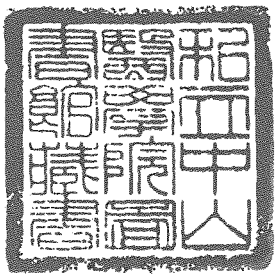
私立中山醫學院醫學研究所

碩士論文

易脆X染色體徵候群分子生物學及細胞遺傳學之研究

Molecular Biology and Cytogenetic Study of Fragile X Syndrome



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中華民國八十三年七月

本論文爲中山醫學院授予理學碩士學位之必備條件之一，經中山醫學院醫學研究所碩士論文考試委員會審查合格及口試通過。

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目　錄

誌　謝

終於，期盼了三年讓我等到今天。我花了五年時間才拿到這學位，這對我個人而言，故然是損失但也是人生一大歷練，使我對事情的看法不再那麼患得患失。三年前，我很無奈的放棄即將得到的學位，這種心境若非當事人及我週遭最親近的人，實在很難能體會。五年前於退伍前夕考上陽明醫學院遺傳學研究所，一個光明人生新里程就在眼前，但是這也註定了我兩年痛苦難熬的日子。一位由陽明外放到中研院分生所的學生，面臨的問題很多也很複雜。當時我的指導老師只重視實驗，非但未教導我如何解決實驗上的問題，更未顧慮我課業上的壓力，加上實驗室內的勾心鬥角及許多不足爲外人道的因素，促成了我後來離開陽明的結局。雖然如此，我仍努力去完成實驗，這期間承蒙中央研究院植物所周德源老師及陳瑞英學姊給我許多指導與幫忙，使我的實驗能順利進行，同時也感謝戴國禎學弟的鼓勵與支持。還要感謝二表哥、表嫂這期間的照顧。

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撐到現在。當然還有學弟紘志、煒翔平日的關心及論文準備上的幫忙，使我實驗能順利進行，論文得以完成。願以本論文紀念我的好友，俊碩學弟，他因血友病於民國八十三年六月十八日病逝台大醫院，我會永遠懷念你！說不盡的謝意，道不盡的感激，對所有曾經給我幫忙與鼓勵的同事及好友。

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第一章

總　論

Introduction

易脆X染色體徵候群之發現，首先見於Lubs於1969年提出之論文"A marker X chromosome" [Lubs, 1969]，此論文描述在一家族中，許多成員的X染色體長臂末端有一細桿連一小結(knob) (Fig. 1)，俱有此種X染色體的男性成員均爲智力不足，然而此報告並未引起人們的注意。1977年Harvey等人(Harvey et. al., 1977)的報告證實了Lubs的觀察。此種標記X染色體就是現在所稱的 "易脆X染色體(fragile X chromosome, Fra(X))"，其易脆位置(fragile site)是在Xq27.3 (圖一)。造成易脆X染色體之顯現(expression)的可能因素之一，是因培養液中葉酸含量很低時，才表現出來[Sutherland 1977, 1979a, 1979b]，但不是每一分裂中期染色體(metaphase chromosome)均能看到。由於易脆X染色體之顯現受到許多因素的影響，包括細胞的組織型態、使用培養液之種類、是否於培養液中加誘導劑(inducer)等，因此本論文的第一主題便是研究出最佳的誘發易脆X染色體顯現方法。

細胞遺傳學診斷易脆X染色體是傳統的診斷方法，但有時會有假陰性或假陽性的結果出現。所以，以傳統方法診斷易脆X染色體是很費時且不是百分之百正確。所幸此困擾拜分子生物學的進步而有了轉機，1991年5月陸續有數個實驗室宣佈選殖到一和易脆X染色體徵候群有關的基因--*FMR-1*的genomic DNA與cDNA [Heitz et. al., 1991; Kremer et. al., 1991; Oberle et. al., 1991; Verkerk et. al., 1991]。研究顯示，此基因共有十七個exons，且從第十個exon開始，有改變接合exon (alternative splicing)的現象(附圖一) [Eichler et. al., 1993; Verkerk et. al., 1993]，cDNA及protein之研究顯示此基因產物爲一RNA結合蛋白(RNA binding protein) (附圖二) [Ashley Jr. et. al., 1993; Gibson et. al., 1993; Siomi et. al., 1993 and 1994]。造成易脆X染色體徵候群的絕大多數原因，是*FMR-1*基因5'端不轉譯區(5' untranslated region, 5' UTR)之CGG trinucleotide tandem repeat [p(CGG)n]發生倍增突變(expansion mutation)，使得*FMR-1*基因之起

動區(promoter)發生超甲基化(hypermethylation)，導致*FMR-1*基因不表現而產生疾病[Bell et. al., 1991; Pieretti et. al., 1991; Sutcliffe et. al., 1992]。其它較罕見的原因尚有基因缺失(deletion) [Gedeon et. al., 1992; Wohrle et. al., 1992; Mornet et. al., 1993; Tarleton, 1993]及點突變(point mutation) [Boulle et. al., 1993]，這些突變不是使得*FMR-1*基因無法表現，不然就是使產物失去功能。簡單地講，由於*FMR-1*基因無法發揮其正常功能，所以產生易脆X染色體徵候群。本論文的第二主題，便是探討*FMR-1* CGG倍增突變於台灣地區中國人之遺傳特性，以及易脆X染色體顯現與CGG序列長度之關係，我們以genomic DNA StB12.3爲探針(probe) (附圖三) [Oostra and Oberle, personal communication]配合*Eag* I與*Eco* RI雙重水解DNA (附圖四)完成這部份實驗。

易脆X染色體徵候群造成的智障，是所有智障原因中第二位，僅次於唐氏症[Gustavson et. al., 1986; Webb et. al., 1986; Li et. al., 1988; Brown et. al., 1990]。然易脆X染色體徵候群爲遺傳性，而唐氏症多爲偶發性，故以優生保健立場而言，易脆X染色體徵候群之篩檢及產前診斷，實有重大意義。雖然Southern analysis能正確分析出*FMR-1*基因有無突變，但是要以此法作產前診斷有其限制: (1) 需大量DNA樣本(5 ~ 10 μg/sample)，(2) 時間花費頗長，約十天左右，因此不適用於大量篩檢及產前診斷。本論文之第三部份，乃探討利用RT-PCR技術以取代Southern analysis作產前診斷，並評估其可行性。

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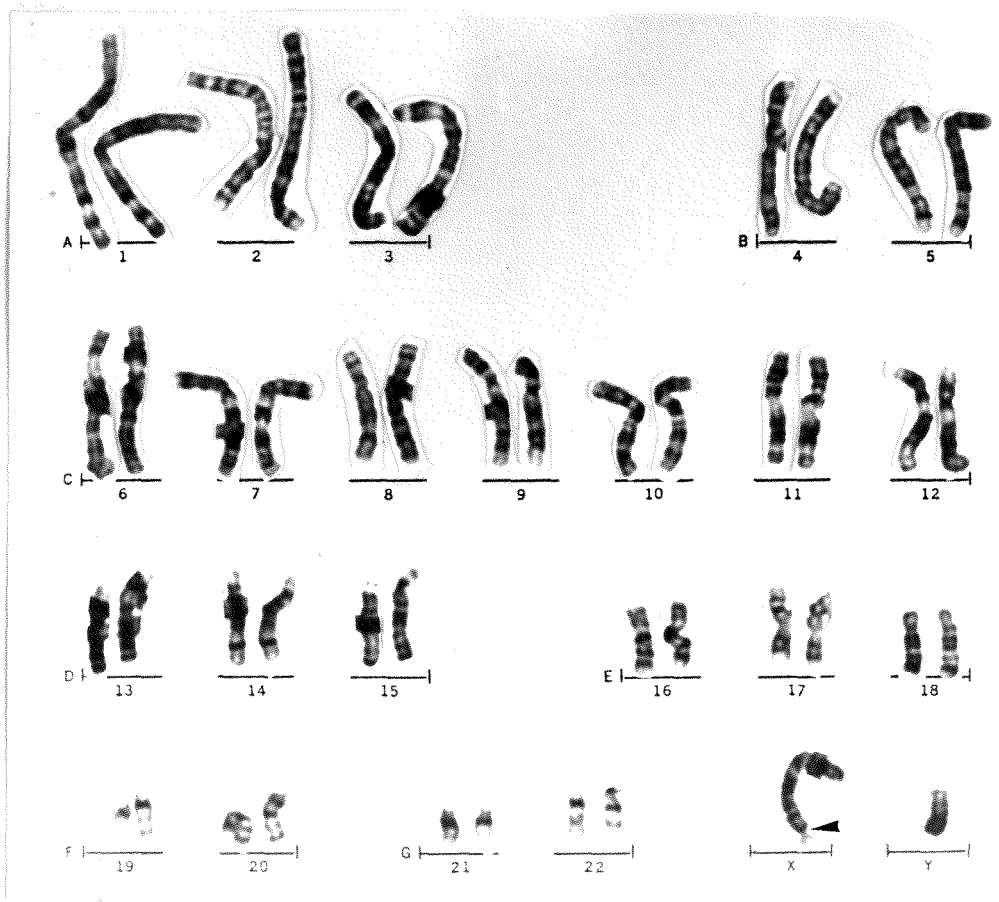
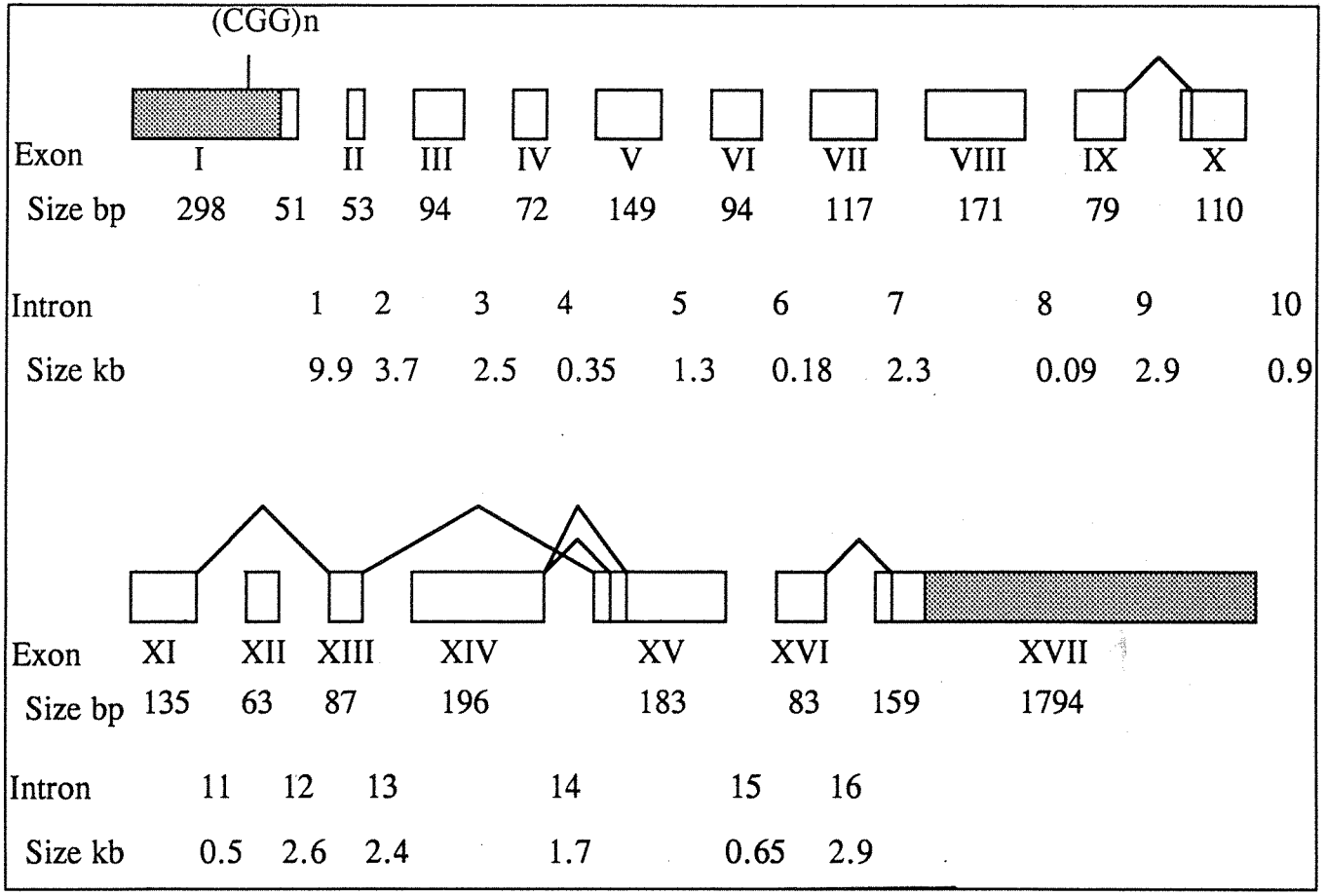
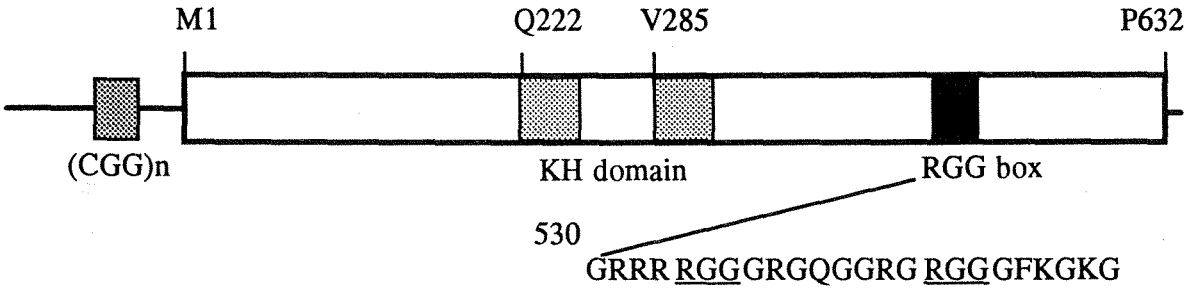


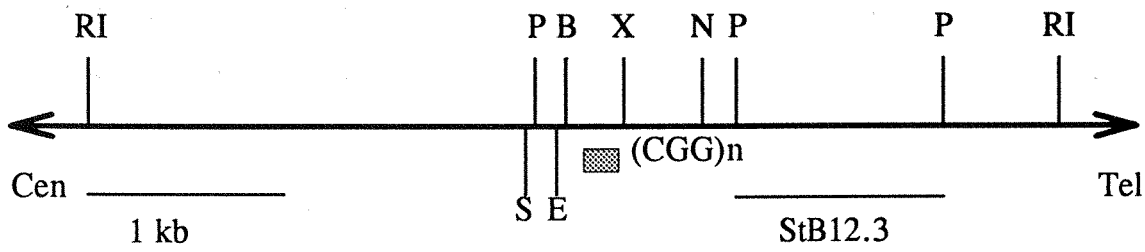
Fig. 1. karyotype of a male with fragile X chromosome. The fragile site is indicated by an arrow.



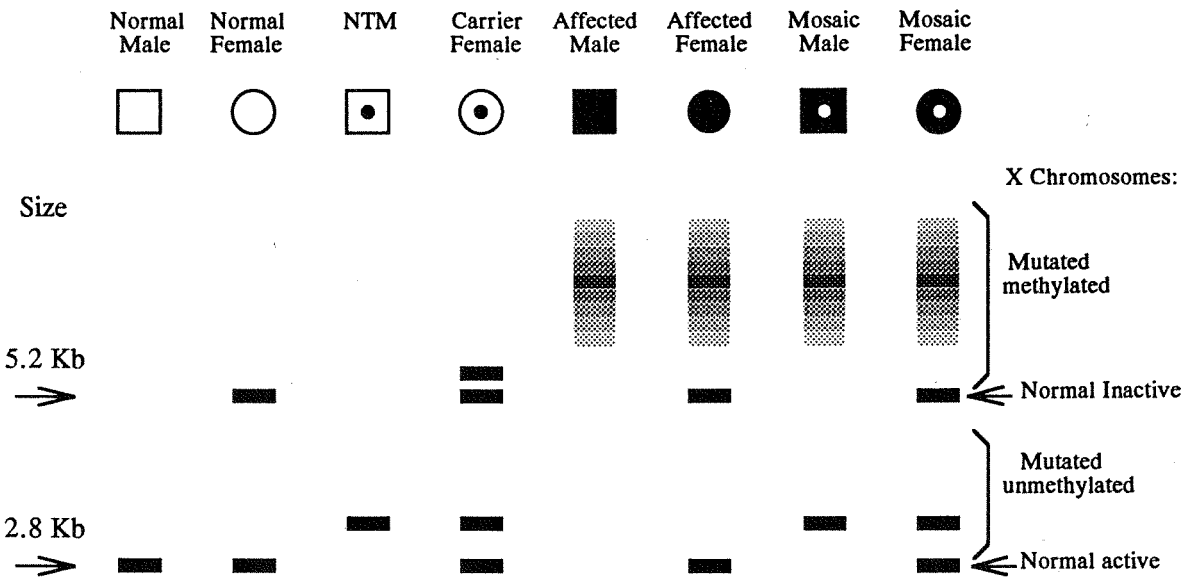
附圖一 Intron/exon distances of *FMR-1*. Exons are depicted as open boxes and numberd with roman numerals, introns are indicated by arabic numerals and sizes are shown below each gap. Shaded portions at the 3' and 5' end signify untranslated regions of the gene. (Eichler et. al., 1993)



附圖二、cDNA structure of *FMR-1* and diagram of FMRP. (Ashley et. al., 1993)



附圖三　Restriction map of Fragile X region and location of probe StB12.3. Shaded box indicates CGG trinucleiotide repeat. B: Bss HII, RI: Eco RI, E: Eag I, N: Nae I, P: Pst I, S: Sac II, X: Xho I.



