Identification of fragile X pre-mutation carriers in the Chinese obstetric population using a robust *FMR1* polymerase chain reaction assay: implications for screening and prenatal diagnosis

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ABSTRACT

Introduction: There is significant morbidity associated with fragile X syndrome. Unfortunately, most maternal carriers are clinically silent during their reproductive years. Because of this, many experts have put forward the notion of preconception or prenatal fragile X carrier screening for females. This study aimed to determine the prevalence of fragile X syndrome pre-mutation and asymptomatic full-mutation carriers in a Chinese pregnant population, and the distribution of cytosine-guanine-guanine (CGG) repeat numbers using a robust fragile X mental retardation 1 (*FMR1*) polymerase chain reaction assay.

Methods: This was a cross-sectional survey in prospectively recruited pregnant women from a university hospital in Hong Kong. Chinese pregnant women without a family history of fragile X syndrome were recruited between April 2013 and May 2015. A specific *FMR1* polymerase chain reaction assay was performed on peripheral blood to determine the CGG repeat number of the *FMR1* gene. Prenatal counselling was offered to full-mutation and pre-mutation carriers.

Results: In 2650 Chinese pregnant women, two individuals with pre-mutation alleles (0.08%, one

in 1325) and one asymptomatic woman with full-mutation (0.04%, one in 2650) alleles were identified. The overall prevalence of pre-mutation and full-mutation alleles was 0.11% (1 in 883). Furthermore, 30 (1.1%) individuals with intermediate alleles were detected. In the 2617 women with normal CGG repeats, the most common CGG repeat allele was 30.

Conclusions: The overall prevalence of pre-mutation and asymptomatic full-mutation carriers in the Chinese pregnant population was one in 883, detected by a new *FMR1* polymerase chain reaction assay.

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New knowledge added by this study
• This study reports the prevalence of fragile X pre-mutation carriers in Chinese pregnant women.
• The prevalence of pre-mutation and asymptomatic full-mutation carriers was one in 883 and disproves the belief that carrier rates in Chinese are extremely low.

Implications for clinical practice or policy
• Maternal fragile X carriers are not rare in a Chinese population. Women should be offered the option of carrier screening during the preconception period or prenatally.

Introduction

Fragile X syndrome (FXS) is the second leading genetic cause of intellectual disability after Down syndrome,[1] affecting one in 4000 males and one in 8000 females.[2] The typical phenotypes include behavioural abnormalities, autism, cognitive impairment, and dysmorphism such as large protruding ears, elongated face, and macroorchidism in male patients. This syndrome is caused by a defective fragile X mental retardation 1 (*FMR1*)

gene located on the X chromosome, where there is an unstable cytosine-guanine-guanine (CGG) trinucleotide repeat in the 5' untranslated region.[3] Normally the number of CGG repeats is less than 44, but if it is more than 200 (full mutation), the *FMR1* gene expression will be 'shut down' due to methylation of its promoter. The protein product, which is essential for normal neurodevelopment, is thus not produced. When the repeat number is between 55 and 200 (pre-mutation),[4] the *FMR1*

gene can be expressed but the repeat number is potentially expandable to full mutation during its transmission to the next generation. Such risk of expansion is increased with the size of the repeat number, and is close to 100% when the size of CGG repeats is 100 or more. In addition, pre-mutation carriers are at risk of developing fragile X–associated primary ovarian insufficiency (FXPOI) and fragile X–associated tremor/ataxia syndrome (FXTAS) in late adult life, although they are mentally normal.[5,6] Intermediate alleles are repeat numbers between 45 and 54, and individuals carrying theses alleles are at risk of expanding into pre-mutation but not into full mutation.[7-9]

Because of the significant morbidity associated with FXS, and since most maternal carriers are clinically silent during their reproductive years, many experts have put forward the notion of preconception or prenatal fragile X carrier screening for females.[10] The prevalence of pre-mutation carriers will directly affect the efficacy and cost-effectiveness of screening, but this varies widely between different ethnic groups and countries. While it is well known that Caucasians and Jews have high carrier rates of 1 in 100-250,[10] many studies in Chinese populations report an extremely low carrier rate.[11-13] Among these studies, the largest included 10 046 newborn boys, but identified only six pre-mutation carriers (1 in 1674).[13] These studies, however, were limited by the fact that the screening methods used were polymerase chain reaction (PCR) assays that were not accurate or were unable to amplify long CGG repeats.[14] In addition, the screening of a low-risk Chinese pregnant population has not been studied.

