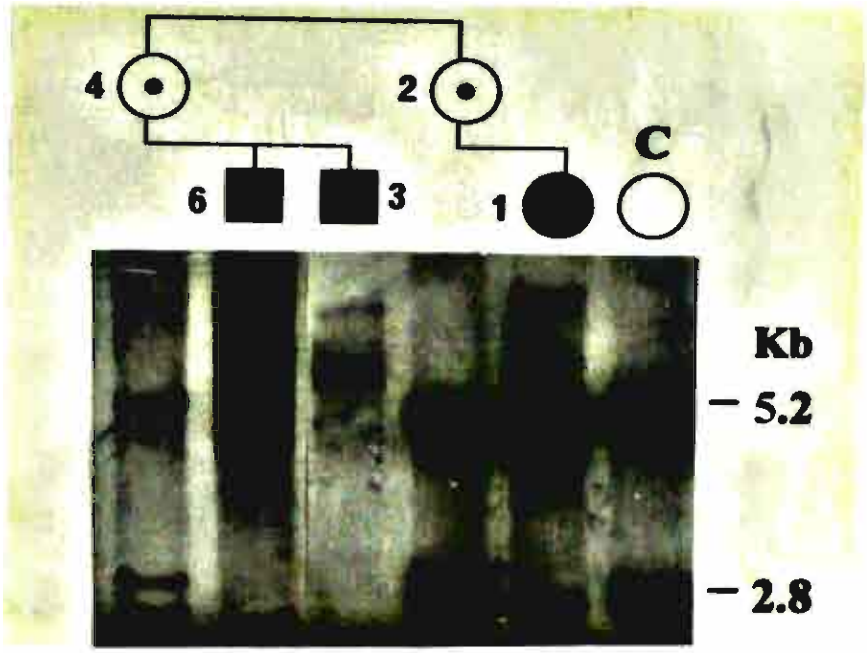


Figure 2.4 Southern blotting after double digestion with EcoRI and EagI restriction enzyme in the same family shown in Fig 2.3. The blot was probed with StB12.3. Individuals are marked with the numbers used to identify the lanes in Fig 2.3. C is a normal control female. Carrier females show different sizes of CGG repeats with random methylation (clearly seen in individual No.4 as 4 bands). All affected individuals show smear bands suggesting different sizes of CGG repeats. The instability of CGG repeats are commonly found in affected individuals.

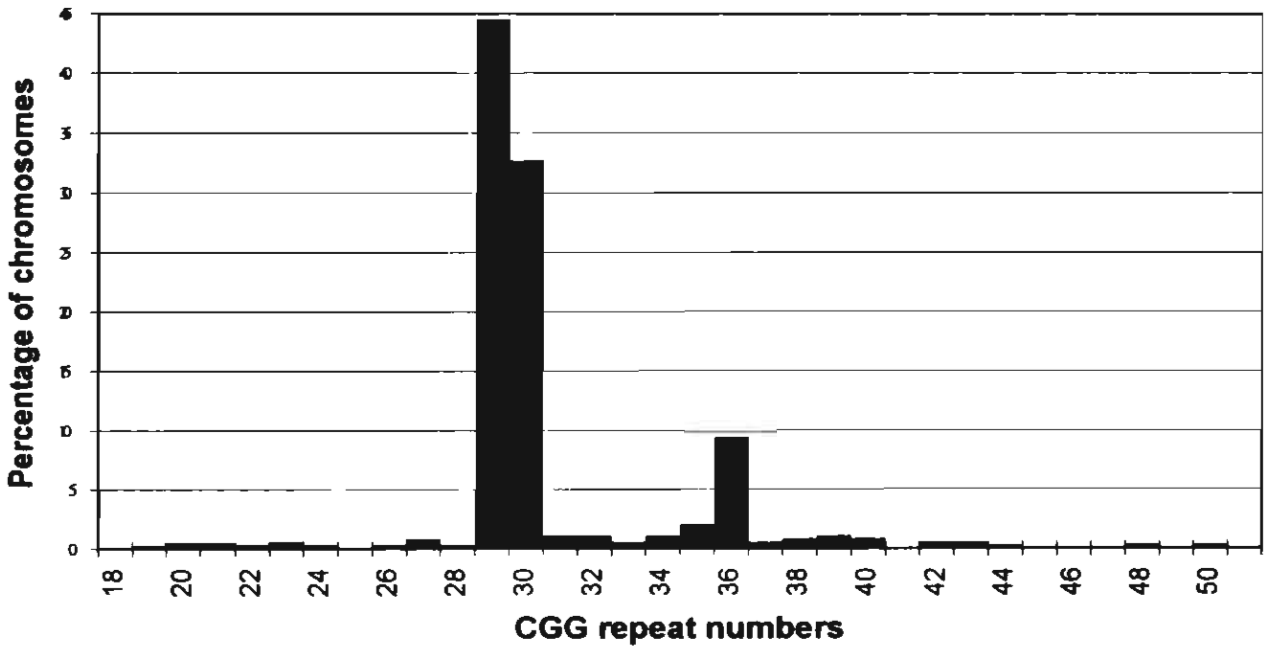


Figure 2.5 The distribution of the normal CGG at the *FMR1* gene in 405 selected Thais.