附圖四　Patterns observed in *Eco* RI + *Eag* I double digestion with probe StB12.3. (Oberle et. al., personal communication)

第 二 章

培養之纖維母細胞中易脆X染色體之分子生物及細胞遺傳學的偵測方法

Molecular And Cytogenetic Detection of the Fragile X Chromosome in Fibroblast Culture

中 文 摘 要

易脆X染色體徵候群是最常見的遺傳性智能不足。此徵候群之產前診斷在臨床之遺傳諮詢上是很重要的。很不幸的，大多數嘗試以胎兒血液淋巴細胞、羊水細胞、或絨毛細胞爲材料，均不能令人滿意地成功完成產前診斷。這部份的研究目的便是想發展一有效、再現性佳及較不俱侵犯性的方法來偵測易脆X染色體，或許以後可應用於產前診斷。我們展示了fluorodeoxyuridine (FudR)或methotrexate (MTX)可誘發培養之纖維母細胞表現出易脆X染色體。並且發現培養於不同培養液加入不同誘發物後，其易脆X染色體之表現度亦有很大的差異。此種誘發易脆X染色體表現之差異，或許就是前人在類似研究上失敗的主要原因。爲確認由染色體的研究所得之結果，我們也使用了一段來自易脆X染色體基因*FMR-1*的片段StB12.3爲探針，來偵測這些來自易脆X染色體徵候群病人的纖維母細胞，其*FMR-1*基因上CpG島甲基化狀態及CGG三聯核苷酸增加的情形。我們很有信心地指出，我們發展出了一套有效的細胞遺傳學偵測方法，並且在產前診斷易脆X染色體徵候群上，俱有應用潛力。

英 文 摘 要 (Abstract)

The fragile X syndrome is one of the most common inherited forms of mental retardation. Prenatal diagnosis of fetus with this syndrome is of essential clinical and counseling importance. Unfortunately, most attempts to use cytogenetic preparations of fetal blood lymphocytes, amniotic cells or chorionic villi as a prenatal diagnostic method were not impressively successful. The present study was thus aimed to develop an unequivocal, reproducible and less invasive diagnosis technique which might be later applied for prenatal diagnosis of the presence of fragile X chromosomes. We demonstrated that the expression of fragile X chromosomes were inducible with treatments of fluorodeoxyuridine (FudR) or methotrexate (MTX) to fibroblasts cultured in different media containing different inducing agents expressed their fragile X chromosomes with great differences. The observed great difference in inducibility of fragile X chromosomes might well be one of the major reasons for failure in similar studies done by others. To validate our data from cytogenetic preparations, we also used the genomic DNA probe StB12.3 from the gene fragile X related mental retardation-1 (*FMR-1*) to detect the methylation status of CpG island and the CGG repeats increased in *FMR-1* in fibroblasts from same patients. A perfect match between data from cytogenetic and molecular detections was observed. With great confidence, we reported the development of a cytogenetic detection method and its potential application for prenatal diagnosis of fragile X syndrome.

序　論 (Introduction)

易脆X染色體徵候群是繼唐氏症之後，最常見的遺傳性智能不足。因易脆X染色體徵候群俱家族遺傳性，而唐氏症大多是偶發的且不是遺傳得到的[Turner et. al., 1983; Fryns et. al., 1984; Li et. al., 1988]。因此，易脆X染色體徵候群之重要性更甚於唐氏症。

依據Gustavson等人在瑞典[Gustavson et. al., 1986]及Webb等人在芬蘭[Webb et. al., 1986]之研究顯示，人類族群裏面，估計每1250名男性中有1名患病的男性；每5000名男性中有1名是不表現徵候的帶因者(carrier)，因此，大約每1000名男性中有1名帶有易脆X染色體。在女性帶因者中，大約三分之一表現出某種程度的智障[Sherman et. al., 1984]。族群中女性的患病率估計在兩千分之一[Nussbaum et. al., 1986]，因此，大約每700名女性中有1名帶有易脆X染色體。根據以上的估計，約每850人中便有1人帶有易脆X染色體[Webb et. al., 1986]。所以，瞭解及偵測易脆X染色體徵候群對於此徵候群之預防及追蹤是必須且重要的。

分子生物技術用於診斷易脆X染色體徵候群，比細胞遺傳學的方法來得便宜及快速。通常，分子生物學的方法可給一較正確的結果，但於產前診斷應用時，仍有許多困難有待克服。首先，南方氏雜合實驗(Southern hybridization)需要大量細胞來純化DNA。再者，對某些前突變(Premutation)的個體而言，其*FMR-1*基因中的CGG重複序列只增加一點點，不容易與正常人的區分出來。因此，一個更有效且更值得信賴的方法是我們一直期盼的。我們實驗室目前正發展以聚合酶鏈鎖反應(PCR)的方法來做產前診斷。

在細胞遺傳學上，並非每一個來自易脆X染色體徵候群病人的細胞都能顯現出易脆X染色體。例如，來自易脆X染色體徵候群病人的淋巴球很少顯現超過50%的易脆X染色體。 影響易脆X染色體顯現的因素有很多，例如細胞的來源、培養液的種類等。要誘發易脆X

染色體顯現，必須培養細胞的培養液中葉酸的含量很低[Brown, 1990; Sutherland, 1977]。要誘發淋巴球顯現易脆X染色體，則必需培養液中不含葉酸或培養液中加入thymidylate synthetase的抑制劑(例如：FudR) [Sutherland, 1979; Glover, 1981; Tommerup et. al., 1981]，或葉酸的拮抗物methotrexate (MTX) [Cantu et. al., 1985; Mattei et. al., 1981]使胸腺嘧啶的合成降低。另一方面，加入大量的胸腺嘧啶將干擾細胞內嘌呤及嘧啶的平衡，亦會誘發易脆X染色體表現[Fonatch, 1981]。前述誘發易脆X染色體顯現的方法，會降低整體細胞的分裂指數(mitotic index; MI)，而增加了分析的困難度。這或許是爲何細胞遺傳學的方法無法廣泛應用在產前診斷易脆X染色體徵候群的原因。最早報告以羊水細胞完成易脆X染色體的產前診斷，是由Jenkins及其夥伴於1981年提出。稍後，Webb與其同事報告他們首度以胎兒血液淋巴球完成了易脆X染色體的產前診斷。在1982年，Shapiro等人成功的展示以羊水細胞完成易脆X染色體的產前診斷。然而，到目前爲止也僅有少數案例報導易脆X染色體的產前診斷[Shapiro et. al., 1982; Jenkins et. al., 1988]，顯示出誘發易脆X染色體顯現的困難。許多實驗室亦曾報告易脆X染色體的產前診斷，但其中有些不確定的僞陰性或僞陽性。所以，長久以來一個簡單且俱再現性誘發易脆X染色體顯現的方法，被大家殷切盼望著。先前，本實驗室已建立好誘發淋巴球顯現易脆X染色體的技術[Li et. al., 1988]，目前本實驗室正發展產前診斷的技術。

一個良好的產前診斷方法必須能同時在羊水細胞及絨毛細胞中勝任愉快。因此，發展誘發纖維母細胞顯現易脆X染色體，似乎是發展產前診斷技術的踏腳石。現階段誘發纖維母細胞顯現易脆X染色體的方法，衆說紛云[Fonatch, 1981; Mattei et. al., 1981]。我們已從五位易脆X染色體徵候群患者，CT-077、CT-119、CT-337、CT-355、及CT-441，建立起皮膚纖維母細胞的初級培養(primary culture) [Li et. al., 1988]。爲了發展產前診斷的基本技術，纖維母細胞首先以MTX

或FudR處理，然後選擇能誘發易脆X染色體顯現最多的方法。由實驗獲得的結果顯示，FudR和MTX均能誘發易脆X染色體顯現，唯易脆X染色體顯現因培養液使用的不同及誘發物加的不同而有極大差異。

爲了印證我們由誘發易脆X染色體顯現之研究所得結果，我們也同時以來自*FMR-1*基因[Oberle et. al., 1991]的一片段StB12.3爲探針，對這五位病人的纖維母細胞進行了*FMR-1*基因的分子分析。我們的結果與前人的報告一致[Verkerk et. al., 1991; Rousseau et. al., 1991]。如同預期的，五位病人的*FMR-1*基因均有CGG核酸序列倍增及CpG島超甲基化(hypermethylation)的現像。我們已發展出一簡便、較不俱侵犯性、及俱再現性誘發纖維母細胞顯現易脆X染色體的細胞遺傳學方法，極俱產前診斷此徵候群的潛力。

材 料 與 方 法 (Materials and Methods)

纖維母細胞的初級培養 (Primary culture of fibroblasts)

初級培養的纖維母細胞是來自這五位易脆X染色體徵候群患者的皮膚，依標準組織培養程序建立。其相對的淋巴球易脆X染色體顯現百分比見(表一)。基本上的培養程序如下：由外科醫師用優碘消毒及局部麻醉，以外科手術方法從每一患者身上取一塊約25-mm^2的皮膚組織，隨即放入含培養液(MEM + 10% FCS + 1% PSN)的小瓶中帶回實驗室。將組織取出以解剖刀切細，置入培養皿(35 mm, Nunc)用collagenase type 1A (2 mg/ml, Sigma)處理，置於37 °C, 5% CO_2恆溫箱1小時，離心後除去上清液，加入2 ml 1X Trypsin-EDTA，置入35 mm培養皿，放在37 °C, 5% CO_2恆溫箱1小時，然後全部轉置於無菌離心管，加入4 ml MEM培養液(含10% FCS, 1% PSN)，離心後除去上清液，再加入2 ml培養液，用帶有19號針頭的注射針筒上下抽

動打散細胞，俟細胞完全分散，加適量MEM培養液，分裝於T-25培養瓶，48小時後觀察其生長狀況，到約九分滿將細胞存於液態氮中。實驗時，將細胞由液態氮中取出，分別培養於含不同濃度胎牛血清的六種不同的培養液(見表二)，包括Medium 199, MEM, RPMI-1640, MEM-FA, Ham's F-10 及M medium。

纖維母細胞易脆X染色體的誘發 (Induction of fra(X) in fibroblast)

爲了誘發纖維母細胞顯現易脆X染色體，纖維母細胞於收穫前24小時加入10^{-7} M的FudR (Sigma)或MTX (Lederle)。於收穫前的4 ~ 5小時加入colcemid (Gibco)，終濃度爲0.05 mg/ml。纖維母細胞隨後收穫、固定並以G-banding方法染色，以方便檢定fra(X)。詳細過程見附錄。所用之培養液及誘發物(inducers)如(表二)。每組均觀察50 ~ 100個G-banding metaphase求其頻率，而分裂指數(mitotic index)是由計數2000個細胞求得的。

*FMR-1*基因的偵測 (*FMR-1* detection)

每一DNA樣本取10 μg，以各50U的*Eco* RI (New England BioLab)及*Eag* I (New England BioLab)於1X NEBuffer #3，37 °C 作用20 ~ 24小時。經由1X TAE buffer 0.8%的洋菜膠體電泳後，DNA以PosiBlot DNA Transfer System (Stratagene)，以90 psi轉印一小時在nitrocellulose (N.C. paper)濾紙上。轉印後的N.C. paper以α-^{32}P標定的DNA探針(probe) StB12.3於6X SSC，0.5% SDS及5X Denhardt's溶液中雜合(hybridization) 18 ~ 20小時。探針先以2X SSC，0.1% SDS洗15分鐘，再以0.2X SSC，0.1% SDS於65 °C洗5 ~ 10分鐘。最後將濾紙以保鮮膜包好，並與X光片(Kodak X-OMAT AR)曝光3 ~ 5天。

結　果 (Results)

Medium 199，MEM及RPMI-1640在FudR或MTX存在下有較高的fra(X)顯現。但是F-10在FudR或MTX存在下，fra(X)的顯現則很低或沒有顯現。然而在F-10組的MI值較高(表三)。CT-355和CT-441在medium 199、MEM、RPMI-1640等三種培養液中於FudR和MTX存在下有較高的fra(X)顯現。雖然CT-077於前述培養液中於FudR存在下有較高的fra(X)顯現，但是在MTX存在下並沒有很高。在Mmedium培養下，不管加入FudR或MTX，CT-355均有較高的fra(X)顯現；但是CT-077及CT-441之fra(X)顯現與分裂指數均很低。CT-119在各組培養液加入FudR條件下，fra(X)的顯現並無明顯差異，但是在F-10及RPMI-1640兩種培養液加入MTX培養下CT-119卻有最高的fra(X)顯現。CT-337在medium 199、MEM於FudR存在下有較高的fra(X)顯現。以F-10加入FudR或MTX來培養細胞時，所有五位病人的纖維母細胞所能誘發fra(X)顯現均很低或無法誘發fra(X)顯現，然而本組之分裂指數是所有組中最高的。

分析這五位病人的纖維母細胞之*FMR-1*基因顯示，CpG島(CpG island)已超甲基化(hypermethylation)且CGG重覆序列有倍增情況(圖一)。結果顯示在正常男性及女性帶有2.8 Kb訊號(此代表正常活化的基因；可在男性及女性中偵測到)以及5.2 Kb訊號(此代表正常不活化的基因；此只能在女性中偵測到)。在四位男性易脆X染色體徵候群患者(圖一之1 ～ 4行)僅顯示出一群不同大小的帶狀訊號且此訊號位置都比5.2 Kb來得高。這些帶狀訊號顯示*FMR-1*基因帶有不同長度之CGG三聯核苷酸重覆序列，以及CpG島有超甲基化。CT-441顯示出兩個不同訊號，一個5.2 Kb的訊號，此代表正常不活化的基因及一個5.8 Kb的訊號，此代表超甲基化及CGG三聯核苷酸重覆序列倍增的突變基因。在第五行中看不到代表正常活化基因的2.8 Kb的訊號，或許是由於女性是嵌紋體(mosaicism)的本質使然。不過，來自

同一病人血液的DNA(第六行)卻可看到此2.8 Kb的限制片段(restriction fragment)。但也有可能lane 5之DNA量太少以致2.8 Kb的訊號看不到。

討　論 (Discussion)

本實驗的結果顯示了來自易脆X染色體徵候群患者的纖維母細胞，在前述培養液中可被FudR和MTX誘發顯現易脆X染色體。然而，值得注意的是，FudR和MTX誘發顯現易脆X染色體的效能在六種培養液中有極大的差異。如我們在表三中顯示的，來自同一病人的纖維母細胞於不同培養液中或不同病人的纖維母細胞於相同培養液中，其fra(X)的表現度(expressivity)有極大的差異。

以纖維母細胞、羊水細胞或絨毛細胞進行例行性的易脆X染色體之染色體檢查，基於下列理由目前還未建立：(1)誘發易脆X染色體顯現之細胞培養條件，往往會降低分裂指數而減少細胞分裂，致使可供診斷的分裂中期(metaphase)細胞數目減少。(2)因爲易脆X染色體顯現之比例通常很低，所以需要大量的細胞才能正確的診斷。(3)一些非特異性的染色體末端結構變化(non-specific telomeric structural changes; TSC)，易造成誤判爲易脆X染色體，導致假陽性的結果(Jenkins, et al., 1986)。不過，在我們發展的方法中，諸如此類的錯誤是可以避免的，因爲本研究中所有染色體均用G-banding染色法染色，可以很清處地判斷X染色體，因而不會將其它TSC誤認爲fra(x)。

Sutherland (1977, 1979)研究淋巴球顯示，唯有培養於不含或只含少量葉酸的培養液，如Medium 199 (含葉酸0.01 mg/l)，方能顯現出易脆X染色體。但是本實驗中，如不加FudR或MTX，只用medium 199或M medium (不含folic acid，hypoxanthine及thymidine)來培養易

脆X染色體患者纖維母細胞，均不會誘發易脆X染色體之顯現。這可能是纖維母細胞與淋巴球之內在性質(intrinsic property)不同或未找到最適當的培養條件。誘發易脆位置(fragile site)顯現之培養液，主要條件是缺乏葉酸，高pH值及低血清量(Sutherland, 1979)。所以，由生化的觀點看來，易脆X染色體之顯現似乎是因dTMP減少，造成DNA複製受到干擾而引起的。圖二中所示的便是易脆X染色體顯現之生化代謝途徑。

於1991年，有幾個實驗室先後宣佈選殖(clone)到易脆X染色體基因(*FMR-1*)的cDNA與genomic DNA [Kremer et. al., 1991; Oberle et. al., 1991; Verkerk et. al., 1991]。Dr. Oberle熱心地提供我們一段genomic DNA探針StB12.3。四位男性患者之染色體DNA，經由*Eco* RI及*Eag* I雙重水解後，顯示出這四位患者*FMR-1*基因的CGG核酸重覆序列有倍增現像，並且*FMR-1*基因5'端的CpG島也發生了超甲基化現象。於女性患者(CT-441)，我們看到了代表正常不活化X染色體上*FMR-1*基因的5.2 Kb訊號，同時也看到了一個比5.2 Kb訊號高，代表突變*FMR-1*基因的訊號。然而，代表正常活化X染色體上*FMR-1*基因的2.8 Kb訊號，卻不見於纖維母細胞的DNA中。一項合理的解釋是，這些纖維母細胞均來自皮膚的一小塊區域，而此區域中的細胞剛好是突變的*FMR-1*基因都在活化的X染色體上；正常的*FMR-1*基因都在不活化的X染色體上。此2.8 Kb片段可在血液的DNA中見到，證明了我們的假設是對的，因爲血液淋巴球俱有異質性(heterogeneity)。另一合理解釋，是lane 5之DNA量太少，所以無法看到2.8 Kb的訊號。

在本研究中，我們展示了以細胞遺傳學方法偵測易脆X染色體有一主要障礙，即易脆X染色體顯現會隨細胞型態(cell type)和培養條件而改變。因此我們建議要診斷易脆X染色體，則必須採用至少兩種誘發方法，以避免假陰性(false-negative)的結果。

雖然分子生物的技術能夠做正確的診斷，但是想要應用在產前診斷仍有些困難。因爲，(1)南方雜合實驗(Southern hybridization)需要大量細胞來製備DNA。(2)對某些前突變(premutation)個體而言，*FMR-1*基因的CGG核酸重覆序列不易與正常的*FMR-1*基因區分出來。因此，需要發展一更有效且更可靠的方法。目前我們實驗室正發展以聚合酶鏈鎖反應(polymerase chain reaction; PCR)來做產前診斷。

以分生技術診斷易脆X染色體徵候群，確實比細胞遺傳學方法來得便宜、快速與正確。然而它卻不能偵測出其它的染色體異常。因此，對一個診斷實驗室而言，分生技術方法與細胞遺傳學方法應同時採用。一旦在一家族中發現了易脆X染色體徵候群患者，其他家族成員就應以分生技術而非細胞遺傳學方法來診斷。

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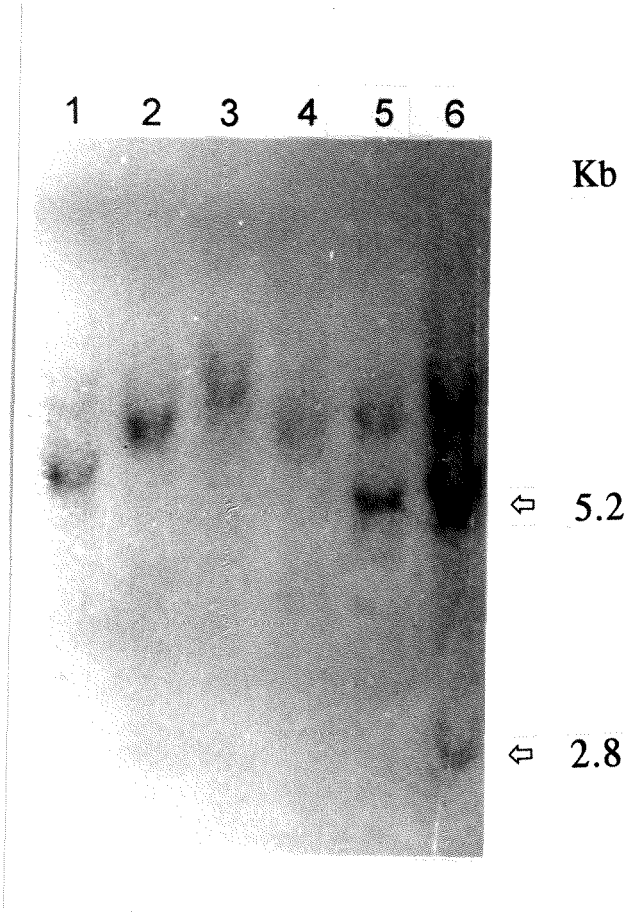

Fig. 1. DNA analysis of *FMR-1* in five fragile X syndrome patients. Lanes 1 ~ 4 (male patients): CT-077, CT-119, CT-337, and CT-355. Lanes 5 ~ 6 (female patient): CT-441. DNA sample of lanes 1 to 5 are come from fibroblast and DNA of lane 6 is originated from blood sample. The corresponding sizes of the signal are shown on the right.