Recently, we have validated a new fragile-X-related–specific PCR assay that utilises a low-cost capillary electrophoresis instrument and the FragilEase reagent kit (PerkinElmer Inc, Waltham [MA], US), and is able to detect CGG repeat numbers at a level as high as 1000.[14] The repeat numbers analysed by this new assay were highly concordant with those obtained from the conventional reference method (PCR + Southern blot) in 112 archived samples, including 25 samples of full mutation (the largest allele size measured at 1380 repeats). The intra-assay (coefficient of variation <2.5%) and inter-assay imprecision was within 1 CGG repeat.[14] The objectives of this study were to determine the prevalence of FXS pre-mutation carriers and the distribution of repeat numbers in the Chinese pregnant population in Hong Kong, using this FXS-specific PCR assay.

Methods

This was a prospective observational study conducted at a university hospital in Hong Kong between April 2013 and May 2015. Chinese pregnant women between 4 and 41 weeks of gestation, at or above

採用強力FMR1基因聚合酶連鎖反應法偵測華籍婦女的X染色體脆折症準突變帶因者：篩查和產前診斷的意義

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引言：X染色體脆折症會帶來很多相關疾病，可惜大多數帶因者於生殖年期內並沒有出現臨床症狀。因此，專家建議在孕前或產前為女性進行X染色體脆折症帶因者篩查。本研究旨在找出華籍孕婦中X染色體脆折症的準突變及全突變帶因者的比率，並採用強力FMR1基因聚合酶連鎖反應法（PCR）偵測三核甘酸（CGG）重複數的分佈。

方法：本橫斷面研究藉着前瞻性研究設計於2013年4月至2015年5月期間招募香港一所大學醫院內的華籍孕婦。她們必須沒有X染色體脆折症的家族史。採用FMR1基因PCR偵測她們外周血的CGG重複數，並為全突變和準突變帶因者提供產前諮詢服務。

結果：2650名華籍孕婦中有兩例具準突變等位基因（0.08%，即1325例中有1例）以及一例無症狀具全突變等位基因（0.04%，即2650例中有1例）。準突變和全突變等位基因的總體發病率為0.11%（即883例中有1例）。研究也檢測到30例（1.1%）具有過渡區等位基因。2617名具正常CGG重複數的婦女中，最常見等位基因的CGG重複數為30。

結論：根據最新的FMR1基因PCR，華籍孕婦中準突變和無症狀全突變帶因者每883例中便有1例。

the age of 18 years who could understand English or Chinese and give informed consent were eligible for the study. Eligible women were approached in the antenatal clinic or the antenatal ward by the research assistant at one convenient time-point once per day and invited to participate in the study. Women with a known family history of FXS were excluded to avoid an over-representation of the pre-mutation carrier rate in the general population, so that data obtained would be more useful in determining whether population-based screening is beneficial. Pre-test counselling was given by a research assistant with a bachelor's and master's degree in human genetics. Printed information about fragile X carrier testing was provided and included information about the clinical features and maternal inheritance of the disease. It was also explained to participants that genetic counselling would be offered if they were found to have an increased CGG repeat number of ≥45. Testing was entirely voluntary, and no payment was received by the participants. Written informed consent was obtained. Two millilitres of maternal blood was collected in an EDTA tube from each participant. The FMR1 CGG repeat result could be obtained within 1 day but study samples were processed in batches so results were available between 1 day and 7 months later. Women were informed prior to consenting that the result might not be available before delivery.

The *FMR1* CGG repeat status of each sample was tested at a screen cut-off value of ≥45.[14] The details of the PCR method are described below. All participants had the right to access personal data and study results. Positive test results (≥45 CGG repeats) would be made known to the participants, and genetic counselling would be provided. Where indicated, prenatal and postnatal diagnoses were offered by means of chorionic villus sampling or amniocentesis and cord blood or neonatal blood respectively, with analysis of CGG repeats in the extracted DNA from the sample using the same PCR method.