Chapter III A clinical checklist for fragile X syndrome screening

Introduction

In our experience, approximately 7% of samples referred for FXS testing, regardless of clinical status, showed positive results on molecular analysis (Limprasert, 1999). Variability of FXS clinical expression showed overlap with other disorders, but some clinical features were commonly found in FXS (Thake et al, 1985; Simko et al, 1989; Nolin et al, 1991). This has emphasized the importance of a clinical checklist for screening purposes. All clinical checklists for FXS so far have only been reported in Caucasian populations (Hagerman 1991; Laing et al, 1991; Bulter et al, 1991; Giangreco et al, 1996; Arvio et al, 1997; Hecimovic S, 1998). Prior to our studies, no standardized FXS clinical checklist for Asian populations has been reported.

We report a FXS clinical checklist in Thai boys with unknown etiology for developmental delay. We used logistic regression model and found that a five-item checklist was the most efficient. Our checklist is the first FXS screening model to assign different weights to each item.

Materials and methods

Subjects

Two hundred and eighty eight selected Thai patients, age $\leq$ 15 years, with developmental delay (DD) of unknown cause were studied. The patients do not have birth asphyxia, CNS infection, hypothyroidism, chromosomal abnormalities using routine karyotyping, deafness nor dysmorphic features of significant syndromes (i.e. Down syndrome). Routine karyotyping and thyroid hormone test will be conducted together with FXS screening if there are indications. The IQ levels will be estimated by methods of the WISC or Stanford Binet. If the patients can not be evaluated (i.e. age < 2 1/2 year, not co-operate), we will estimate from speech development. The patients attended two major medical centers in Songkhla and Bangkok which are located southern and central Thailand, respectively. The project was approved by the faculty ethics committee. The patients were divided into 2 groups. Group A consisted of 92 cases who were tested between June 1991- December 1996 by cytogenetic methods and were now re-tested by molecular methods. Group B consisted of 196 cases prospectively screened who were tested between January 1997- October 1999 using molecular methods.

Clinical checklist

We used a six-item clinical checklist modified from the report of Giangreco et al (1996) as shown in Table 1. These included family history (FH), long and narrow face (F), prominent and large ears (E), attention deficit/hyperactivity (AH), autistic like behaviors (AT), and testicular volume (T). These are the following criteria:

1. Family history included learning difficulties, developmental delay and mental retardation.

2. A narrow and long face was based on clinical impression of long jaw and high forehead.

3. Prominent ears were considered to be present when the angle of the ear and face was approximately 90 degrees. The longest axis of the ears were measured and compared to the standard scale using the 95 percentile as the threshold (Butler et al, 1992).

4. Attention deficit and hyperactivity was scored according to DSM- IV criteria (1994).

5. Autistic-like behaviors were scored as positive when one of the following behaviors was present: tactile defensiveness, hand flapping, hand biting (excluding nail biting), delayed or perseverative speech and poor eye contact (Hagerman, 1991).

6. Testicular volume was measured with an orchidometer as milliliters and compared to a modified standard scale (Butler et al, 1992).

Age group	Testicular volume	Score
≤ 8 years	1-2 ml	0
	3 ml	1
	> 3 ml	2
> 8 years	95 percentile - 2 ml	0
	95 percentile +/- 1 ml	1
	95 percentile + 2 ml	2

All DD patients were physically examined by pediatricians before the report of laboratory tests. In addition, the laboratory personnel did not see the checklist results. Due to some missing data (i.e. orphans or non-cooperative physical examinations) or unavailable information from retrospective cases, we used the subjects' data only when at least five items of the checklist were available.

DNA testing

PCR and/or EcoRI/EagI digestion with StB12.3 probe hybridization in the CGG repeat region of the *FMR1* gene were done in all patients as described in chapter I.

Statistics analysis

We used a logistic regression model to analyze the data. Logistic regression has been commonly used to describe the probability of developing some diseases over specified period as a function of certain risk factor (Kleinbaum et al, 1988). We adopted this model for our study since it was based on a similar concept. Likewise, we used six items of the clinical checklist as a predictor of a DD child being a FXS case. The logistic regression module of the Stata version 5.0 program (1997), logit command, was used for data analysis to determine the weight to assign to each item and to test the significance of each item in the model.