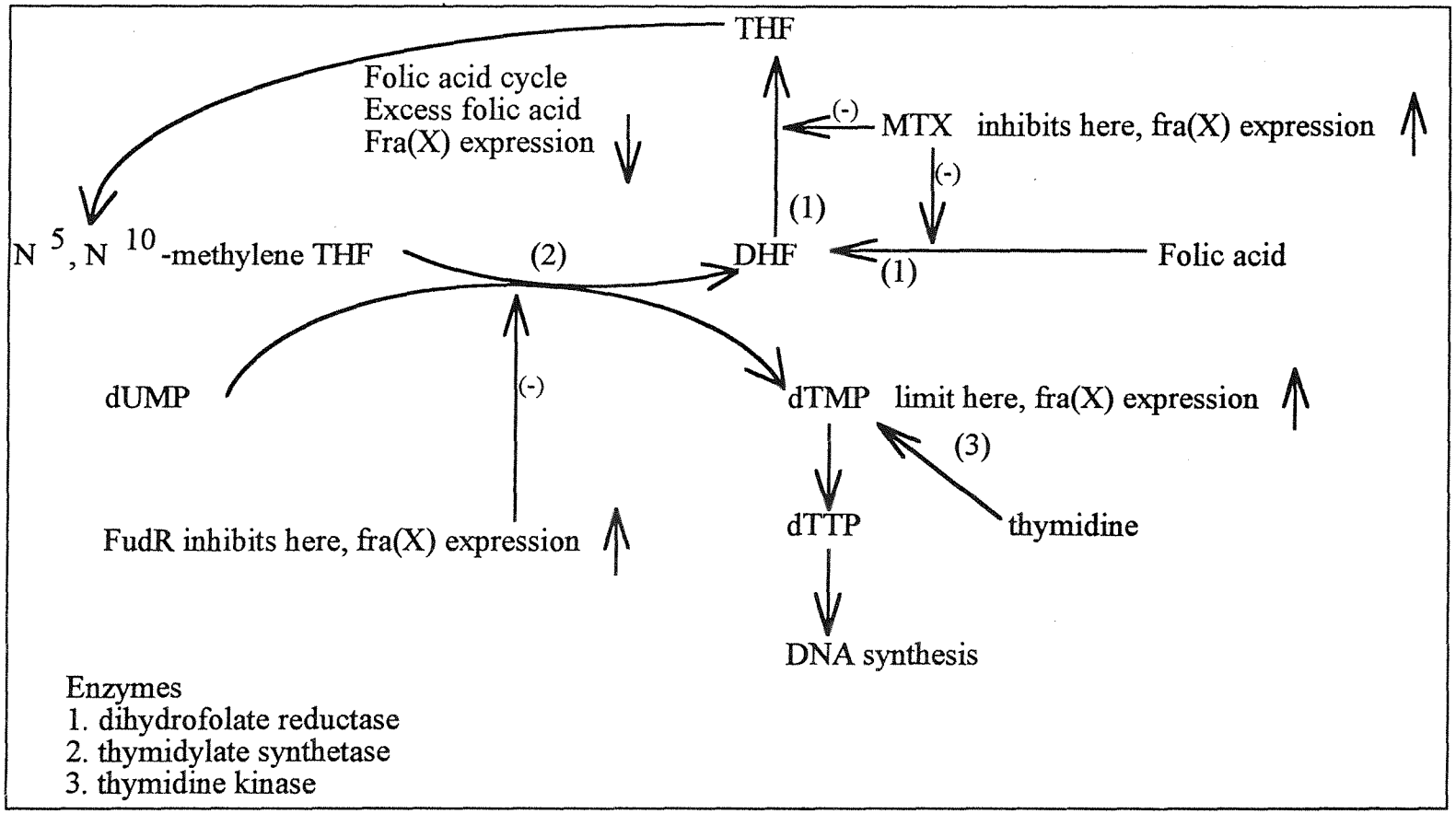

Fig. 2. The area of metabolism affected by inducers and inhibitors of fragile X expression.

Table 1. Percent breakage at Xq27.3 in lymphocyte culture

Subject	Sex	Fra(X)/total cells	Fra(X) %
CT-077	Male	14/100	14
CT-119	Male	8/250	3.2
CT-337	Male	5/178	2.8
CT-355	Male	15/200	7.5
CT-441	Female	10/100	10

Table 2. Media and reagents used in Fra(X) induction

No.	Medium	Reagents (final 24 hours)
1	Medium 199[a] + 5% FCS	0.1 μM FudR or 0.1 μM MTX
2	MEM + 10% FCS	0.1 μM FudR or 0.1 μM MTX
3	RPMI-1640 + 10% FCS	0.1 μM FudR or 0.1 μM MTX
4	MEM-FA[b] + 10% FCS	0.1 μM FudR or 0.1 μM MTX
5	HAM's F-10 + 20% FCS	0.1 μM FudR or 0.1 μM MTX
6	M medium[c] + 4.5% FCS	0.1 μM FudR or 0.1 μM MTX

a: Medium 199 contains folic acid 0.01 mg/ml.

b: MEM-FA without folic acid.

c: M medium without hypoxanthine, folic acid, and thymidine.

Table 3. Expression of fra(X)(27.3) in fibroblasts from five affected individuals

Case No.	Culture media*	Control			FudR			MTX		
		Fra(X) cells	Fra(X) %	MI** %	Fra(X) cells	Fra(X) %	MI %	Fra(X) cells	Fra(X) %	MI %
077	1	0/100	0.00	1.4	13/15	25.49	0.7	1/59	1.69	0.5
	2	0/100	0.00	3.9	4/56	7.14	0.4	2/59	3.39	0.8
	3	0/100	0.00	4.5	9/100	9.00	0.2	0/50	0.00	0.2
	4	0/100	0.00	2.8	1/63	1.59	0.3	0/63	0.00	0.3
	5	0/100	0.00	5.5	0/100	0.00	3.7	1/50	0.00	0.8
	6	0/100	0.00	3.6	5/100	5.00	0.8	2/100	2.00	0.8
119	1	0/100	0.00	3.6	5/100	5.00	0.8	2/100	2.00	0.8
	2	0/100	0.00	3.0	6/100	6.00	0.7	5/100	5.00	0.6
	3	0/100	0.00	5.0	5/100	5.00	0.6	10/100	10.00	0.8
	4	0/100	0.00	3.0	6/100	6.00	0.4	3/100	3.00	0.5
	5	0/100	0.00	5.0	0/100	0.00	4.0	4/100	4.00	3.0
	6	0/100	0.00	4.0	8/100	8.00	0.5	2/100	2.00	0.4
337	1	0/100	0.00	2.5	6/100	6.00	0.6	2/100	2.00	0.5
	2	0/100	0.00	4.5	5/100	5.00	0.6	3/100	3.00	0.7
	3	0/100	0.00	5.0	2/100	2.00	0.5	1/100	1.00	0.6
	4	0/100	0.00	3.0	3/100	3.00	0.4	1/100	1.00	0.5
	5	0/100	0.00	5.6	1/100	1.00	4.0	2/100	2.00	3.0
	6	0/100	0.00	4.0	2/100	2.00	0.6	1/100	1.00	0.5
355	1	0/100	0.00	1.7	31/100	31.00	0.5	13/100	13.00	0.9
	2	0/100	0.00	3.2	28/100	28.00	0.6	12/100	12.00	0.8
	3	0/100	0.00	3.2	25/61	40.98	0.8	6/55	10.90	0.2
	4	0/100	0.00	2.6	19/100	19.00	0.6	6/71	8.45	0.3
	5	0/100	0.00	5.9	1/100	1.00	4.5	0/100	0.00	2.0
	6	0/100	0.00	2.5	16/100	16.00	0.5	16/100	16.00	0.4
441	1	0/100	0.00	4.7	11/46	24.00	0.2	21/100	21.00	0.6
	2	0/100	0.00	5.4	15/100	15.00	0.4	27/100	27.00	0.6
	3	0/100	0.00	4.0	16/100	16.00	1.4	21/78	24.14	0.6
	4	0/100	0.00	3.3	8/58	13.79	0.6	6/50	12.00	0.4
	5	0/100	0.00	5.6	0/100	0.00	5.4	1/100	1.00	4.0
	6	1/100	1.00	4.0	5/38	13.16	0.2	1/100	1.00	0.2

* See Table 2.

** Mitotic index was based on the analysis of at least 2,000 cells/culture.

第 三 章

於一大家族中易脆X染色體顯現與*FMR-1*基因p(CGG)n倍增現象之研究

A study of fragile X chromosome expression and p(CGG)n amplification in *FMR-1* gene in a large family

中 文 摘 要

易脆X染色體徵候群是最常見的遺傳性智能障礙。和此徵候群有關的基因，fragile X mental retardation-1 (*FMR-1*)，已被找到。在此基因5'端未轉譯區(5' untranslated region, 5' UTR)內的CGG核酸重覆序列發生倍增突變(amplification mutation)是造成此徵候群的原因。在一易脆X染色體徵候群大家族中，我們對其中二十三位家族成員做了易脆X染色體顯現的染色體分析，同時也分析了*FMR-1*基因CGG核酸重覆序列的倍增情形。這些成員當中，十一位是正常，三位僅發現*FMR-1*基因CGG核酸重覆序列有倍增情形，而有九位是CGG核酸重覆序列有倍增情形且顯現出易脆X染色體。本研究中我們展示了*FMR-1*基因CGG核酸重覆序列的倍增程度和易脆X染色體顯現程度，有正相關性，並且在此大家族中也觀察到了期望現象(anticipation phenomenon)。

英 文 摘 要 (Abstract)

The fragile X syndrome is the most common cause of hereditary mental retardation. The gene, fragile X mental retardation -1 (*FMR-1*), has been identified to be associated within this syndrome. Amplification mutations in CGG repeats in the 5' untranslated region (5' UTR) of the *FMR-1* give rise to this phenotype. The purpose of this study was aimed to test the phenomenon of Sherman paradox and to check the relationship between the fra(X) expression and p(CGG)n expansion. In a large fragile X family, twenty three individuals were cytogenetically examined for fra(X) expression and molecular analyses of p(CGG)n amplification in *FMR-1* were also performed. Of these individuals, eleven are normal, three individuals have only CGG amplification in *FMR-1*, and nine of them have both CGG amplification and fra(X) positive. We demonstrated that the DNA increased in CGG repeat has positive correlation with fra(X) expression, and the anticipation phenomenon been also seen in this large family.

序　論 (Introduction)

易脆X染色體徵候群是造成智障的主要原因之一，僅次於唐氏症[Gustavson et. al., 1986; Webb et. al., 1986; Li et. al., 1988; Brown et. al., 1990]。然唐氏症多爲偶發性(sporadic)，而易脆X染色體徵候群爲遺傳性，故本徵候群之重要性甚於唐氏症。易脆X染色體徵候群是一不尋常的X-linked疾病，因爲30%帶有此突變基因的女性會顯現出某種程度的智障，且20%帶有此易脆X染色體的男性其表現型爲正常[Sherman, et.al., 1984; Sherman et. al., 1985]。分子選殖的進展已在跨越脆裂位置(fragile site)找到一個基因 -- *FMR-1*，此基因和易脆X染色體徵候群有強烈的關聯性。*FMR-1*基因的核酸序列分析顯示，此基因的第一個exon有CGG核酸重覆序列，此序列在易脆X染色體患者中不安定[Verkerk et. al., 1991; Oberle et. al., 1991; Heitz et. al., 1991; Fu et. al., 1991; Kremer et. al., 1991]。此CGG核酸序列重覆次數表現出與突變之傾向有關，於正常基因重覆次數在6 ~ 54之間，於前突變(premutation)基因重覆次數在52 ~ 200之間，而完全突變(full mutation)的基因此重覆次數在200以上。

帶有前突變基因的男性在外表上是正常，稱爲〝正常傳遞男性〞(normal transmitting males; NTMs)並且細胞遺傳學上是誘發不出易脆X染色體。NTMs所帶的突變基因可傳給其女兒，她們也常是外表正常的女性帶因者(female carrier)並且細胞遺傳學上是誘發不出易脆X染色體。但是NTMs的孫子往往發病，*FMR-1*基因的CGG重覆序列戲劇性的增加，使得此基因由前突變成爲完全突變。在細胞遺傳學上可誘發出易脆X染色體。女性帶有完全突變基因時的情況就較爲複雜，有的可誘發出易脆X染色體，有的則不能，且表現出的智能狀況可能是智障或正常[Jacobs, 1991]。然而，在易脆X染色體徵候群家族中，智障的危險似乎是依據成員在家族族譜中的位置而定。也就是說，族譜中代數越後面的其智障程度也越嚴重。此現象首先由

Sherman注意並辨識出來，這就是後來大家所熟知的anticipation phenomenon [Sherman et. al., 1984; Sherman et. al., 1985]。

造成易脆X染色體之易脆性的確實機轉，以及*FMR-1*基因產物的功能在智障中扮演何種角色，都還有待進一步瞭解。不論如何，已有許多研究報告指出，變長了的p(CGG)n將會特別地誘發緊鄰此序列5'端的CpG島甲基化。在此CpG島甲基化將使*FMR-1*基因不表現[Bell et. al., 1991; Pieretti et. al., 1991; Sutcliffe et. al., 1992]。這似乎顯示出*FMR-1*的CGG倍增突變誘導此區域的甲基化，降低調節(down regulate)*FMR-1*的表現而造成疾病。

易脆X染色體之顯現很少超過50%。我們最近的觀察[Wang and Li, 1993]以及他人之研究[Sutherland, 1977; Sutherland, 1979; Glover, 1981; Tommerup et. al., 1981; Mattei et. al., 1981; Fonatch et. al., 1981; Sutherland, 1986]均顯示出以細胞遺傳學方法偵測易脆X染色體，有一重大障礙就是依賴細胞種類(cell type)及培養條件。另一研究易脆X染色體徵候群的明顯困難是，要找到一成員衆多的完整大家族並不容易。在我們近來研究的案例當中，剛好有此難得機會對一易脆X染色體徵候群大家族中的三代二十三位成員，驗證Sherman paradox及進行CGG擴大(expansion)與易脆X染色體顯現間相關性之研究。

在這同一族譜中的二十三位成員，均做了易脆X染色體顯現之細胞遺傳學診斷，及用探針StB12.3對他們的*FMR-1*基因進行偵測。這些成員當中，十一位是正常，三位僅發現*FMR-1*基因CGG核酸重覆序列有倍增情形，而有九位是CGG核酸重覆序列有倍增情形且顯現出易脆X染色體。由此家族獲得的結果，我們觀察到了期望現象(anticipation phenomenon)。以線性迴歸分析，我們亦觀察到此兩種表現型有強烈的正相關性，即成員CGG序列重覆次數越高者，其易脆X染色體顯現的頻率也越高($P < 0.005$)。

材 料 與 方 法 (Materials and Methods)

研究對象(Examined subjects)

本研究之研究對像均來自同一大家族(見圖一)。二十三位受檢家族成員分別是：II-2, II-4, II-6, III-1, III-3, III-5, III-7, III-9, III-11, III-13, III-14, III-15, III-17, III-18, III-19, IV-1, IV-2, IV-3, IV-5, IV-6, IV-7, IV-10及IV-11。

周邊白血球易脆X染色體的誘發 (Induction of fra(X) in peripheral blood cell)

以含有肝素(heparin)的針筒抽血10 ml，5 ml以M medium作培養觀察易脆X染色體，另5 ml作DNA之純化(方法詳見附錄)。細胞收穫前4～5小時家入colcemid (Gibco)，至終濃度爲0.05 mg/ml。細胞隨後收穫、固定並以G-banding方法染色，以方便檢定fra(X)。每一個例均至少檢定一百個分裂中期(metaphase)細胞。

*FMR-1*之偵測 (*FMR-1* detection)

每一DNA樣本取10 μg，以各50U的*Eco* RI (New England Biolab)及*Eag* I (New England BioLab)於1X NEBuffer #3，37 °C 作用20 ～ 24小時。經由1X TAE buffer，0.8 %的洋菜膠體電泳後，DNA以PosiBlot DNA Transfer System (Stratagene)，以90 psi轉印一小時在nitrocellulose (N.C. paper)濾紙上。轉印後的N.C. paper以α-^{32}P標定的DNA探針(probe)StB12.3於6X SSC，0.5 % SDS及5X Denhardt's溶液中雜合(hybridization)18 ～ 20小時。探針先以2X SSC，0.1 % SDS洗15分鐘，再以0.2X SSC，0.1 % SDS於65 °C洗5 ～ 10分鐘。最後將濾紙以保鮮膜包好，並與X光片(Kodak X-OMAT AR)曝光3～5天。

結　果 (Results)

二十三位成員均以細胞遺傳學方法做易脆X染色體之檢驗，並且對其*FMR-1*基因做了CGG擴張突變(expansion mutation)之分析。經由IV-10使得我們找到此大家族。在這二十三人當中，十一位是正常，九位患者，三位帶因者。每一成員的易脆X染色體顯現百分比及CGG重覆序列增加的長度(以Kb爲單位)，詳見(表一)。全部分生結果均依據同一片膠體上marker DNA所分佈位置，以半對數表(semi-log plot)作圖後來計算。因此，即使長度上只增加一點點，我們也能精確估計每一個訊號長度(圖二)。

II-2，II-4和II-6無法誘發出易脆X染色體顯現。她們都帶有一前突變基因並傳給她們的後代，此CGG重覆序列在這些後代中均戲劇性地增加，使得他們患病。一個值得注意的例外是III-5，她其中一個基因增加了1 Kb，以分生的角度來說，應歸類在完全突變，且她的血球有10%易脆X染色體顯現率。然而，和其它完全突變個體比起來，她只表現出輕度的智障。

從族譜中我們觀察到，CGG重覆序列長度一代比一代增加。唯一例外是成員IV-10，其增加長度(以"Δ"表之；$\Delta = 2$ Kb)反而比他母親($\Delta = 3$ Kb)減少。由這些數據我們證實Sherman所指的anticipation現象。迴歸分析顯示CGG重覆序列長度的增加與易脆X染色體顯現率之間有重要的線性關係($P < 0.005$) (圖三)。

有趣的是，成員III-19有一比正常大0.2 Kb的訊號及一平均增加1.8 Kb的拖曳帶狀訊號(圖二)。表示此個體的某些細胞俱有前突變基因，而其它的細胞俱有完全突變基因，顯示出此人是一嵌紋體(mosaic)。

討　論 (Discussion)