Approval for the study was obtained from the institutional review board of the Joint-Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee (CRE-2013.055).

Polymerase chain reaction–only assay for detection of CGG repeats in *FMR1* gene

DNA preparation

Genomic DNA was extracted from peripheral blood samples using DNeasy Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) or using the automatic system, chemagic Prepito-D, following the manufacturer's protocol (PerkinElmer, Turku, Finland).

Polymerase chain reaction analysis of fragile X mutations

The *FMR1* repeat region of each DNA sample was amplified using the FragilEase PCR reagent kit following the manufacturer's protocol (PerkinElmer). It involved a forward (TCA GGC GCT CAG CTC CGT TTC GGT TTC A) and a reverse primer (FAM-AAG CGC CAT TGG AGC CCC GCA CTT CC) anneal to *FMR1*-specific sequence upstream and downstream of the trinucleotide repeat region, respectively. Thermal cycle amplification of the highly GC-rich trinucleotide repeat region produced fragments whose size was directly related to the number of trinucleotide repeats present in the DNA sample. Two female reference DNA samples (one pre-mutation carrier [30/80 repeats] and one individual with full mutation [20/200 repeats]) for evaluating the analytical performance of the assay were obtained from the Coriell Institute for Medical Research (Camden, US). The known repeat sizes of the reference samples were concurrently amplified and used to calculate the CGG repeat numbers of the unknown samples.

Purification of the polymerase chain reaction product

Polymerase chain reaction products were purified using NucleoFast 96 PCR plate (MACHEREY-NAGEL GmbH & Co KG, Germany) or the PureLink PCR Micro Kit (Invitrogen, Carlsbad [CA], US). Purification procedures were performed according to the manufacturer's instructions with a final elution volume of 20 µL.

Fragment sizing with microfluidic capillary electrophoresis

The fragment size for the sample was analysed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara [CA], US). In this study, 3 µL of the purified PCR product and 3 µL of the 7500-size marker reagent (from an Agilent DNA 7500 kit) were loaded into each of the 12 wells of the Bioanalyzer chip. A standard curve was constructed from the two female reference samples (Coriell NA20240 [30/80 CGG repeats] and NA20239 [20/200 CGG repeats]) with known repeat size. This allowed the determination of CGG repeat size with higher accuracy.

Report of FMR1 fragment size

FraXsoft analysis software (PerkinElmer) was used to calculate the CGG repeat lengths by utilising the base pair size data exported from the bioanalyser. Fragment sizes that were below 200 CGG repeats were interpolated from their base-pair electrophoresis result using a linear regression constructed between the four allele values of the two Coriell female reference samples. Larger repeat sizes (>200 repeats) were calculated by extrapolation along the same regression line.[14]

Results

A convenient sample of 2650 Chinese pregnant women was recruited between 4 and 41 weeks of gestation. *FMR1* allelic expansion was screened in each subject, and two pre-mutation carriers (0.08%, one in 1325) and one asymptomatic individual with full mutation (0.04%, one in 2650) who were unrelated were identified. The overall prevalence was therefore one in 883 (0.11%) or 11 per 10 000 (95% confidence interval, 3-36 per 10 000 using the Wilson method with continuity correction). These three women are described in detail below. There were also 30 (1.1%) women with CGG repeats who fell into the intermediate category. The remaining 2617 women screened were found to have normal CGG repeats, and the most common CGG repeat allele was 30. This distribution of allele frequencies for CGG repeats in the *FMR1* gene in the population with normal CGG repeat numbers is shown in the Figure.

