The fragile X syndrome (FXS) is the leading heritable form of mental retardation (MR). The disorder-causing mutation is the amplification of a CGG repeat in the promoter region of FMR1 gene located at Xq27.3.\(^1,2\) Based on the copy number of CGG-repeat, the FMR1 is classified as normal (below 54), premutation (55-200), or full mutation (above 200). The normal form is usually transmitted from parent to child uneventfully. In individuals with premutation are generally asymptomatic, but the mutant allele is unstable and tends to expand when passing into the next generation through a carrier mother to her children. The repeat size of a female carrier is positively related to the risk of expansion into full mutation in her offspring.\(^3,4\) Once an FMR1 gene has expanded to full mutation range, its CGG-repeat and surrounding CpG islands become hypermethylated and transcriptionally inactive.

Specific treatment for FXS is not yet feasible to day, however, this disorder can be prevented. Because the mutant FMR1 in all FXS patients is maternally derived, these mothers would be carriers of premutation or full mutation. It is thus very important to effectively identify carrier women, who could then take necessary preventive measures. Screening related to FXS patients is an effective approach. However, there may be difficulties in obtaining cooperation from related families at risk, as opposed by others and ourselves.\(^5,6\) Screening of pregnant women with family history of mental deficiency of undetermined etiology.

**Background.** Carrier detection before or at early pregnancy through a wide screening program may be a practical approach to prevent the fragile X syndrome. However, prior to implementation of such a program, the carrier prevalence in a population and availability of effective screening tests should be evaluated.

**Methods.** One thousand and two pregnant women were randomly selected from our obstetric clinic and screened for FMR1 mutation. Each woman was examined individually using a simple non-radioactive PCR, as well as in pool with two other women using high-resolution Southern blot hybridization.

**Results.** One third of women could be excluded from carrier status by PCR alone, while the rest had to be screened using Southern-blot hybridization in pool with two other women. This screening strategy was reliable and suitable for large-scale screening. Among the 1002 women, no carrier of either premutation or full mutation was found. A lele with intermediate CGG-repeat between 40 and 52 was found in only 22 women (2.2%).

**Conclusions.** Estimation of female fragile X carrier rate in Taiwan could not be made in the present study, due to insufficient sample size. However, the results in this study clearly show that the rate in Taiwan was significantly lower than that of Israel (0/1002 vs. 1/113, \(p = 0.003\)) and also lower than those from other western countries (1/186-259, \(p = 0.020-0.049\)). We doubt that the cost-efficiency of such a wide screening program in Taiwan is acceptable. However, the effective screening strategy proposed in this study would be very helpful for women with family history of mental deficiency of undetermined etiology.

Received: August 7, 2002. Accepted: January 21, 2003.

Correspondence to: Ching-Cherng Tzeng, MD, Genetics Laboratory, Department of Pathology, Chi-Mei Foundation Hospital, Tainan 710, Taiwan.
Fax: +886-6-251-3215; E-mail: tzeng-tainan@yahoo.com.tw
screening for fragile X carriers in women before or at their early pregnancy will initially avoid any miss of detection.\textsuperscript{9,10}

Be fore implementation of a population-based screening program is considered, the prevalence of carrier women in a population should first be determined. Such prevalence had been estimated between 1/186 and 1/259 in the United States, Finland, and Canada, and as high as 1/113 in Israel.\textsuperscript{7,10-12} Such screening was also demonstrated, based on de-tailed calculation, to be rather cost-effective in Israel.\textsuperscript{10} According to our previous study and others',\textsuperscript{8,13} we believed that the prevalence of FXS in Taiwan might not be different from those reported from many other western countries, mostly for Caucasians of northern European descent.\textsuperscript{14} However, for more strict and prudent consideration, initiation of a pilot screening strategy was proposed to screen women first with undetermined carrier status by Southern blot assay.\textsuperscript{6} Theoretically, such women were unlikely fragile X carriers. Southern blot hybridization is still the gold standard of study of \textit{FMR1} gene mutation. With greatly improved resolution, our non-radioactive procedure could detect all kinds of \textit{FMR1} mutation in a DNA mixture from three women. To establish a cost-effective screening strategy, we proposed to screen women first using the simple PCR, followed by examining the rest with undetermined carrier status by Southern blot as say.