借由此易脆X染色體大家族之便，我們在易脆X染色體之顯現及p(CGG)n於每一代中之擴張，觀察到所謂的anticipation phenomenon。即，有一可預期的傾向：由一代到一代，其易脆X染色體徵候群病徵的嚴重性將增加。再者，我們發現p(CGG)n的長度似乎和易脆X染色體之顯現及智障程度有正相關性。

本研究中我們找到了三位女性帶因者，II-2，II-4，及II-6。她們都無易脆X染色體之顯現，但是CGG重覆序列長度都有一點點增加。在此我們要指出III-5是一完全突變者而非帶因者(前突變)。雖然她表現出近乎正常的智能狀態，但她卻表現出大多數易脆X染色體徵候群的基本要件：易脆X染色體之顯現與p(CGG)n倍增現象。這是一單一且可理解的個例，因爲帶有完全突變基因的女性約80%而非100%會表現出智障[Sherman et. al., 1984; Sherman et. al., 1985]。

突變基因中的p(CGG)n長度，一代比一代增加。減數分裂的不安定性(meiotic instability)或許是造成此結果的主因。另一可能造成的因素是〝創立者染色體效應〞(founder chromosome effect) [Chakravarti, 1992; Richards et. al., 1992]，但是其它的機轉像是非對等交換(unequal crossing over)也可能扮演了重要角色。有一值得注意的例外是IV-10，我們看到了p(CGG)n長度減少。III-19這個個案我們認爲他是嵌紋體的男性(mosaic male)。由DNA分析知道，他的體細胞中分別帶有前突變及完全突變的基因。這現象表示了在卵子形成過程中CGG重覆序列發生擴張，接著在胚胎早期發生了非對等交換，導致嵌紋現象。更進一步，有絲分裂不安定性(mitotic instability)也發生了，造成了在7 Kb的地方有一拖曳狀(smear)帶狀訊號(Δ = 1.8 Kb)。造成有絲分裂不安定性的機轉可能是〝滑動的DNA合成〞(slippery DNA synthesis) [Kunkel, 1993; Strand et. al., 1993]。整個導致嵌紋現象的流程詳見圖四。

III-19表現出輕度的智障，他身上完全突變的基因也絕大多數都甲基化。已有文獻報告指出*FMR-1*的甲基化，會降低調節(down regulate)它的表現而產生疾病[Bell et. al., 1991; Pieretti et. al., 1991; Sutcliffe et. al., 1991]。然而對嵌紋體的人來講，*FMR-1*的甲基化狀態和其易脆X染色體徵候群徵狀之不同程度的表現有關[McConkie-Rosell et. al., 1993]。帶有突變基因的女性，將表現出選擇性的差異性甲基化(selectively differential methylation)，就像我們在II-6觀察到的一樣(圖二)。她的正常基因大多數位於活化X染色體，突變基因大多數位於不活化X染色體，這可由比較分別來自正常活化基因與正常不活化基因的2.8 Kb與5.2 Kb的訊號強度而得知。相似的結果也發生在III-5，細胞遺傳學及分子生物學的方法均診斷爲患者，但易脆X染色體徵候群之臨床表現卻很難看到。在女性的這種選擇性甲基化，或許是經由X染色體不活化作用而隨機發生。因此，X染色體不活化作用在完全突變女性的易脆X染色體徵候群之臨床表現上，扮演著重要角色。

在此大家族中我們看到了anticipation現象，其中III-5是不完全表現(incomplete penetrance)。因此，從期望現象的觀點，對一位已懷孕的易脆X染色體帶因者而言，產前診斷是很重要的。目前用來偵測易脆X染色體的方法，要應用在產前診斷仍有許多臨床上的困難。然而，本實驗室近來使用RT-PCR的方法結合PCR偵測*SRY*基因作胎兒性別決定，爲一位已懷孕的易脆X染色體帶因者(經探針StB12.3證實)，成功地完成了一次產前診斷。在下一章中有詳細的探討。

Yu等人[Yu et. al., 1992]曾報告p(CGG)n長度與易脆X染色體顯現間有正相關性。我們的結果也證實了這點，然而她們並未解釋爲何易脆X染色體之顯現很少超過50%，以及易脆X染色體之易脆性。Hansen等人[Hansen et. al., 1993]報告，在突變的*FMR-1*發生的超甲基化將延遲涵蓋此基因180 Kb區域的DNA複製。有可能此易脆X染色體延遲的DNA複製，反過來影響到有絲分裂時之染色體濃縮，造

成我們在分裂中期看到的結果。還有一可能機轉是GC雙核苷酸交替改變序列，有傾向產生Z-DNA的特性[Rich et. al., 1984]。相似的情形，p(CGG)n是100% GC組成的DNA序列，所以很有可能誘使產生左旋的DNA結構。然而，Z-DNA如何影響染色體的濃縮，且如果眞是這樣，Z-DNA經由何種機轉達到其影響，還有待進一步證明。

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(本章內容已被*J. Genet. Mol. Biol.* 接受，發表時之原稿附於本論文之後)

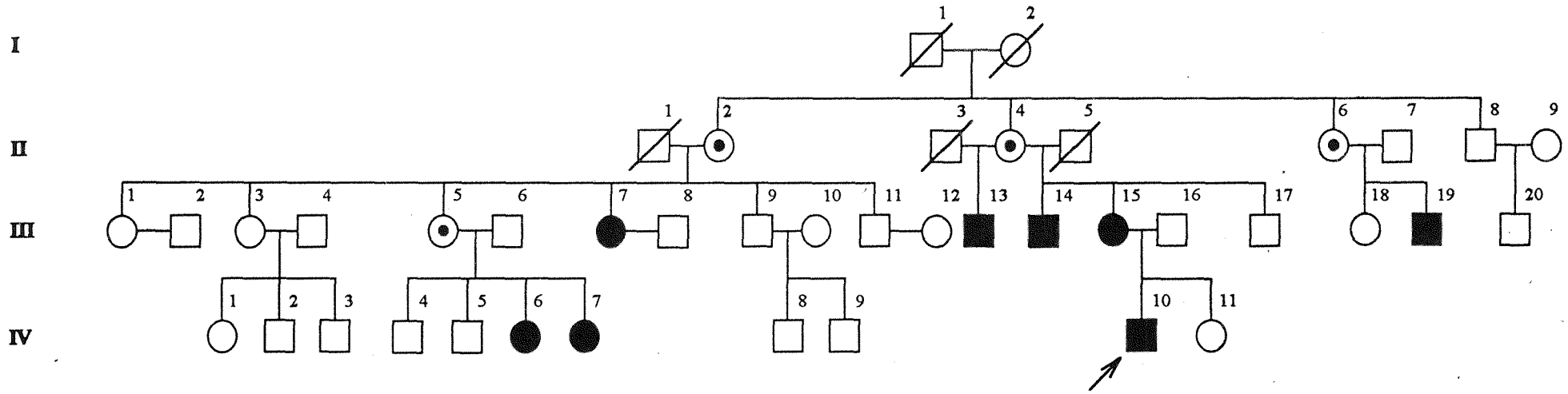


Figure 1. Pedigree of the large family in this study. Open boxes and circles indicate normal individuals, and doted symbols indicate these individuals are carriers (premutations). Solid symbols indicate affected individuals, with exception that III-19 is referred as a mosaic male (for detail see text).

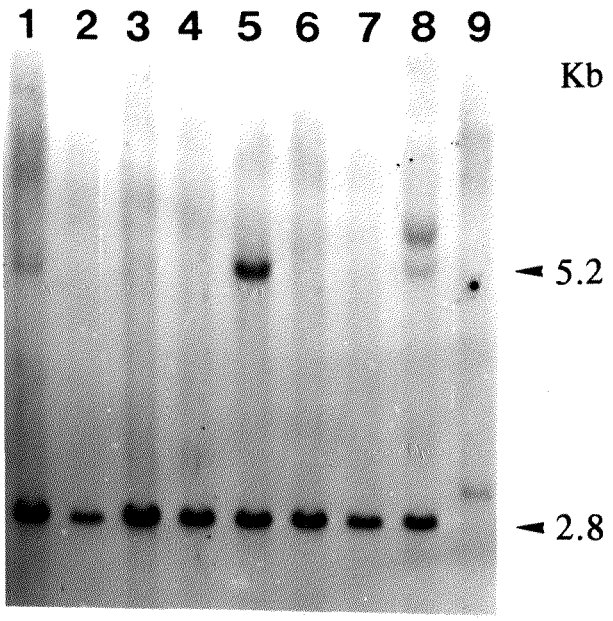


Figure 2. DNA analysis of *FMR-1*. Only partial individuals of this large family are shown. The individuals in lane 1 to 9 (from left to right) are: III-7, III-9, III-11, IV-5, III-3, IV-2, IV-3, II-6, and III-19. The corresponding sizes of the signal are shown on the right. The 2.8 Kb indicates normal active allele and the 5.2 Kb indicates normal inactive alleles. It should be noticed that premutation allele of III-19 is unmethylated (lane 9).

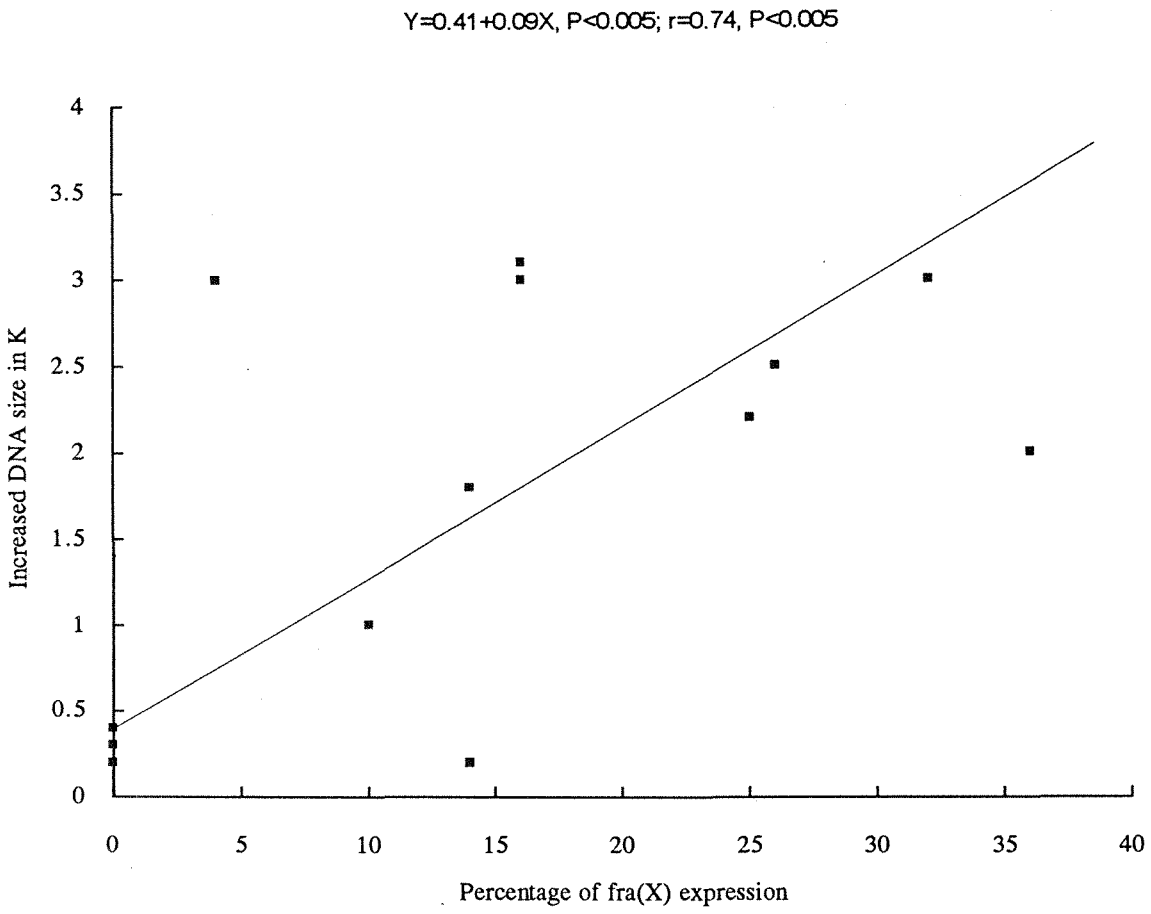
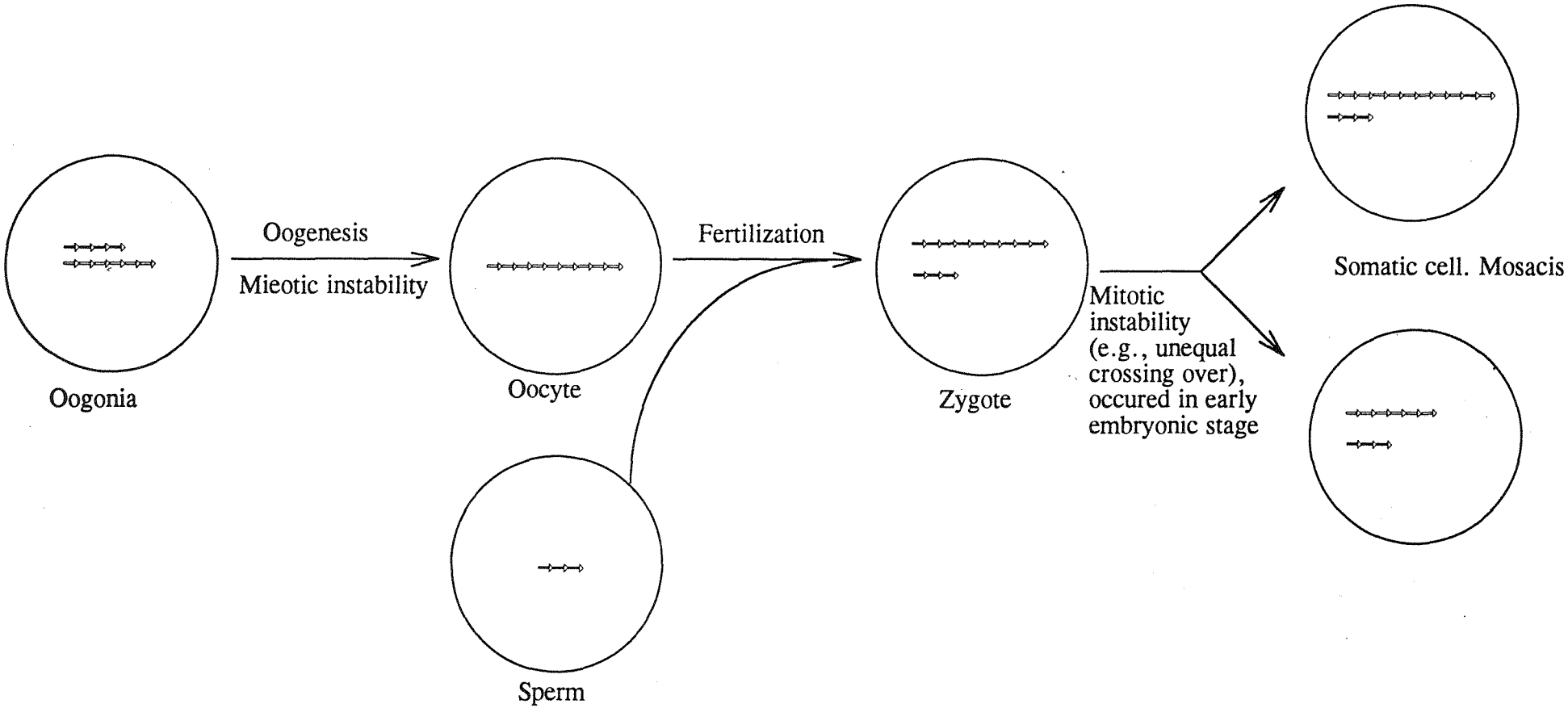


Figure 3. Correlation between p(CGG)n length increased and the percentage of cells that express the fragile X chromosome in peripheral blood. For the mosaic individuals (III-19), two bands were both scored.



圖四 嵌紋體形成之可能原因。箭頭代表CGG核酸重覆序列

Table 1. Percentage of fra(X) expression and DNA increased in kilobase of each individual.

Position of individuals in pedigree	Increased DNA size in Kb	Percentage of fra(X) expression
II-2	0.2	0
II-4	0.3	0
II-6	0.4	0
III-1	0	0
III-3	0	0
III-5	1	10
III-7	2.5	26
III-9	0	0
III-11	0	0
III-13	3	4
III-14	3	32
III-15	3	16
III-17	0	0
III-18	0	0
III-19	0.2/1.8*	14
IV-1	0	0
IV-2	0	0
IV-3	0	0
IV-5	0	0
IV-6	2.2	25
IV-7	3.1	16
IV-10	2	36
IV-11	0	0

* indicates this subject is a mosaic male.

第 四 章

反轉錄-聚合酶鏈鎖反應於易脆X染色體徵候群產前診斷應用之研究

The Study of RT-PCR in Prenatal Diagnosis of Fragile X Syndrome

中 文 摘 要

易脆X染色體徵候群於遺傳性智障中，佔有重要比例。傳統上以細胞遺傳學方法診斷此徵候群，須培養大量細胞，不僅耗時且準確性不是百分之百。Southern analysis固然能做正確診斷，但需要大量細胞來純化DNA，所以這兩種方法都不適用於檢體細胞少的產前診斷工作上。因此，發展出一套快速、準確、經濟的診斷方法，一直是我們長久以來渴望的。我們參考前人的方法，以RT-PCR來做易脆X染色體徵候群的診斷，進而應用於產前診斷。我們已用RT-PCR完成了兩例的產前診斷，證實胎兒爲帶有正常*FMR-1*基因之男性。希望經由不斷的努力改良，終能使得RT-PCR進入實用的臨床診斷階段。

英 文 摘 要 (Abstract)

Fragile X syndrome is the most common form of inherited mental retardation. Cytogenetic diagnosis is the traditional method for detecting this syndrome. However, this method needs large amount of cells to examine. Furthermore, it is time consuming and false negative results may occur in some cases. Southern analysis can give more accurate result. This method also requires a lot of cells to obtain DNA. Thus, both cytogenetic and Southern analysis methods are not suitable for prenatal diagnosis, because the specimens are not sufficient for prenatal diagnosis. Therefore, to develop a fast, accurate, and economic diagnosis method is highly desirable and long overdue. We have used RT-PCR for prenatal diagnosis of fragile X syndrome. We used this technique to perform two prenatal diagnostic cases and identified that both fetuses are normal males. By our effort, we hope that RT-PCR can be routinely used in clinical diagnosis.