One asymptomatic subject with full-mutation allele

The woman had *FMR1* gene testing carried out during the third trimester and was found to have a

FIG. Distribution of allele frequencies for the number of CGG repeats in *FMR1* gene in the 2617 normal pregnant women
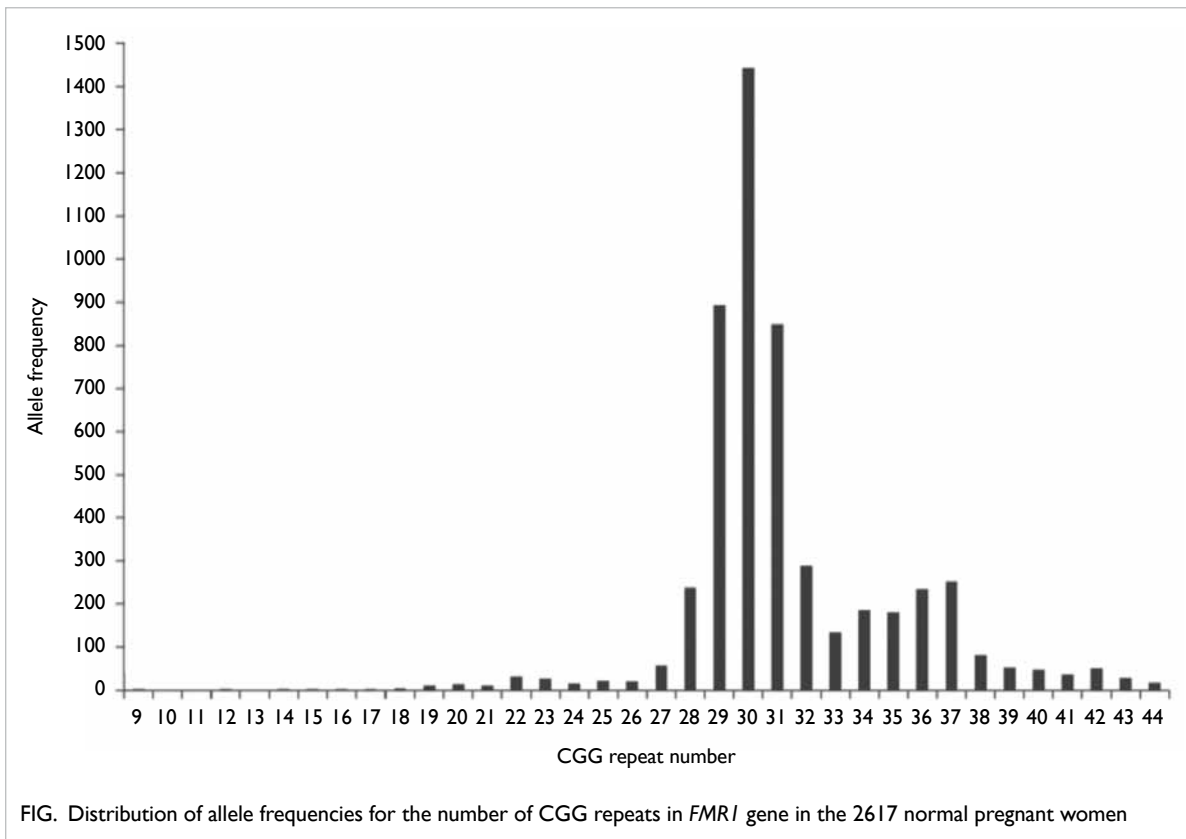
full mutation with CGG repeat number of 35/401. She was phenotypically normal. She had completed junior high school education and was working in a fast food restaurant. Upon detailed questioning, she suspected that her brother and one of her maternal male cousins might have some autistic features, but she was not aware of any mental retardation, or any formal genetic diagnosis in either of these two relatives. Genetic counselling was given. Prenatal diagnosis was not performed since the woman had been tested during the third trimester and termination of pregnancy was not an option. She subsequently delivered a healthy baby girl. The parents were counselled about testing the baby for FXS but they declined and preferred to observe the development of their child first. The child was referred for follow-up of her development. The woman's other family members also declined fragile X screening because they were phenotypically normal with no current reproductive plans.

Two subjects with pre-mutation allele

The first pre-mutation carrier was a 31-year-old nulliparous female with no features associated with FXS or family history of intellectual disability or autism. Testing of the *FMR1* gene was done at 13 weeks and 4 days, and the result was available at 14 weeks and 4 days indicating a CGG repeat number

of 30/70. The woman requested prenatal diagnosis for her female fetus following genetic counselling. Amniocentesis was performed at gestation 16 weeks and 4 days. The PCR analysis result was available at 17 weeks and 2 days, and revealed that the maternal pre-mutation allele had been transmitted to the fetus and expanded to CGG repeat number of 30/579, indicating the female fetus carried a full-mutation allele. Genetic counselling was provided and the couple opted for termination of pregnancy and planned for pre-implantation genetic diagnosis in future.