Results

We analyzed 206 cases, from total 288 tested cases (~72%), that had completed at least 5 items of the clinical checklist. Of 65 cases from group A, 52 cases were unrelated non-FXS cases and 13 cases were FXS cases from 7 families. Of 141 cases from group B, 127 cases were unrelated non-FXS cases and 14 cases were FXS cases from 11 families. Figure 3.1 shows schematic of the cases studied. There were 28 missing item-records described as following: 2 age, 1 FH and 23 T in the non-FXS group, 1 FH and 1 T in the FXS group. The mean age of the FXS positive group was 7.9 years (N = 27, range 8 months to 13.6 years). The mean age of the non-FXS group was 6.5 years (N = 177, range 8 months to 14.8 years). There was no statistical difference between the ages of the FXS and non-FXS groups (t-test, equal variance, P = 0.07). We used logit command with cluster analysis in the FXS group to reduce bias from related FXS cases (average scores from the same families were analyzed by the Stata program). We tested each item as an univariate. We found that AT was not statistically significant (P = 0.5). We analyzed the remaining five items using logit command with cluster and found coefficient as 1.59FH, 0.93F, 0.69E, 1.41AH and 1.20T. We first used a simulated model without weight, FH + F + E + AH + T= total score. We found that with a threshold total score of 2, 68.4% (specificity) of the non-FXS cases could be eliminated (total score $\leq$ 2). In addition all FXS cases would have been detected (sensitivity = 100%, total score > 2). When we used the coefficient as a multiplicative weight in the model, the specificity was improved. All five-item checklist models had 100% of sensitivity at different threshold scores. The model, 2FH + F + 0.5E + 2AH + T = total score, showed the

best specificity (78.7%) with a threshold score of 4 from a total score of 13. Figure 3.2 shows a graph comparing non-FXS and FXS groups using the best weight model. We observed that the unavailable data was usually the testicular volume. Therefore, we also tried to use two four-item models (FH + F + E + AH and 2FH + F + E + 2AH). The summary of the models is shown in Table 3.2.

Discussion

Although FXS testing has been recommended for both males and females with mental retardation of unknown etiology (Curry et al, 1997), this might not apply to many developing countries with limited facilities. Therefore, a clinical checklist for FXS screening is still applicable for many clinical settings. Table 3.3 is a comparison among clinical checklists studies for FXS. Our six-item checklist was a modification of the FXS checklist by Giangreco et al (1996). Three FXS clinical checklist reports were based on cytogenetic methods Hagerman, 1991; Laing et al, 1991; Butler et al, 1991). However, our study and the other three reports (Giangreco et al, 1996; Arvio et al, 1997; Hecimovic et al, 1998) were based on molecular methods. Molecular methods replaced cytogenetic methods since the identification of the *FMR1* mutation. We prospectively and retrospectively studied male cases with developmental delay or mental retardation of unknown cause, but the report of Giangreco et al (1996) and Hecimovic et al (1998) retrospectively studied both male and female cases with or without mental retardation. All reported clinical checklists, except for the report of Arvivo et al (1997), studied in pediatric subjects. We compared non-FXS and FXS males because only positive FXS cases from index males were found (data on screened females not shown). However, we recommended that FXS testing should be done in female patients with family history of mental retardation or suspected clinical features.

Autistic-like behaviors was not a significant item as reported in a previous study (Giangreco et al,1996). Therefore, it was discarded from the model. Our checklist retained five clinical items. The standard curves of ear length and testicular volume have not been studied in the normal Thai population. For this reason, we used standardized normal curves from the report of Butler et al (1992). Although these standardized curves came from Caucasian subjects, we found that these two items had statistical significance between non-FXS and FXS groups. These findings reveal that FXS patients tend to have much longer ear length and much larger testicular volume than normal children as seen in the report of Butler et

al (1992) . We suggest that using the Caucasian data may assist a population with lack of these standardized curves.

For the purpose of screening, we need to have a checklist with 100% sensitivity and the highest possible specificity. We proposed different models as shown in Table 3.2 because these might be beneficial for a similar study. Our study is the first report on a FXS clinical checklist in Asians. In addition, this study is the first report on a FXS clinical checklist with multiplicative weight assigned to each item. We propose this clinical checklist for male FXS, particularly from Asian population settings. However, we suggest that a clinical checklist may not be applicable to all clinical settings, but individual settings may need to modify it according to their experiences.

Table 3.1 Six-item checklist for FXS screening modified from Giangreco et al (1996)

Clinical items	Score		
	0	1	2
Family history*	None	Unidentified	X-linked
Narrow/long face	None	Borderline	Present
Prominent/large ears	None	Either	Both
Attention deficit/Hyperactivity	None	Either	Both
Autistic-like behaviors	None	1 behavior	> 1 behavior
Macro-orchidism	None	Borderline	Present

* Mental retardation, Developmental delay and Learning difficulty