序　論 (Introduction)

造成易脆X染色體徵候群的原因，主要是*FMR-1*基因第一個exon內5'端不轉譯區(untranslated region) CGG三聯核苷酸發生擴增突變(expansion mutation) [Fu et. al., 1991; Kremer et. al., 1991; Nakahori et. al., 1991; Verkerk et. al., 1991; Yu et. al., 1992]，使得起動區(promoter region)的CpG島(CpG island)發生超甲基化(hypermethylation) [Oberle et. al., 1991; Rousseau et. al., 1991; Steinbach et. al., 1993]，導致*FMR-1*基因的不表現[Bell et. al., 1991; Pieretti et. al., 1991; Sutcliffe et. al., 1992]。

以往要診斷易脆X染色體徵候群，主要是靠細胞遺傳學方法，檢視分裂中期染色體(metaphase chromosome)有無易脆X染色體(fragile x chromosome; fra(X))顯現。此法有許多技術上之困難無法克服: (1) 並非每一分裂中期細胞都能表現出易脆X染色體[Turner and Jacobs, 1983]，(2) 易脆X染色體之誘發，須將細胞培養在葉酸含量很低[Sutherland, 1977; Sutherland, 1979]或含thymidylate synthetase抑制劑FudR[Cantu et. al., 1985; Glover, 1981; Tommerup et. al., 1981]或含葉酸拮抗物Methotrexate (MTX) [Mattei et. al., 1981; Fomatch, 1981]，但這些藥物都會降低細胞分裂指數(mitotic index, MI) [Wang and Li, 1993]，增加分析上的困難，(3) 對於*FMR-1*基因之前突變者，很難觀察到易脆X染色體顯現。基於上述之理由，細胞遺傳學方法要應用在產前診斷上，著實有其困難。到了1991年，發現*FMR-1*基因後，以分子生物學的方法，提供了更精確的診斷途徑：以Southern hybridization可以更容易診斷出前突變的帶因者，唯此法需約10 μg DNA，如此大量的DNA很難直接由產前診斷的細胞樣本中獲得，除非細胞再經過培養，但這也相對的降低了診斷的時效性。再者，Southern hybridization雖然能夠診斷出前突變的基因，但是對於CGG重覆次數只增加一點點的前突變基因，則很難作正確判斷。

利用PCR來診斷易脆X染色體徵候群確實是一快速、經濟的方法，特別是應用在產前診斷上。Fu等人及Pergolizzi等人[Fu et. al., 1991; Pergolizzi et. al., 1992]已完成應用此法作易脆X染色體徵候群之診斷，並確定出正常人的*FMR-1*基因CGG序列之重覆次數介於6～54次之間，前突變基因則介於52～200次之間[Fu et. al., 1991]。但是PCR方法亦有其先天上的限制，因CGG序列本身不易複製，使得PCR不易成功。爲了克服此困難，PCR反應中常以7-deaza-dGTP取代正常的dGTP，但是含7-deaza-dGMP之DNA無法以ethidium bromide染色[Latimer et. al., 1991]，所以必須以α-^{32}P-dCTP標定DNA，最後以自動放射顯影得知產物大小。由於放射性同位素之使用無法避免，使得實驗多了一項放射性廢棄物要處理，增加實驗室之負擔。基於上述之理由，發展一與PCR同樣方便、快速、準確的方法有其實際上之需要性，所以我們參照前人的方法[Pai et. al., 1994]以RT-PCR方法看*FMR-1*基因之表現，同時也以看*HPRT*之表現作爲實驗內在控制組(internal control)。運用RT-PCR技術，我們已成功地完成了兩例的易脆X染色體徵候群之產前診斷。本章中將詳細討論PCR與RT-PCR，在實際產前診斷應用上的注意事項與可能的改進方法。

材 料 與 方 法 (Materials and Methods)

RNA之純化 (RNA Purification)

培養於T-25 flask之羊水細胞或絨毛細胞，於長至八分滿時，以ULTRASPEC™ RNA Isolation System (Biotexc Inc.)純化RNA。純化過程儘量於冰上操做，方法如下：

一、將培養瓶中之培養液儘可能倒乾。

二、加1 ml ULTRASPEC™ RNA至T-25培養瓶。

三、以微量吸管上下抽打數次，務使細胞溶解。

四、將溶液吸至1.5 ml微量離心管，置冰上5 ~ 10分鐘。

五、加200 μl chloroform，強烈振盪15秒，置冰上5分鐘。

六、於4 °C以12 Krpm離心15分鐘。

七、小心地將水層(上層)吸到新管，切勿吸到界面的雜質。

八、加入等體積之異丙醇(2-propanol)，搖勻，置冰上10分鐘。

九、於4°C以12 Krpm離心15分鐘。

十、沉澱塊(pellet)以1 ml 75%酒精洗一次，再以1 ml絕對酒精洗一次。

十一、陰乾沉澱塊。

十二、溶沉澱塊於50 μl DEPC-H_2O。

*FMR-1*之RT-PCR(RT-PCR of the *FMR-1*)

RT-PCR反應分別先完成反轉錄(reverse transcription, RT)部份，再完成PCR部份。所有引子(primer)的位置見附圖一。PCR部份又可分爲1st round PCR與2nd round PCR。

(1) RT反應如下: 反應總體積爲20 μl。2 μg total RNA，RT引子爲 5 pmol FMR-1(#4) 及 5 pmol HPRT#2 (HPRT primer; internal control)，200 μM dNTPs，1X M-MLV RT buffer，混合好後90 °C 5 min.，再加5 units RNasin (RNase inhibitor, Promega)，最後才加10 units M-MLV reverse transcriptase (Promega)，37 °C 60 min.。以95 °C 5 min.終止反應，隨即把樣本插在冰上。

(2) 1st round PCR: 1st round PCR總體積爲40 μl，反應如下: 20 μl cDNA (RT產物)，200 μM dNTPs，1X *Taq* DNA polymerase buffer，2.5 mM $MgCl_2$，5 pmol of each primer (FMRUS#1, FMR-1#4, HPRT#1, HPRT#2, 請參照附圖一)，1 unit *Taq* DNA polymerase

(Promega)。熱週期條件爲95 °C 8 min.; 95 °C 1 min.，55 °C 1 min.，72 °C 1.5 min. 30 cycles; 72 °C 10 min.。

(3) Second round PCR: 第二次PCR總體積爲20 μl，反應如下: 1 μl DNA (1st round PCR產物)，200 μM dNTPs，1X *Taq* DNA polymerase buffer，2.5 mM $MgCl_2$，5 pmol of each primer (FMRUS#5, FMRLS#6, HPRT#3, HPRT#4, 請參照附圖一)，1 unit *Taq* DNA polymerase (Promega)。熱週期條件同Nest PCR。產物以6 % 聚丙烯醯胺膠體(polyacrylamide gel)，1X TBE buffer電泳分離，最後膠體以ethidium bromide染色，並以Polaroid type 57底片照像存檔。

結　果 (Results)

在以RT-PCR作產前診斷之前，我們做了幾次預實驗(preliminary study)。實驗的材料爲培養的絨毛細胞或羊水細胞。在這些預實驗當中，我們找出最佳的反應條件，發現過多的reverse transcriptase及dNTPs反而反應不好，最適的條件爲10U的reverse transcriptase及200 μM dNTPs (圖一)。在最適的條件下，*FMR-1*及*HPRT*均可被RT-PCR放大出來，但是由血液純化的RNA則很難把*HPRT*放大出來 [Pai et. al., 1994]，即使在兩例的產前診斷中，由血液純化之RNA仍無法把*HPRT*放大出來(圖二)及(圖三)。所有的結果當中，*FMR-1*之nest PCR及2nd round PCR產物均可在膠體上輕易看到(206 bp及137 bp)。

討　論 (Discussion)

以RT-PCR來作易脆X染色體之篩檢，確實有其快速、方便的好處。我們在發展此技術的過成中，發現高濃度的M-MLV reverse transcriptase (100 units)及dNTPs (800 μM)，會影響反轉錄的DNA合成(圖一A)，最適當的條件則是10 units的reverse transcriptase及200 μM的dNTPs(圖一C)，可見過量的dNTP會抑制酵素的活性，且過量的酵素也影響自身的活性。然而在本實驗中作爲內在控制組的*HPRT*卻不易放大出來，可能的解釋是週邊血球細胞很少或不表現*HPRT*。這樣的結果容易有問題：當RNA來源爲血液，恰巧此個體*FMR-1*突變不表現，又*HPRT*也不在血球細胞中表現，此時分析膠體電泳結果將會造成困擾，因爲我們將無法區別是酵素反應出了問題或是此個體眞的是*FMR-1*基因發生突變。爲了解決內在控制組要百分之百做成功，我們設計了一組放大*RPS4X* (ribosomal protein small subunit 4 protein, in X chromosome locus)基因的引子[Fisher et. al., 1990]，目前正積極尋找反應條件。

易脆X染色體徵候群是X-linked疾病，如同其它X-linked疾病一樣，在產前診斷時，胎兒性別的偵測有其必要性，這有助於遺傳諮詢時能提供更正確的資訊。以本章之產前診斷爲例，很幸運的兩例中之胎兒均爲男性(分別經*SRY* PCR及染色體檢查確定，data not shown)。若不知胎兒性別時，我們無法判斷胎兒是正常男性或女性或是完全突變的女性，因完全突變的女性仍有一正常的*FMR-1*基因會表現，所以RT-PCR仍會做出產物。也許有人會爭論，認爲完全突變的女性其RT-PCR產物比正常女性的產物少，所以可由膠體上判斷出來。但是我們必須考慮一點，因*FMR-1*基因位於X染色體，因此受到X染色體不活化作用的影響(X inactivation)。所以，當胎兒是完全突變的女性時(實際上不清楚胎兒是否帶有完全突變基因)，僅憑RT-PCR產物量正常便說胎兒是正常女性，將是很危險的舉動。因爲很

有可能其突變基因大多位於不活化X染色體，正常基因多位於活化X染色體，就像上一章中II-6一樣，這種情況下RT-PCR之結果可能和正常人一樣。雖然此情況對個體影響不大，但是我們誤判的結果將使她以後因輕忽產前診斷，而生下有此徵候群的小孩，造成家庭不幸。綜合來講，RT-PCR要正確診斷出完全突變的女性，還是有其技術上的困難，但胎兒爲男性時，則無上述困擾，因帶有完全突變基因的男性，RT-PCR是做不出產物的。當然，連胎兒性別都不知到時，要判斷胎兒是否正常則是難上加難。

除了完全突變基因於產前診斷時，遇上胎兒是女性會有問題外，另一普遍性的問題是RT-PCR無法偵測出前突變的基因。由於前突變的基因其CGG序列只增加一點點，並未使*FMR-1*的起動區(promoter)發生甲基化，所以*FMR-1*仍有轉錄作用，因此RT-PCR有產物。要解決此問題可以靠PCR放大包含CGG序列的DNA片段(簡稱爲〝CGG PCR〞)，我們可分析胎兒*FMR-1*基因CGG序列之重複次數來判斷。但是面對完全突變基因時，我們無法僅由膠體結果分辨出胎兒是正常男性或是帶完全突變基因的女性，因爲這兩者的電泳形態(pattern)完全一樣。這再一次顯示胎兒性別的判斷，在產前診斷上的重要性。

本實驗室目前正朝CGG PCR發展中，我們須先統計出國人正常及前突變之*FMR-1*基因CGG序列之重複次數範圍，這樣才能實際應用於產前診斷。由於要建立起此項資料需要分析大量的alleles，因此這部份未能放於本論文中。

由於CGG PCR及RT-PCR在診斷上仍有其限制，因此，當知到胎兒爲女性時，應進一步以Southern analysis做驗證。我們建議CGG PCR及RT-PCR應結合運用，因CGG PCR可排除女性胎兒是帶正常或完全突變基因的困擾。即使如此，這兩種技術仍無法知到DNA的甲基化狀態，因此會失掉嵌紋體之個體(mosaic individuals)，同時也

很難對帶完全突變基因的女性胎兒做良好的預後判斷。所以，以Southern analysis做進一步驗證有其必要性。

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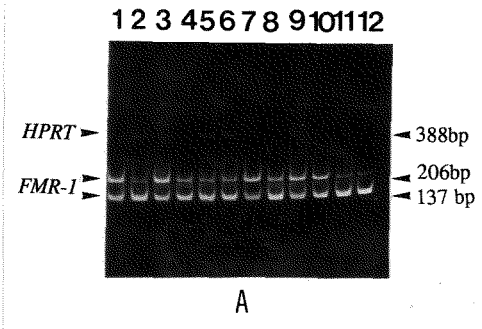
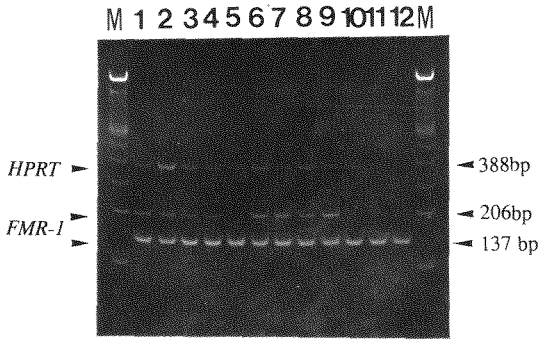


A



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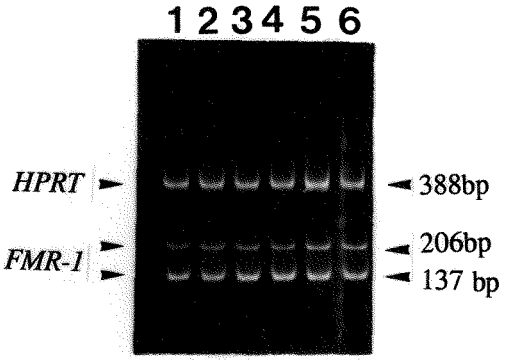
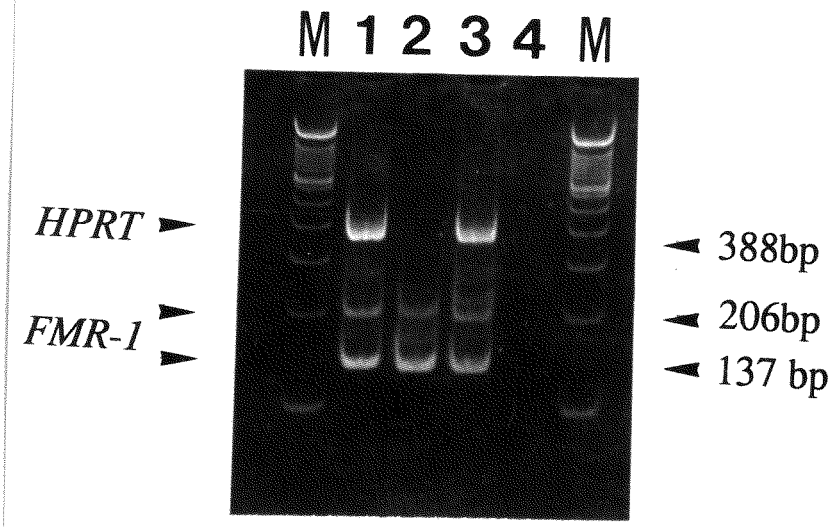
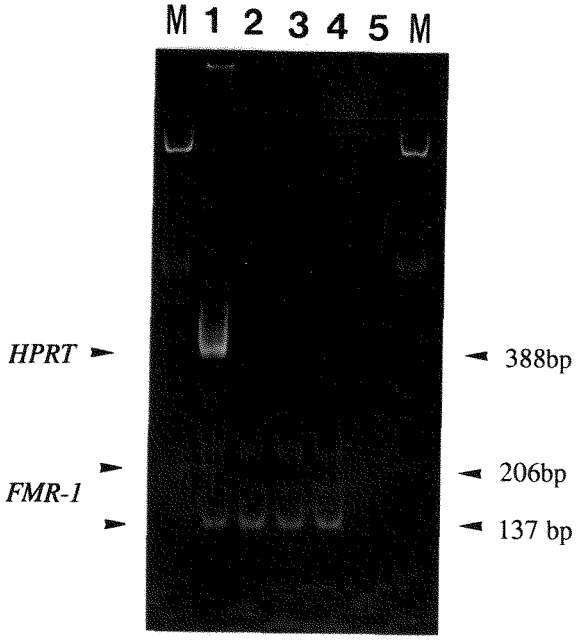


C

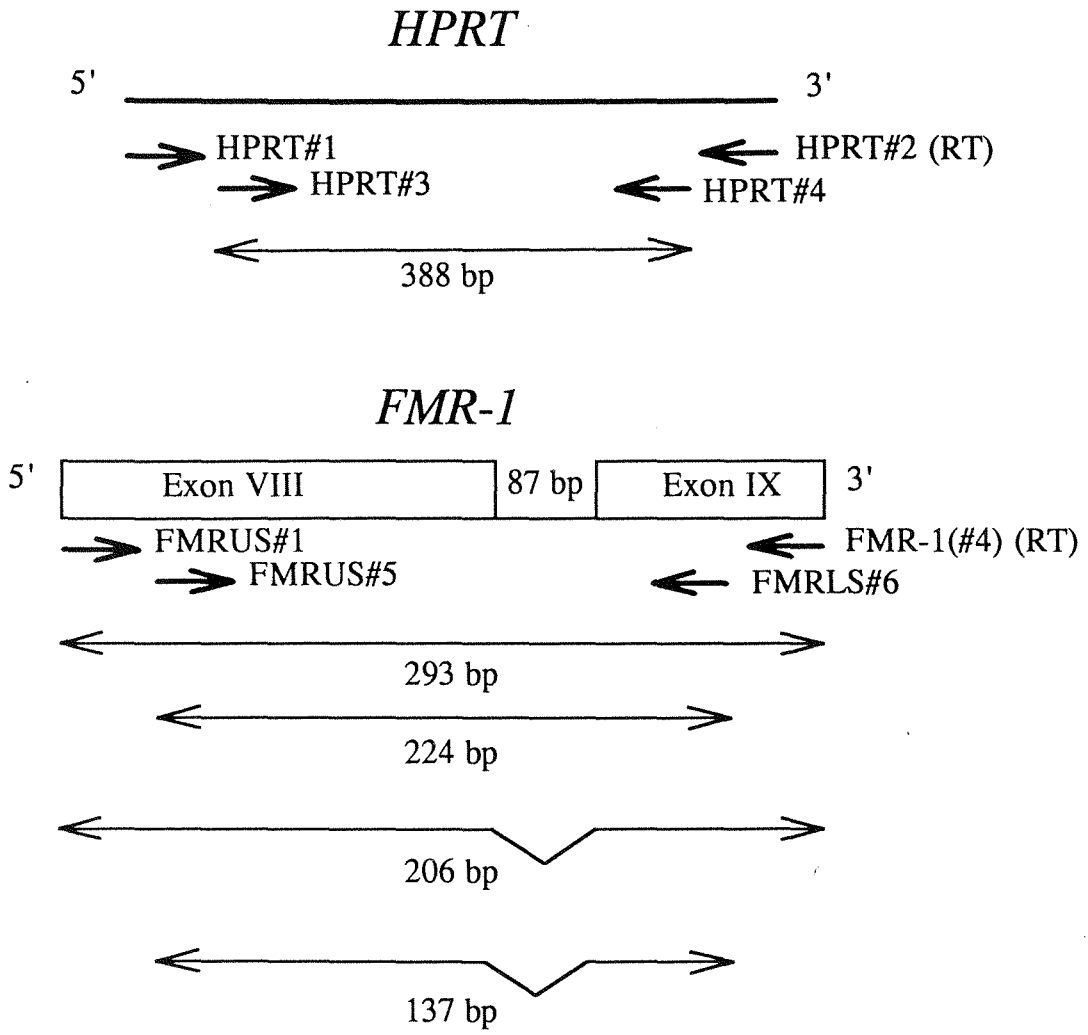
圖一 RT-PCR之預實驗。Panel (A): 100U R.T., 800 μM dNTPs; lane 1 ~ 9, cultured amniotic cells; lane 10 ~ 12, cultured villous cells. Panel (B): 100U R.T., 200 μM dNTPs; lane 1 ~ 10, cultured amniotic cells; lane 11 ~ 13, cultured villous cells. Panel (C): 10U R.T., 200 μM dNTPs; lane 1 ~ 6, cultured amniotic cells. 以Panel (C)效果最好。