The second carrier was a 39-year-old female with normal phenotype and no family history of intellectual disability or autism. She had testing of the *FMR1* gene at 20 weeks and 6 days of gestation. The result was available at 21 weeks and 2 days of gestation showing a CGG repeat number of 31/64, and her fetus was female. Following genetic counselling, the parents decided not to have any prenatal or postnatal diagnosis owing to the variable and unpredictable phenotype in full-mutation females.

Discussion

This is the largest study of the prevalence of fragile X pre-mutation carriers in Chinese pregnant women, as well as the largest one using this new FXS-specific

PCR assay (FragilEase) in the Chinese population. The combined prevalence of pre-mutations and full mutations of FXS in normal asymptomatic pregnant Hong Kong Chinese women was as high as 0.11% (1 in 883). We included also one case of full mutation in our estimation of prevalence because some women with full mutations are apparently asymptomatic but are at risk of transmitting FXS to their offspring. Therefore, the combined prevalence would reflect more precisely the overall risk of transmitting FXS in the general population. Our finding is consistent with the recent publication by Huang et al,[15] who identified one pre-mutation carrier in their population of 1113 Han Chinese (534 males and 579 non-pregnant females). Our finding also refutes the belief that FXS pre-mutations are extremely rare in Chinese.[11-13] In fact, the incidence in Chinese is comparable with some of the incidences reported from Korea (1 in 1090).[16]

A standard capillary analyser is only capable of detecting and sizing *FMR1* PCR products with less than 200 CGG repeats. Thus differentiating full mutations with greater than 200 CGG repeats from apparently homozygous normal female samples, and confirming full mutations, has historically required the Southern blot reflex test. The Southern blotting assay, however, is expensive, labour intensive, and requires a large amount of DNA making its use in screening impractical.[13] The advantage of this FragilEase PCR assay is the ability to detect up to 1000 CGG repeats, so that even asymptomatic full-mutation individuals can be identified during routine screening, as shown in one of our cases. It has high throughput and high sensitivity of 99%.[14] The cost for each fragile X assay is estimated to be only US$44, deduced from a parallel run of a minimum of four samples plus two reference standards on each Bioanalyzer chip, and this cost includes that of FragilEase reagent kit, DNA extraction kit, PCR-related consumables, Bioanalyzer kit, equipment maintenance, and staff costs. Processing of each chip takes 1 hour and a maximum of nine samples per chip can run. The low cost of this test is beneficial as a screening test.

Our study showing an incidence of one pre-mutation or full mutation of FXS in 883 pregnant Chinese women has important implications for counselling and implementation of a FXS carrier screening programme in Hong Kong and in China, as well as in countries where Chinese immigrants are numerous. It remains controversial whether FXS should be screened for, and which model should be adopted. Some experts advocate universal prenatal screening[17] as it is much more effective in identification of pre-mutation carriers compared with case finding followed by cascade screening. The latest UK Health Technology Assessment (HTA) review[18] indicated that the maximal rate of detection of female pre-mutation carriers by population screening is 60% whereas only 6% of carriers will be identified by active cascade screening. In their simulation model, the additional number of births of FXS children that could be avoided each year was estimated to be 15 with cascade screening compared with 39 with prenatal screening. Since family size tends to be small in many developed countries now, the effectiveness of cascade screening has become very limited. In Hong Kong, the average number of children per household is only 1.24.[19] In mainland China, until 2015, families were allowed to have only one child. The chance of revealing a positive family history with affected siblings or close relatives is thus low. Furthermore, even though parents might be planning their second child, the first affected child would not have been diagnosed with FXS if very young. Such diagnosis is particularly difficult and is delayed in Hong Kong, China, or other Asia-Pacific regions where clinical genetic services are inadequate.[20] The variable phenotypes of FXS may also be masked by the mixed education levels of the population in different geographical regions. Indeed, the patient in our study who carried a full mutation is a very good example of the limitation of cascade screening with an uncertain family history or without a formal genetic assessment.