圖二 產前診斷例一。lane 1, normal control (cultured amniotic cells); lane 2, BC-1080, mother of AF-134 (peripheral blood cells); lane 3, AF-134, the cultured amniotic fluid cells used in this prenatal diagnosis. M: 100 bp ladder DNA marker. 值得注意的是由血液純化的RNA無法複製出*HPRT*產物(388 bp)。



圖三 產前診斷之例二。Lane 1, AF-468, cultured amniotic cells used in this prenatal diagnosis; lane 2, mother of AF-468 (peripheral blood cells); lane 3, normal male (peripheral blood cells); lane 4, normal female (peripheral blood cells). 來自週邊血球之RNA均無法複製出*HPRT*產物。



附圖一 RT-PCR中所使用各組引子之位置與其產物之大小(Pai, et, al., 1994)。

第 五 章

總　結

Conclusion

本論文從易脆X染色體誘發方法研究開始，探討了影響易脆X染色體顯現之各種因素，並得到結論: 以細胞遺傳學方法診斷易脆X染色體時，至少要用兩種不同的誘發方法，以避免假陰性(false-negative)的結果。接著我們又探討了易脆X染色體顯現與*FMR-1*基因CGG核酸序列長度的關係，我們發現易脆X染色體顯現與CGG核酸序列長度有正相關性($P < 0.005$)。同時也對易脆X染色體徵候群之分子遺傳機制做了探討，我們的結果與前人的報告一致，不僅驗證了所謂的Sherman paradox -- anticipation phenomenon，並且對*FMR-1*基因CGG核酸序列長度，於各代傳遞間的變化，做了儘可能之詳細解釋。*FMR-1*基因甲基化狀態(methylation status)與易脆X染色體徵候群之外表型(phenotype)間，有著密切的關係，尤其是女性完全突變者(full mutation)，其外表型(智障程度)端看正常*FMR-1*基因位於活化X染色體比例而定，這也就說明了爲何帶完全突變基因之女性中，近三分之一外表正常。

本論文之最後部份，則是探討RT-PCR於易脆X染色體徵候群產前診斷可行性之評估。我們很幸運地完成了兩例的產前診斷，說幸運是因爲胎兒是男性，只帶一個X染色體，使得判讀上單純許多。我們討論了RT-PCR、CGG PCR與Southern analysis在產前診斷應用上之優缺點，並說明了胎兒性別偵測在產前診斷上的重要性。RT-PCR所能給的資訊較CGG PCR來得少，但是技術上較簡單，也沒有放射性廢棄物的問題，適合做大量篩檢及產前診斷工作。因此，我們覺得實際運用上，可先以RT-PCR過濾掉大部份的檢體，剩下的再由Southern analysis做進一步分析。

未來本實驗室除了在易脆X染色體徵候群之家族追蹤調查、產前診斷技術改良之外，還要繼續探討*FMR-1*突變的機轉，以及*FMR-1*之致病機轉。若能找出*FMR-1*突變的機轉，相信有助於我們瞭解其它和trinucleotide expansion mutation有關疾病基因(例如: Myotonic dystrophy, *DM*; Huntington disease, *HD*; Androgen receptor, *AR*;

Spinocerebellar ataxia type 1, *SCA1*)的突變原因。而瞭解其致病機轉，則可提供將來治療此疾病的方向。

附 錄 一　實 驗 步 驟

DNA探針StB12.3的製備 (Probe StB12.3 preparation)

探針StB12.3乃選殖在pBlueScrip SK-中，並以*E.coli* JM101繁殖。StB12.3的製備過程如下：挑選單一菌落(single colony)培養於3 ml LB/ampicilin borth (ampicilin 終濃度爲 50 μg/ml, Sigma) overnight，然後取1 ml加入50 ml LB/ampicilin broth中培養12 ~ 14小時。菌收穫後，以5000 rpm離心五分鐘，倒掉上清液，隨即以Qiagen column(Qiagen)進行純化，方法如下:

1. Resuspend the bacterial pellet in 10 ml of buffer P1.
2. Add 10 ml of buffer P2, mix, and incubate at room temperature for 5 min.
3. Add 10 ml of chilled buffer P3, mix, and incubate on ice for 20 min.
4. Centrifuge at 4°C for 30 min at ≧ 30,000xg.
   Remove suspernatant promptly.
5. Centrifuge at 4°C for 15 min at ≧ 30,000xg.
6. Equilibrate a QAIGEN-tip 500 with 10 ml of buffer QBT.
7. Apply the suspernatant from step 4 onto the QAIGEN-tip 500.
8. Wash the QAIGEN-tip 500 with 2 x 30 ml of buffer QC.
9. Elute the DNA with 15 ml of buffer QF.
10. Precipitate the DNA with 0.7 volumes of isopropanol.
    Centrifuge at ≧ 15,000xg at 4°C for 30 min.
11 Wash the DNA with 15 ml of cold 70% ethanol, air dry for 5 min, and redissolve in a suitable volume of buffer.

純化好的plasmid取20 μg以20U的*Pst* I於37°C水解2小時(2X overcutting)。水解後的DNA以1 % low melting point agarose gel，20

volts 0°C電泳分離，將StB12.3所在的膠體挖下，置於eppendorf離心管中。把eppendorf離心管置於60 °C水浴，待膠體溶解後加入四分之一體積的5 M NaCl，vortex，再置回60 °C水浴。以等體積的buffer saturated phenol(含0.2 % β-mercaptoethanol)粹取一次，離心14 Krpm 10分鐘，將上層吸到新管，再以等體積的phenol/chloroform(含0.2 % β-mercaptoethanol)粹取一次，以14 Krpm離心，再將水層換到新管，最後以chloroform粹取一次。DNA(水層)以0.8倍體積的異丙醇(2-propanol)沉澱，DNA以70 %及100 %酒精各洗一次，陰乾，溶DNA於100 μl TE buffer (10 mM Tris-Cl pH 8.0, 1 mM Na_2-EDTA)。

纖維母細胞DNA的純化 (Fibroblast DNA purification)

DNA的純化步驟如下：細胞培養於含有α-MEM的T-80培養瓶，於九分滿時將細胞刮下，隨即使其懸浮於10 ml solution I (10 mM Tris-Cl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$)中。以1500 rpm離心，倒掉上清液，再加入10 ml solution I。震盪，使細胞懸浮。此時加入120 ml的Nonidet P-40(NP-40, Sigma)，搖勻使細胞膜破裂。以2000 rpm離心，倒掉上清液，將細胞核(沉澱物)懸浮於300 ml solution I中，並將其轉換到eppendorf離心管，加入300 ml solution II (10 mM Tris-Cl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 1 M NaCl, 1 % SDS, 4 mM Na_2-EDTA)搖勻。待全部細胞核溶解後，以等體積的buffer saturated phenol(含0.2 % β-mercaptoethanol)粹取一次，粹取時離心管不可劇烈搖動以免DNA嚴重斷裂，以14 Krpm離心，再將水層以切口的tip轉換到eppendorf離心管，小心不要吸到界面的變性蛋白。此時以等體積的phenol/chloroform(含0.2 % β-mercaptoethanol)萃取一次，以14 Krpm離心，再將水層以切口的tip轉換到eppendorf離心管，小心不要吸到界面的變性蛋白。最後以chloroform粹取一次。DNA(水層)以0.8倍體積的異丙醇(2-propanol)沉澱，DNA以70 %及

100 %酒精各洗一次，陰乾，溶DNA於200 μl TE buffer。溶好的DNA以光度比色儀(Spectrophotometer; DU-640, Beckman)測波長260 nm的相對吸光值及260 nm/280 nm吸光比值，以便曉得DNA的質與量。

週邊血球細胞DNA的純化 (Peripheral blood cell DNA purification)

5 ml血液加5 ml solution I (10 mM Tris-Cl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$)，搖勻。加120 μl Nonidet P-40，搖勻使血球之細胞膜破裂。以下步驟同纖維母細胞DNA純化方法。

StB12.3放射性同位素標定 (Isotope labeling of StB12.3)

純化好的StB12.3以光度比色儀(Spectrophotometer; DU-640, Beckman)測波長260 nm的相對吸光值及260 nm/280 nm吸光比值，以便曉得DNA的質與量。取20 ~ 40 ng StB12.3，50 μCi α-^{32}P-dCTP放射性同位素以Nick translation Kit (Promega)，於15 °C 1小時完成標定。最後以Sephadex G-50 (Pharmacia) column分離標定之DNA。

南方氏轉印 (Southern Blot)

我們將傳統方法中的第一步驟depurination省略。Agarose gel先以0.5 N NaOH, 1.5 N NaCl處理三十分鐘，再以3 M NaCl, 0.5 M Tris-Cl pH7.0處理三十分鐘。全部過程均以shaker緩慢搖動。膠體內之DNA以PosiBlot DNA transfer System經20X SSC以90 psi，1小時，轉印在Nitrocellulose paper (S & S Inc.)。

附錄二　藥品製備

Solution	Method of preparation
10% Ammonium persulfate	To 1 g of ammonium persulfate, add H_2O to 10 ml.
0.5 M EDTA (pH 8.0)	Add 186.1 g of disodium ethylenediaminetetra-acetate • $2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.
Ethidium bromide (10 mg/ml)	Add 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.
5 M NaCl	Dissolve 292.2 g of NaCl in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
10% Sodium dodecyl sulfate (SDS)	Dissolve 100 g of SDS in 900 ml of H_2O. Heat to 68 °C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.
20X SSC	Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 liter with H_2O. Dispense into aliquots. Sterilize by autoclaving.

1 M Tris	Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl. pH HCl 7.4 70 ml 7.6 60 ml 8.0 42 ml Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.
50X TAE	Dissolve 242 g of Tris base in 800 ml of H_2O. Add 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA. Adjust volume to 1 liter with H_2O.
10X TBE	Dissolve 108 g of Tris base and 55 g of boric acid in 800 ml of H_2O. Add 40 ml of 0.5 M EDTA. Adjust volume to 1 liter with H_2O.
10X DNA loading dye	50% glycerol, 12.5 mM Tris-Cl pH 8.0, 60 mM EDTA pH 8.0, 0.01% bromophenol blue, 0.01% xylene cyanol FF.
20X SSC	175.3 g NaCl, 88.2 g Sodium Citrate.
Denhardt's solution (100X stock)	2g Ficoll (Type 400, Pharmacia), 2g BSA (Pentax Fraction V, Sigma), 2g polyvinylpyrrolidone. Add distilled water to make 100 ml.

Phenol 之再蒸餾	Phenol(和光，試藥級)以163 °C蒸餾，冷凝管以70 °C熱水冷凝，防止Phenol結晶。收集瓶預先秤重(W1)，收集後的瓶子再秤重(W2)，求出Phenol體積[(W1-W2)/1.071]。加入1/10 X體積的蒸餾水，標示好Phenol體積及日期，儲存於-80 °C冰箱。
Phenol/ Chloroform	68 °C水浴溶解Phenol，分裝至50 ml離心管，每管15 ml。加chloroform 14.4 ml，isoamyl alcohol 600 μl。加β-mercaptoethanol (β-MSH)至終濃度爲0.2%。
Buffer saturated phenol	精製好的Phenol以68 °C水浴溶解。以等體積的1 M Tris-Cl, pH 8.0萃取一次，再以等體積的0.1 M Tris-Cl, pH 8.0萃取三次，直到水層之pH>7.6。加β-mercaptoethanol (β-MSH)至終濃度爲0.2%，並保留部分buffer覆蓋Phenol。
LB borth (Luria-Bertani medium)	Per liter: To 950 ml of deionized H_2O, add: bacto-tryptone 10 g bacto-yeast extract 5 g NaCl 10 g Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume to 1 liter with deionized H_2O.

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附 錄 三　儀 器 設 備

CO_2 incubator	Nuaire
Laminar flow hood	Bellco Glass Inc.
DNA Thermal Cycler	Perkin-Elmer Cetus Inc., Ericomp.
Power Supply	Pharmacia LKB Inc.
Horizotal Gel Electrophoresis Tank	BRL
Vertical Gel Electrophoresis Tank	BRL
PosiBlot DNA Transfer System	Stratagene
Microscope	Zeiss
Microfuge	Eppendorf
Microfuge with refrigerator	Hitachi

附 錄 四　本論文第二章已發表部份之摘印

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MOLECULAR AND CYTOGENETIC DETECTION OF THE FRAGILE X CHROMOSOME IN FIBROBLAST CULTURE

YI-CHUN WANG, AND SHUAN-YOW LI

The fragile X syndrome is one of the most common inherited forms of mental retardation. Prenatal diagnosis of fetus with this syndrome is of essential counselling importance clinically. Unfortunately, most attempts to use cytogenetic preparations of fetal blood lymphocytes, amniotic cells or chorionic villi as a prenatal diagnostic method were not impressively successful. The present study was thus aimed to develop an unequivocal, reproducible and less invasive diagnosis technique which might be later applied for prenatal diagnosis of the presence of fragile X chromosomes. We demonstrated that the expression of fragile X chromosomes were inducible with treatments of fluorodeoxyuridine (FudR) or methotrexate (MTX) to fibroblasts cultured in different media containing different inducing agents expressed their fragile X chromosomes with great differences. The observed great difference in inducibility of fragile X chromosomes might well be one of the major reasons for failure in similar studies done by others. To validate our data from cytogenetic preparations, we also used the genomic DNA probe StB12.3 from the gene fragile X related mental retardation-1 (FMR-1) to detect the methylation status of CpG island and the CGG repeats increased in FMR-1 in fibroblasts from same patients. A perfect match between data from cytogenetic and molecular detections was observd. With great confidence, we reported the development of a cytogenetic detection method and its potential application for prenatal diagnosis of fragile X sydrome.

Key Words: Fragile X chromosome, fra(X), mitotic index, FMR-1, CpG island, CGG repeat, prenatal diagnosis

INTRODUCTION

The fragile X syndrome, also known as the Martin-Bell syndrome, is the most common inherited form of mental retardation, second only to Down syndrome. Since the fragile X syndrome has familial inheritance whereas the majority of Down syndrome are sporadic and not inherited [1, 2, 3]. Thus the importance of fragile x syndrome is more significant than that of the Down syndrome.

According to population surveys conducted in Sweden and Fingland [4, 5], the prevalance of affected males has been estimated as approximately 1 in 1250, while that of nonpenetrant males has been estimated as 1 in 5000, leading to an overall prevalence of about 1 in 1000 among males. Among female carrier, approximately one-third have some degree of mental impairment or mental retardation [6]. The population prevalence of affected females is approximately 1 in 2000 [7]. Thus the overall prevalence of female

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carriers is estimated to be about 1 in 700. Based on these estimations, it appears that about 1 in 850 people carries the fra(X) chromosome [8]. Therefore, the understanding and detection of fragile X syndrome are of essential important for prevention and tracing of this syndrome.

Molecular techniques in diagnosing the fragile X syndrome are cheaper and faster than cytogenetic techniques. Usually, they give more accurate results, but some difficulties remain to be overcome when used for prenatal diagnosis. Firstly, large amount of cells are needed when preparing genomic DNA for Southern hybridization. Secondly, for some premutation individuals, the increased CGG repeats of *FMR-1* gene can not be easily distinguished from normal *FMR-1* gene. Therefore, a more efficient and reliable method is still wanted. Our laboratory is now developing polymerase chain reaction (PCR) for prenatal diagnosis.

Cytogenetically not every cell in metaphase will show its fra(X) chromosome even if they are from a patient with the syndrome. For example, lymphocytes from fragile X patients rarely express fra(X) chromosomes over 50% [1]. Factors such as tissue origins, culture medium all have been implicated to affect the frequency of fra(X) expression. To induce fra(X) expression by cytogenetic method, the medium should be low in folic acid [9, 10]. For fra(X) induction in lymphocytes, the depletion of folic acid from culture medium or the addition of thymidylate synthetase inhibitor (e. g., FudR) [11, 12, 13] or folic acid antagonist methotrexate (MTX) [14, 15] will reduce thymidine synthesis. On the other hand, addition of a large amount of thymidine will interfere the balance between purine and pyrimidine and thus induces fra(X) [16]. The efficacy in inducing fra(X) expression of methods described above, however, is greatly tarded off by their reduction of overall mitotic index (MI) which increase the difficulty of analysis. This might be accounted for the reason why it is not widely used in prenatal diagnosis. The earliest report on using amniotic fluid of prenatal diagnosis of fra(X) was by Jenkins and coworkers in 1981. Later, Webb and his colleagues reported that they had performed the first time prenatal diagnosis of fra(X) by using fetal blood lymphocytes. In 1982, Shapiro et al. [17] successfully demonstrated the prenatal diagnosis of fra(X) using amniotic cells. However, only few reports on fra(X) prenatal diagnosis have been published since then [18, 19], reflecting difficulties of fra(X) induction. Several laboratories had demonstrated the prenatal diagnosis of fra(X) with uncertainties of false negative or false positive results. Thus, a simple and reproducible fra(X) induction method is highly desirable and long overdued. Previously our laboratory has established the techniques for fra(X) induction in lymphocytes [3], and we are now in developing the process of developing techniques for prenatal diagnosis of fra(X). An ideal method for prenatal diganosis should work with amniotic fluid cells and chorionic villi cells. Therefore, induction of fra(X) expression in fibroblast seems to be the first touch-stone in developing prenatal diagnosis. There are several methods avialable for inducing fra(X) in fibroblast [14, 15, 20]. We established primary cultures of dermal fibroblasts from five fragile X patients, CT-077, CT-119, CT-337, CT-355, and CT-441 [3]. To develop basic techniques for prenatal diganosis, the fibroblasts were first treated with MTX and FudR, then the method which induced the highest frequency of fra(X) expression was chosen. Results obtained from our studies show that FudR and MTX would induce fra(X) expression equally well in fibroblast but the inducibility and expression of fragile X chomosomes differs greatly from one to each other based on difference in culture media used and inducing agents added.

To confirm our obtained from studies on fra(X) induction, we also conducted parallel molecular assay using the probe StB12.3[from Mandel; 21], a genomic DNA sequence from *FMR-1* [22], to examine these five samples. Our data are consistent with previous reports [23; 24]. As expected, all five samples have increased CGG repeats and hypermethylation in CpG island of *FMR-1*. We have developed an easy, less invasive and reproducible cytogenetic method to induce expression of fra(X) in fibroblasts for potential use in prenatal diagnosis of the syndrome. Details of our studies are reported herein.

MATERIALS AND METHODS

Primary culture of fibroblasts Primary culture of fibroblasts were initiated from skin biopsy samples from five patients with the fragile X syndrome (see Table 1 for the corresponding percentage of fra(X) at Xq27.3 as shown by lymphocyte karyotyping) according to standard tissue culture procedures. In brief, a piece of skin tissue, about 25-mm^2, was excised aseptically from each patient. Sample tissues were further cut into smaller pieces with scalpel blades. The stromal portions were digested with collagenase type 1A in α-MEM (2 mg/ml, Sigma) at 37°C, with 5% CO_2 atmosphere and high relative humidity for 1 hour. To ensure better yield of single cell, suspensions, a second 1-hr incubation in 2ml 0.05% Trypsin-0.53 mM EDTA in Hank's balanced salt solution, was carried out under the same condition. Following the incubation, the tissues sluries were dispersed through a 19 Gauge needle. Single cell suspensions were then plated out in Nunc T25 tissue culture flasks. Once primary cultures reached confluency, fibroblasts were subcultured and maintained in six different culture media, including Medium 199, MEM,

Table 1. Percent breakage at Xq27.3 in lymphocyte culture

Subject	Sex	Fra(X)/total cell	Fra(X)%
CT077	Male	14/100	14
CT-119	Male	8/250	3.2
CT-337	Male	5/178	2.8
CT-355	Male	15/200	7.5
CT-441	Female	10/100	10

Table 2. Media and reagents used in Fra(X) induction

No.	Medium	Reagents(final 24 hours)
1	Medium 199[a] + 5% FCS	0.1 μ M FudR or 0.1 μ M MTX
2	Mem + 10%FCS	0.1 μ M FudRor 0.1 μ M MTX
3	RPMI-1640 + 10% FCS	0.1 μ M FudR or 0.1 μ M MTX
4	MEM-FA[b] + 10% FCS	0.1 μ M FudR or 0.1 μ M MTX
5	HAM's F-10 + 20% FCS	0.1 μ M FudR or 0.1 μ M MTX
6	M medium[c] +4.5% FCS	0.1 μ M FudR or 0.1 μ M MTX

a: Medium 199 contain folic acid 0.01 mg/ ml.

b: MEM-FA without folic acid.

c: M medium without hypoxanthine, folic acid, and thymidine.

RPMI-1640, MEM-FA, Ham's F-10 and M medium each supplemented with various concentration of fetal bovine serum (see Table 2 for detail).

Induction of fra(X) in fibroblast To induce breakage at Xq27.3, fibroblasts from each patient were treated with either 10^{-7} M of FudR or 10^{-7} M MTX 24 hours before harvest. About 4~5 hours before harvest, colcemid was added to a final concentration of 0.05 μg/ml. Fibroblasts were then harvested, fixed, and processed for Giemsa karyotyping as described elsewhere. Approximately 50~100 G-banded chromosomes were examined and mitotic index of each sample was obtained by counting 2000 cells.

***FMR-1* detection** To verify the cytogenetic data on fragile X expression in fibroblasts grwon in different culture media as described above, molecular detection of correlation between copies of CGG repeats and degree of methylation in CpG island was also conducted by Southern blotting of corresponding DNAs molecules. Briefly, DNAs were purified from cultured fibroblasts and 10 μg of DNA of each sample was digested with *Eco* RI and *Eag* I. After fractionated in a 0.8% agarose gel, the DNAs were transferred onto a nitrocellulose membrane. The blot was hybridized with ^{32}P-labeled StB12.3 genomic DNA probe overnight in 6X SSC, 0.5% SDS, and 5X Denhardt's solution. The blot was washed to the final concentration of 0.2X SSC, 0.1% SDS at 65℃.

RESULTS

Medium 199, MEM, and RPMI-1640 with the addition of FudR or MTX have higher fra(X) expression, but the fra(X) expressivity is low or has no fra(X) induction in F-10 with addition of FudR or MTX, however, the MI is higher in F-10 group (Table 3). CT-355 and CT-441 have higher fra(X) expression in medium 199, MEM, and RPMI-1640 with addition of FudR and MTX. CT-077 also has higher fra(X) expression in medium 199, MEM and RPMI-1640 with addition of FudR but it does not with addition of MTX. In M medium culture, CT-355 has higher fra(X) expression either with addition of FudR or MTX, but CT-077 and CT-441 fra(X) expression and MI are both very low. CT-119 fra(X) expression has no remarkable change in media with addition of FudR, except F-10 medium, and with RPMI-1640 plus MTX, CT-119 has the highest fra(X) expression. CT-337 in medium 199, MEM with addition of FudR has higher fra(X) expression. In F-10 with addition of FudR or MTX, all five patients' fibroblasts fra(X) induction is very low or have no induction, however, the MI is higher than other groups.

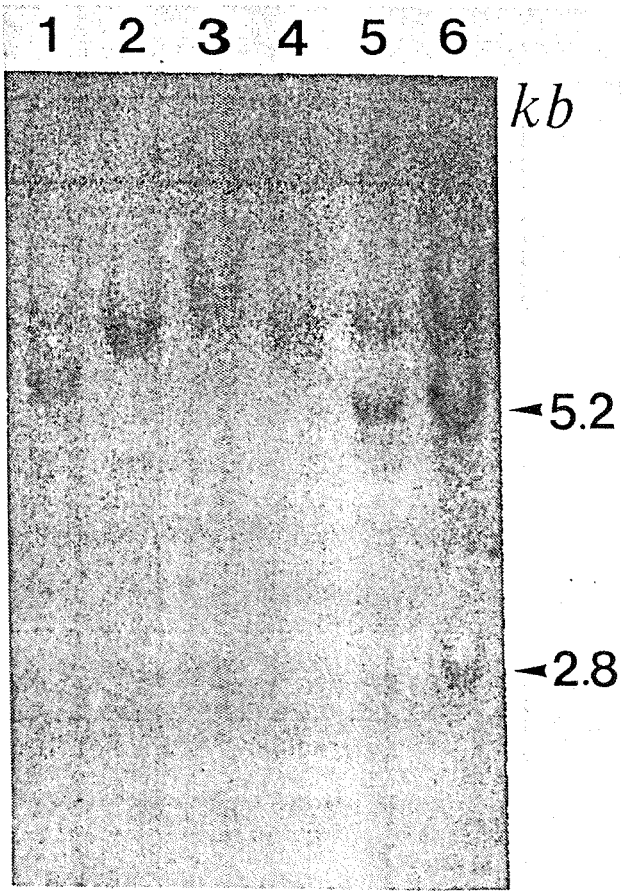

Fig. 1. DNA analysis of *FMR-1* in five fragile X syndrome patients. Lanes 1-4 (male patients): CT-077, CT-119, CT-337, and CT-335. Lanes 5-6 (female patient): CT-441. DNA sample of lanes 1 to 5 are come from fibroblast and DNA of lane 6 is originated from blood sample. The corresponding sizes of the signal are shown on the right.

Table 3. Expression of fra(X)(q27.3) in fibroblasts from five affected individuals

Case No.	Culture media*	Control			FudR			MTX		
		Fra(X) cells	Fra(X) %	MI** %	Fra(X) cells	Fra(x) %	MI %	Fra(x) cells	Fra(X) %	MI %
077	1	0/100	0.00	1.4	13/15	25.49	0.7	1/59	1.69	0.5
	2	0/100	0.00	3.9	4.56	7.14	0.4	2/59	3.39	0.8
	3	0/100	0.00	4.5	9/100	9.00	0.2	0/50	0.00	0.2
	4	0/100	0.00	2.8	1/63	1.59	0.3	0/63	0.00	0.3
	5	0/100	0.00	5.5	0/100	0.00	3.7	1/50	0.00	0.8
	6	0/100	0.00	3.6	5/100	5.00	0.8	2/100	2.00	0.8
119	1	0/100	0.00	3.6	5/100	5.00	0.8	2/100	2.00	0.8
	2	0/100	0.00	3.0	6/100	6.00	0.7	5/100	5.00	0.6
	3	0/100	0.00	5.0	5/100	5.00	0.6	10/100	10.00	0.8
	4	0/100	0.00	3.0	6/100	6.00	0.4	3/100	3.00	0.5
	5	0/100	0.00	5.0	0/100	0.00	4.0	4/100	4.00	3.0
	6	0/100	0.00	4.0	8/100	8.00	0.5	2/100	2.00	0.4
337	1	0/100	0.00	2.5	6/100	6.00	0.6	2/100	2.00	0.5
	2	0/100	0.00	4.5	5/100	5.00	0.6	3/100	3.00	0.7
	3	0/100	0.00	5.0	2/100	2.00	0.5	1/100	1.00	0.6
	4	0/100	0.00	3.0	3/100	3.00	0.4	1/100	1.00	0.5
	5	0/100	0.00	5.6	1/100	1.00	4.0	2/100	2.00	3.0
	6	0/100	0.00	4.0	2/100	2.00	0.6	1/100	1.00	0.5
355	1	0/100	0.00	1.7	31/100	31.00	0.5	13/100	13.00	0.9
	2	0/100	0.00	3.2	28/100	28.00	0.6	12/100	12.00	0.8
	3	0/100	0.00	3.2	25/61	40.98	0.8	6/55	10.90	0.2
	4	0/100	0.00	2.6	19/100	19.00	0.6	6/71	8.45	0.3
	5	0/100	0.00	5.9	1/100	1.00	4.5	0/100	0.00	2.0
	6	0/100	0.00	2.5	16/100	16.00	0.5	16/100	16.00	0.4
441	1	0/100	0.00	4.7	11/46	24.00	0.2	21/100	21.00	0.6
	2	0/100	0.00	5.4	15/100	15.00	0.4	27/100	27.00	0.6
	3	0/100	0.00	4.0	16/100	16.00	1.4	21/78	24.14	0.6
	4	0/100	0.00	3.3	8/58	13.79	0.6	6/50	12.00	0.4
	5	0/100	0.00	5.6	0/100	0.00	5.4	1/100	1.00	4.0
	6	0/100	1.00	4.0	5/38	13.16	0.2	1/100	1.00	0.2

*See Table 2.

**Mitotic index was based on the analysis of at least 2,000 cells/ culture.

FMR-1 analyzed in these five patients' fibroblasts revealed that the CpG island is hypermethylated and the CGG repeats increased (Fig. 1). The result showed that the normal male and female carry the normal signal at 2.8 Kb (which represents the normal active allele; it can be detected in both male and female) and 5.2 Kb (which repressents the normal inactive allele; it can be detected only in female). The four fragile X syndrome males (lane 1 to lane 4) only showed one band with various sizes, however, all of them are larger than 5.2 Kb. Those bands represented the hypermethylation (inactivated) genes with different CGG trinucleotide repeats. The female patient showed two bands. One 5.2 Kb which represented the normal inactive allele and a 5.8 Kb band, which represents the hypermethylated and increased in CGG repeats in mutated allele. The absence of 2.8 Kb signal in lane 5, which represents the normal active allele may be due to the nature of female mosaicism. However, blood DNA of the same patients (lane 6) represents the 2.8 Kb restriction fragment.

DISCUSSION

Our studies have demonstrated that both FudR and MTX, under the conditions described above, are capable of inducing expression of fragile X chromosomes in fibroblasts from patients with the syndrome. However, it should be noted that the efficacy of inducing fra(X) expression of FudR and MTX seems to be highly variable among the six culturing media used. As shown in Table 3, the expressivity of fra(X) differs greatly when fibroblasts from the same patients cultured in different media or when fibroblasts from different patients cultured in the same medium.

Routine cytogenetic procedures for inducing fra(X) expression in fibroblast, amniotic cells, or chorionic villi cells has not been established for the following technical reasons. (1) The culture condition for fra(X) expression usually slows down cell groth with decreasing MI and thus fewer cells in metaphase are available for examing to start with. (2) Since the fra(X) expression is usually very low, it takes a large number of cells for an accurate diagnosis. (3) Some non-specific telomeric structural changes (TSC) might be misdiagnosed as fra(X) leading to false-positive results. Nevertheless, mistakes of this kind are circumvented in the method developed by us, since by G-banding of metaphase chromosomes we can examine X chromosome clearly.

It is generally true that media not containing folic acid, hypoxanthine, and thymidine such as M medium alone are not very effective in inducing fra(X) expression in fibroblast. In contrarst, for fra(X) expression in lymphocytes, only the cells cultured in medium with very low content of folic acid or even without it, e. g. medium 199 and M medium, are inducible [9, 10]. This difference might be due to the intrinsic property of fibroblasts and lymphocytes, or simply we have not yet find the optimum culture conditions to reconcile the differences. Ideal media for fragile sites induction are principally deprived of folic acid, having high pH and low in serum[10]. Thus, from the view of biochemistry, it seems to be the DNA synthesis in a restricted dTMP pool cause fra(X) expression. The overall biochemical mechanism of fra(X) induction is shown in Fig. 2.

In 1991, several laboratories had cloned both the cDNA and genomic DNA of fragile X gene, *FMR-1* (21; 22; 26). We got the genomic DNA probe StB12.3 kindly provided by Dr.Oberlé By using *Eco* RI and *Eag* I double digestion of genomic DNA, we demonstrated that all four male subjects' *FMR-1* have increased CGG repeats number and hypermethylation in the *FMR-1* 5' CpG island also. The female subject showed a band in 5.2 Kb that corresponds to normal inactive X and a higher smear-like band that represents the mutated *FMR-1*. However, the 2.8 Kb band corresponding to normal active X was absent in fibroblast DNA. One reasonable explanation to the fact is the cells were from a tiny region in skin area and the cells in this region are all mutated *FMR-1* in active X and normal *FMR-1* in inactive X. Blood DNA has this 2.8 Kb fragment that confirms our assumption beacuse the lymphocytes are heterogeniety.

In this study we demonstrated that the cyto-

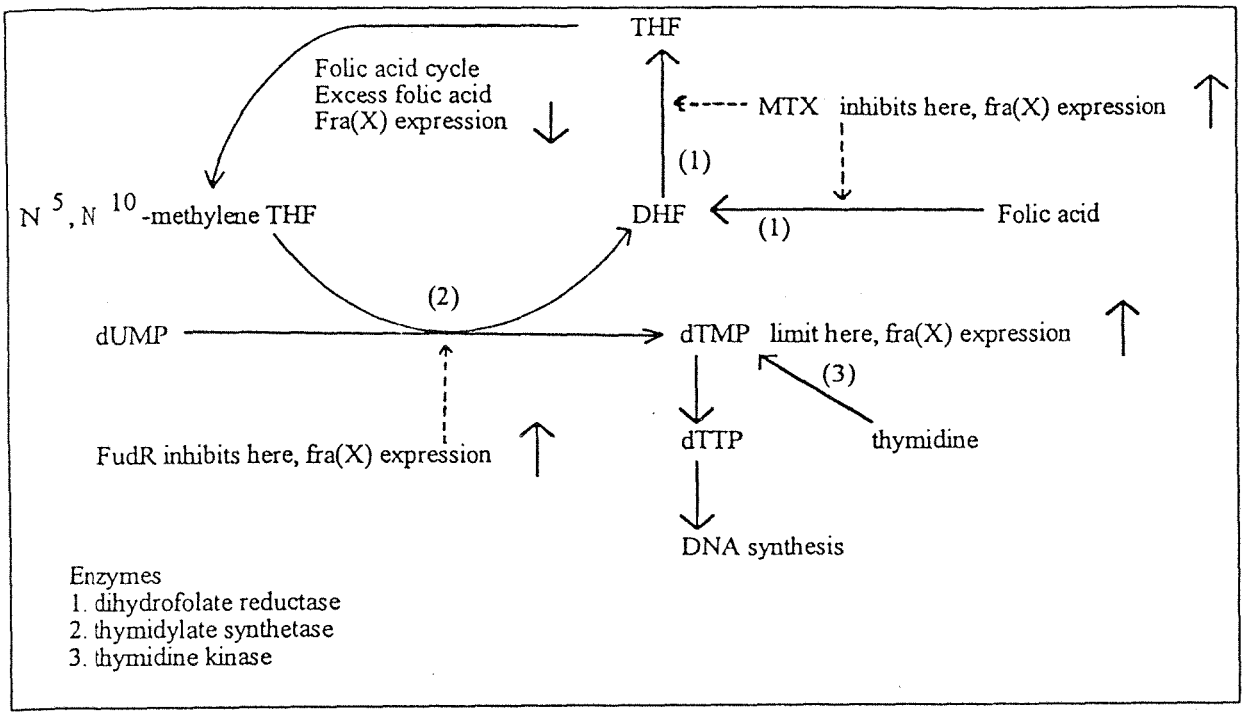

Fig. 2. The area of metabolism affected by inducers and inhibitors of fragile X expression.

genetic method to detect fragile X has a major bias that depends on the cell type and culture condition. We suggest that for diagnosing fra(X) should take at least two induction methods to avoid false-negative results. Molecular thecniques can get accurately diagnose, but there are some difficulties for prenatal diagnosis. First, Southern hybridization needs large amount of cells for genomic DNA preparation; second for some premuation persons, the increased CGG repeats of *FMR-1* gene could not be easily distinguished from normal *FMR-1* gene. Therefore, a more efficient and reliable methods should be developed. Our laboratory is now developing polymerase chain reaction (PCR) for prenatal diagnosis.

Molecular techniques in diagnosis of the fragile X syndrome are indeed cheaper, faster, and more accurate than cytogenetic techniques, however, it can fail to detect the other cytogenetic abnormalities. Thus, diagnostic laboratories should take these two methods at least. Once the fragile X syndrome has been detected in a proband, the family members should be diagnosed by molecular methods rather than cytogenetically.

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附 錄 五　本論文第三章已發表部份之摘印

A study of fragile X chromosome expression and p(CGG)n amplification in *FMR-1* gene in a large family

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Abstract

The fragile X syndrome is the most common cause of hereditary mental retardation. The gene, fragile X mental retardation -1 (*FMR-1*), has been identified to be associated within this syndrome. Amplification mutations in CGG repeats in the 5' untranslated region (5' UTR) of the *FMR-1* give rise to this phenotype. The purpose of this study was aimed to test the phenomenon of Sherman paradox and to check the relationship between the fra(X) expression and p(CGG)n expansion. In a large fragile X family, twenty three individuals were cytogenetically examined for fra(X) expression and molecular analysis of p(CGG)n amplification in *FMR-1* were also performed. Of these individuals, eleven are normal, three individuals have only CGG amplification in *FMR-1*, and nine of them have both CGG amplification and fra(X) positive. We demonstrated that the DNA increased in CGG repeat has positive correlation with fra(X) expression, and anticipation phenomenon also seen in this large family.