Unlike first-trimester combined Down syndrome screening that requires intensive training and effort in ultrasound measurement and a stringent algorithm in risk calculation to achieve a 90% detection rate with a 5% false-positive rate,[21-24] screening for fragile X carriers is relatively simple by a maternal blood test and is thus acceptable to most women. It can also be done before conception. Furthermore, once a carrier is identified, other carriers may be found through family screening. Hence the potential utility of this screening can be profound. In the validation study of FragilEase by Kwok et al,[14] 78 samples tested positive, of which one was classified as false positive. This sample was tested to have a CGG repeat number of 55 (pre-mutation) by FragilEase whereas Southern blot analysis determined the repeat number to be 53 (intermediate). This gives a false-positive rate of 1.3%. The false-positive result occurred because the CGG repeat number was at the lower limit of the pre-mutation range, such that a difference of 2 repeats led to an intermediate allele being classified in the pre-mutation range. We believe that this false-positive rate is overestimated, as the majority of the pre-mutation carriers do not have a repeat number at this lower limit that could lead to such a false-positive result. Although the positive predictive value calculated was 8.0%, assuming a fragile X pre-mutation carrier prevalence of one in 883, the performance of the test should be better because the positive predictive value was underestimated due to

the overestimated false-positive rate.

There are no current data on the health care costs of caring for a FXS patient in Hong Kong or Asia. In the UK, the lifetime cost for each FXS patient is estimated to be UK$380 000 and the HTA model expects population screening to be a cost-effective strategy.[18] In fact, it has been shown that health care professionals and families of patients with FXS are in favour of preconception or prenatal screening.[25,26] Detection of pre-mutation alleles also offers information about the women's own health, as they are at increased risk of FXPOI and FXTAS.[27] The above factors may affect a woman's fertility planning and allow informed choices not only in this pregnancy but also subsequent pregnancies.

Despite this, the UK National Screening Committee and the American College of Obstetricians and Gynecologists do not advocate universal screening,[28,29] but rather screening in those with a family history of congenital intellectual disability, autism, or premature ovarian failure. There are concerns over the counselling about complex genetic mechanisms and the psychological impact of FXS when population screening is offered. The difficulties in counselling include (1) the variable phenotypes (eg female fetuses with full mutation) associated with FXS, and (2) identification of individuals with pre-mutation allele may lead to anxiety in these individuals because both FXPOI and FXTAS have no specific treatment. Another factor that limits population screening for fragile X is the access to prenatal care and screening, especially in rural areas of mainland China.

The strength of this study lies in its size. It is the largest study of the prevalence of fragile X pre-mutation carriers performed in the Chinese pregnant women. This study also demonstrated the feasibility of this validated FXS-specific PCR-based method (FragilEase) in the Chinese population.[14] One limitation is that not all pregnant women were approached for the study and the study participants were recruited by convenient sampling. During the study period, approximately 13 600 Chinese women attended our hospital but only 2650 women were recruited. Women were recruited each day at one convenient time-point by the research assistant in the antenatal ward or clinic. This was not a true random sample and hence has doubtful representativeness. Nonetheless, as the largest obstetric hospital in Hong Kong with participants recruited from both antenatal clinic and obstetric wards, and a large sample size of 2650, we aimed to include a group most typical of the general obstetric population. Another limitation is that our cohort represented mainly the Southern Chinese population and not the entire Chinese population. Despite this, the findings of our study should provide a strong foundation for further large-scale national studies that may benefit our

understanding of the carrier frequencies in different parts of China and the Asia-Pacific region. Further studies are also required to look into the different models of carrier screening programmes and their cost-effectiveness in our locale to determine which screening strategy is the most appropriate in the Chinese pregnant population.

Conclusions

The prevalence of pre-mutation and full-mutation alleles altogether in the asymptomatic Southern Chinese population was one in 883, and that for pre-mutation alleles alone was one in 1325. These figures are higher than those reported previously in small-scale studies, and indicate that FXS is a clinical condition not to be overlooked in our locale. Further studies of the prevalence in different areas of Asia may be beneficial to direct future screening strategies.

Acknowledgements

Declaration

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