Key words

Fragile X syndrome, *FMR-1*, CpG island, CGG repeat, normal transmitting male, anticipation

INTRODUCTION

The fragile X syndrome is the most common form of hereditary mental retardation and the second most frequent cause of mental retardation after Down's syndrome [1, 2, 3]. It is an unusual X-linked disorder, since 30% of females who carry the mutated allele show some degree of mental retardation and 20% of males who carry the fragile X chromosome are phenotypically normal [4, 5]. Further study of genomic DNA spanning the fragile site has identified a gene, *FMR-1*, that strongly associated within this syndrome. Sequence analysis of *FMR-1* gene has also been shown that there are unstable tandem repeats of CGG trinucleotide in the first exon of *FMR-1* in fragile X patients [6, 7, 8, 9, 10]. The number of p(CGG)n repeats appears to be correlated with the extent of mutagenicity, ranging from 6 ~ 54 in normal alleles to 52 ~ 200 in premutation alleles, while repeats greater than 200 copies were found in full mutation alleles.

The males with small p(CGG)n expansion (premutation) are phenotypically normal (normal transmitting males; NTMs) and cytogenetically fra(X) negative. The mutated allele of NTMs can pass through to their daughters who also are nonpenetrant (female carriers) and cytogenetically fra(X) negative with no significant p(CGG)n expansion. But grandsons of these NTMs are often affected, the size of CGG repeat enlarged dramatically (full mutation), and cytogenetically fra(X) positive. The situation in females with a large expansion is more complicated, it may be fra(X) positive or negative and mentally impaired or normal [11]. However, the risk of mental impairment in fragile X pedigrees is likely upon position of individuals in pedigrees. That is, the later generation in pedigree the more severe mental impairment. This was first noticed and discerned by Sherman and became known as the anticipation phenomenon [4, 5].

The exact mechanism(s) causing the fragility of the fra(X) chromosome and functional roles of *FMR-1* gene product in mental retardation remain to be elucidated. However, it has been demonstrated that the lengthened p(CGG)n will

specifically induce methylation in a CpG island 5' adjacent to these repeats. Methylation at this CpG island correlates with loss of expression of the *FMR-1* mRNA [12, 13, 14]. It appears likely that the *FMR-1* CGG amplification mutation induces methylation of the region that down regulates *FMR-1* expression, resulting in disease.

The expression of the fra(X) chromosome is rarely over 50%. Our recent observation [15] and data from others [16, 17, 18, 19, 20, 21, 22, 23] have demonstrated that the cytogenetic methods used to detect fragile X chromosomes have a major bias that depends on the cell type and culture conditions. Another obvious difficulty in the study of fragile X syndrome is that a complete pedigree with adequately large numbers of individuals having similar genetic background is relatively uncommon. In one of our recently studied cases, we took the rare opportunity of an affected large family with all twenty three members of four generations available for investigation to test the Sherman paradox and correlation between CGG expansion and fra(X) chromosome expression.

Twenty three individuals in the same pedigree were examined cytogenetically for fra(X) expression and p(CGG)n expansion in their *FMR-1* genes were also detected by probe StB12.3. Of the twenty three subjects, eleven are normal, three have only CGG amplification in *FMR-1*, and nine of the rest have both CGG amplification and fra(X) positive. From the data obtained in this large family, we observed the anticipation phenomenon with incomplete penetrance. Using linear regression analysis, we also observed a strong positive correlation ($P < 0.005$) between these two phenotypes, i.e. individuals having increased DNA length in CGG repeats will have increased frequency of fra(X) expression.

MATERIALS AND METHODS

Examined subjects

The subjects in this study are from the same large family (Fig. 1). Twenty three individuals we examined were II-2, II-4, II-6, III-1, III-3, III-5, III-7, III-9, III-11, III-13, III-14, III-15, III-17, III-18, III-19, IV-1, IV-2, IV-3, IV-5, IV-6, IV-7, IV-10, and IV-11.

Induction of fra(X) in peripheral blood cell

10 ml peripheral blood was withdrawn in a heparin-containing syringe. 5 ml blood was cultured in M medium. About 4～5 hours before harvest, colcemid was added to a final concentration of 0.05 μg/ml. Peripheral blood cells were then harvested, fixed, and processed for Giemsa karyotyping as described elsewhere. At least 100 G-banded metaphases were examined.

FMR-1 detection

DNAs were purified from 5 ml freshly withdrawn blood. 10 μg of DNA of each sample was digested with *Eco* RI and *Eag* I. After being fractionated in a 0.8% agarose gel, the DNAs were transferred onto a nitrocellulose membrane. The blots were then baked at 80 °C, 1 hour and hybridized with ^{32}P-labeled Stb12.3 genomic DNA probe, a gift from Oberle [24] overnight in 6X SSC, 0.5% SDS, and 5X Denhardt's solution. The blots were washed to the final concentration of 0.5X SSC, 0.1% SDS at 65 °C. The blots were autoradiographed between two intensifying screens under -80 °C for about 3～5 days.

RESULTS

Twenty three persons of a large family were cytogenetically examined for fra(X) expression and their DNAs were also analyzed for *FMR-1* CGG expansion mutation (Fig. 1). Of these individuals, eleven are normal, nine are affected, and three are carriers. The proband, IV-10, is the fragile X patient leading to the recognition of this family. Table 1 showed the percentage of fra(X) expression and CGG repeats increased in kilobase of each individual. All molecular data were calculated according to the semi-log plot of marker DNA fractionated on the same gel. Therefore, even a very small increase in size can be noticed and estimated the size of each band accurately (Fig. 2).

II-2, II-4, and II-6 are cytogenetically fra(X) negative. They have one premutation allele that passed through to their offspring and the p(CGG)n repeats in their offspring increased dramatically, causing them been affected. One remarkable exception is that subject III-5 has one allele with 1 Kb expansion (data not shown) which is scored as full mutation in terms of molecular biology, and her blood cells showed 10% fra(X) expression as well. However, only mild mental impairment is observed as compared with other full mutation individuals.

In this pedigree, the extent of p(CGG)n repeats becomes greater and greater through generations. One exception to this aspect was the subject IV-10 who has less p(CGG)n repeats (Δ = 2 Kb) than the mutated allele from his mother (Δ = 3 Kb). From these data, we confirmed the anticipation phenomenon as referred by Sherman. Regression analysis showed a significant linear relationship between CGG repeats increased and percentage of fra(X) expression ($P < 0.005$) (Fig. 3).

Interestingly, the individual III-19 has a band with 0.2 Kb increasing and an additional smear band in average size of 1.8 Kb (Fig. 2). This individual shows one premutation allele in some cells and full mutation allele in others indicated that he is a mosaic.

DISCUSSION

Taking advantages of the availability of a large family with members diagnosed as having fragile X syndrome, we observed the so-called Sherman paradox in the pattern of fra(X) expression and p(CGG)n expansion among generations. Namely, there is an almost predictable tendency of increasing severity of mutated alleles taking place from generation to generation. Furthermore, we found that the length of tandem repeats of p(CGG)n seems to be positively correlated with the fra(X) expression and the severity of mental impairment.

In the present study we found three female carriers, II-2, II-4, and II-6 who are fra(X) negative but with small increase size in CGG repeats (termed "Δ"). We would like to refer the individual III-5 as a full mutation rather than a carrier (premutation). Since she has manifested most of the basic criteria for fragile X syndrome: fra(X) expression and p(CGG)n amplification, albeit the subject shows nearly normal mental status. This is a unique but understandable case, since only about 80%, not 100% of females carrying the full mutation *FMR-1* allele have shown mental retardation [4, 5].

The p(CGG)n repeats in mutated allele are lengthened through generation to generation. The meiotic instability might be responsible for this consequence, but the mechanism is still unknown. Another possible causing factor is the founder chromosome effect [25, 26], but still other mechanisms such as unequal crossing over might also play a role or two. One noticeable exception is that decreasing in the size of p(CGG)n repeats was observed in IV-10. In the case of subject III-19, we considered him as a mosaic male. From DNA analysis, he has both premutation and full mutation alleles in different cells suggesting that the p(CGG)n repeat expansion occurred during oogenesis and subsequent unequal crossing over took place in early embryonic stage, resulting in mosaicism. Moreover, mitotic instability in this repeat was also occurred, resulting a smear band in blot with

mean size of 7 Kb (Δ = 1.8 Kb). The mechanism of mitotic instability may be due to slippery DNA synthesis [27, 28].

III-19 shows mild mental retardation as the most full mutation allele are methylated. It had been demonstrated that methylation in *FMR-1* will down regulate its expression, resulting in disease [12, 13, 14]. However, in mosaic individuals, the methylation status is responsible for variable phenotypic expression of the fragile X syndrome [29]. In females, who carry the mutated allele, will exhibit selectively differential methylation just like we observed in II-6 (Fig. 2). The normal alleles are mostly located on active X chromosome and mutated alleles are mostly located on inactive X chromosome (compare signal intensity of 2.8 and 5.2 Kb from normal alleles). Similar result also occurred in III-5 while she is an affected individual as examined by cytogenetic and molecular methods, but the clinical expression of fragile X is barely detectable. The selective methylation may be happened by chance via X chromosome inactivation in females and thus, X chromosome inactivation may play an important role in fragile X syndrome phenotype expression in full mutation females.

Anticipation was seen in this large family with one individual of incomplete penetrance (III-5). Therefore, from the view point of anticipation, it is important for a fra(X) female carrier that she should take prenatal diagnosis for this syndrome if she has pregnancy. The methods currently used for fragile X detection still remain several clinical difficulties for prenatal diagnosis. However, our laboratory recently use RT-PCR combined with sex determination by PCR amplification of *SRY* gene, successfully performed a prenatal diagnosis (Wang and Li, unpublished data) in which the mother is fragile X carrier (premutation, confirmed by probe StB12.3).

Yu et. al. [30] had demonstrated that there was strong positive correlation between the length of p(CGG)n repeats and fra(X) expression. Our data confirmed their finding, However, they did not explain why the fra(X) expression is rarely

over 50% and the fragility of fra(X) chromosome. Hansen et. al. [31] reported that hypermethylation in mutated *FMR-1* will delay the replication of a segment of DNA over 180 Kb spanning these repeats. It is possible that the delayed DNA replication in fra(X) chromosome, in turn, affects chromosome condensation during mitosis and subsequently results fra(X) as we seen in metaphase preparations. Another possible mechanism is that GC dinucleotide alternative sequence has the property prone to Z-DNA conformation [32]. Similarly, p(CGG)n is 100% GC rich DNA stretch that may induce left-handed DNA conformation. However, whether Z-DNA how to influences chromosome condensation, and if so, by what mechanism Z-DNA exerts its influence should be further proved.

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Figure Legends

Figure 1. Pedigree of the large family in this study. Open boxes and circles indicate normal individuals, and doted symbols indicate these individuals are carriers (premutation). Solid symbols indicate affected individuals, with exception that III-19 is referred as a mosaic male (for detail see text).

Figure 2. DNA analysis of *FMR-1*. Partial individuals of this large family are shown. The individuals in lane 1 to 9 (from left to right) are: III-7, III-9, III-11, IV-5, III-3, IV-2, IV-3, II-6, and III-19. The corresponding sizes of the signal are shown on the right. The 2.8 Kb indicates normal active allele and the 5.2 Kb indicates normal inactive alleles. It should be noticed that premutation allele of III-19 is unmethylated (lanes 9).

Figure 3. Correlation between p(CGG)n length increased and the percentage of cells that express the fragile X chromosome in peripheral blood. For the mosaic individuals (III-19), two bands were both scored.

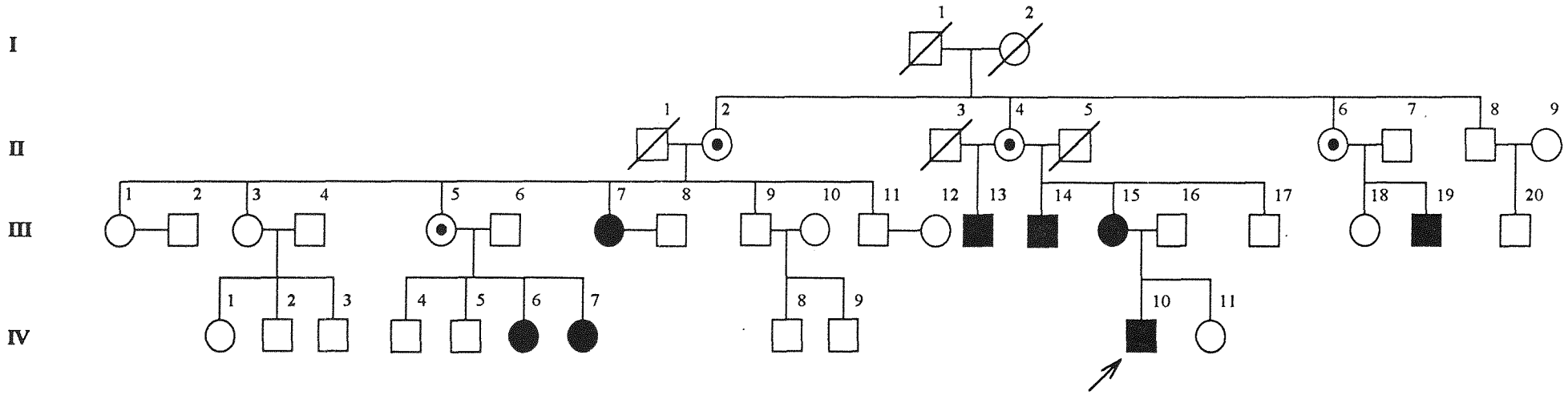
I
II
III
IV
1 2
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
1 2 3 4 5 6 7 8 9 10 11

Fig. 1

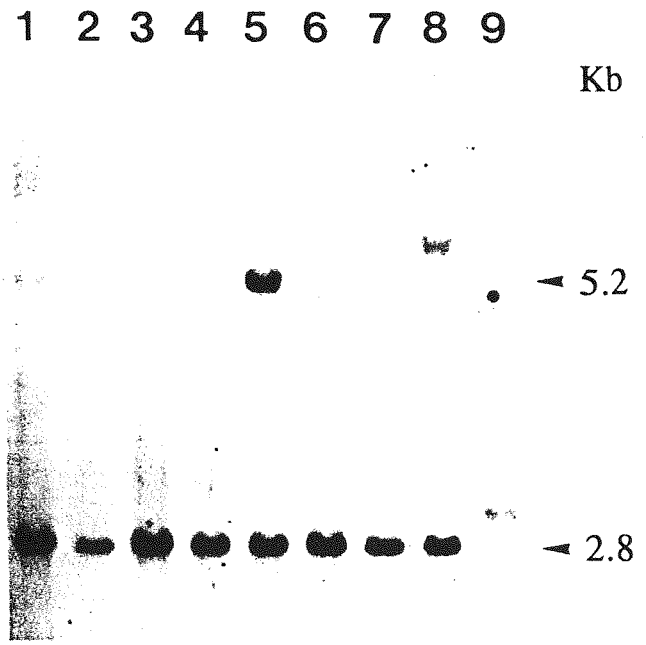

Fig. 2

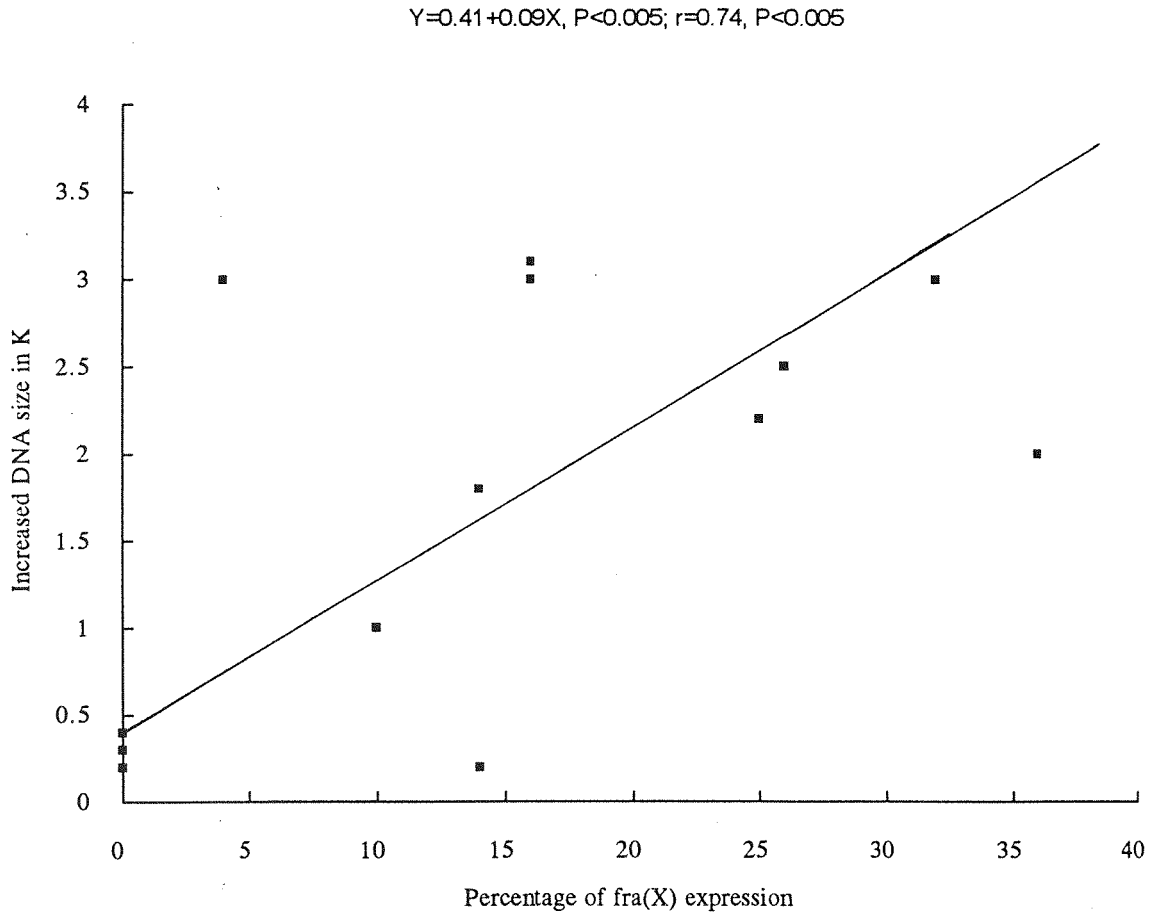
Y=0.41+0.09X, P<0.005; r=0.74, P<0.005
Increased DNA size in K
Percentage of fra(X) expression
0
0.5
1
1.5
2
2.5
3
3.5
4
0
5
10
15
20
25
30
35
40

Fig. 3

Table 1. Percentage of fra(X) expression and DNA increased in kilobase of each individual.

Position of individuals in pedigree	Increased DNA size in Kb	Percentage of fra(X) expression
II-2	0.2	0
II-4	0.3	0
II-6	0.4	0
III-1	0	0
III-3	0	0
III-5	1	10
III-7	2.5	26
III-9	0	0
III-11	0	0
III-13	3	4
III-14	3	32
III-15	3	16
III-17	0	0
III-18	0	0
III-19	0.2/1.8*	14
IV-1	0	0
IV-2	0	0
IV-3	0	0
IV-5	0	0
IV-6	2.2	25
IV-7	3.1	16
IV-10	2	36
IV-11	0	0

* indicates this subject is a mosaic male.

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