In this study, we attempted to estimate the female carrier rate in general population of Taiwan. Therefore, we conducted a pilot study selecting randomly from our obstetric clinic. In our previous study and ours', prenatal diagnosis was offered at their choice. All patients were released to their obstetrician-in-charge before submitting 2 mL of blood for analysis. Test results were generally released to their obstetrician-in-charge within two weeks. For those proved as fragile X carriers, prenatal diagnosis was offered at their choice. All molecular tests provided in this study were free of charge.

Blood DNA was extracted using a commercial kit (Puregene, Minneapolis, MN, USA) and resolved in TE buffer to a concentration of 0.25 mg/mL. Each sample was pooled with two others, followed by examination with non-radioactive Southern blot hybridization to exclude women from being carriers. Similar amounts of digested DNA from three subjects, about 1.25 µg each, were then mixed to gether, followed by separation on a 2% agarose gel for 24 hours. The blot was hybridized using digoxigenin-labeled probe StB12.3 (with courtesy of Professor J.L. Mandel, Strasbourg, France), and detected with a chemiluminescent kit (Roche Boehringer Mannheim, Mannheim, Germany). The control DNA mixture was prepared from three EBV-lymphoblastoid cell lines transformed from a male with 52-CGG, a woman with a 122- and 30-CGG allele, and an FXS woman with a methylated full mutation allele and a 29-CGG allele. In each test, the gap of 2.8-Kb hybridization signals between the 52-CGG allele and 30/29-CGG alleles in the control mixture had to be distinctive.

All samples were also analyzed in duplicate using a simple non-radioactive PCR as described elsewhere.\textsuperscript{6} Any woman with two \textit{FMR1} alleles different in the CGG repeats up to 5 or more could reliably be detected. These women were unlikely fragile X carriers. The reliability of PCR test for excluding women from being carriers

\section*{METHODS}

This screening study of estimation of fragile X carrier rate in pregnant women was approved by the Institutional Review Board of our hospital and carried out between November 2000 and July 2001. All pregnant women in the first trimester who at tended our obstetric clinics during above period were provided with a leaflet describing the purpose of this study. No clinical information was used as criteria for sample selection. All women had to provide a written informed consent before submitting 2 mL of blood for analysis. Test results were generally released to their obstetrician-in-charge within two weeks. For those proved as fragile X carriers, prenatal diagnosis was offered at their choice. All molecular tests provided in this study were free of charge.

Blood DNA was extracted using a commercial kit (Puregene, Minneapolis, MN, USA) and resolved in TE buffer to a concentration of 0.25 mg/mL. Each sample was pooled with two others, followed by examination with non-radioactive Southern blot hybridization to exclude women from being carriers. Similar amounts of digested DNA from three subjects, about 1.25 µg each, were then mixed to gether, followed by separation on a 2% agarose gel for 24 hours. The blot was hybridized using digoxigenin-labeled probe StB12.3 (with courtesy of Professor J.L. Mandel, Strasbourg, France), and detected with a chemiluminescent kit (Roche Boehringer Mannheim, Mannheim, Germany). The control DNA mixture was prepared from three EBV-lymphoblastoid cell lines transformed from a male with 52-CGG, a woman with a 122- and 30-CGG allele, and an FXS woman with a methylated full mutation allele and a 29-CGG allele. In each test, the gap of 2.8-Kb hybridization signals between the 52-CGG allele and 30/29-CGG alleles in the control mixture had to be distinctive.

All samples were also analyzed in duplicate using a simple non-radioactive PCR as described elsewhere.\textsuperscript{6} Any woman with two \textit{FMR1} alleles different in the CGG repeats up to 5 or more could reliably be detected. These women were unlikely fragile X carriers. The reliability of PCR test for excluding women from being carriers

\section*{RESULTS}
was further confirmed by comparing with the results of the above Southern blot hybridization. For women displaying allele(s) around 40-CGG or larger, measurement of the exact CGG-repeat size of both alleles was carried out using standard PCR-sequencing gel electrophoresis as described elsewhere.

Chi-square test was used to evaluate the statistical significance of difference between the prevalences of fragile X female carrier reported by other countries and ours.

RESULTS

During this study period, all women who received the leaflet describing the purpose of this screening study had never heard of fragile X syndrome, and appreciated the opportunity of receiving free tests to examine their FMR1 genes. A total of 1002 women were included in this study.

As depicted in the lanes M of Fig. 1, different alleles in the control mixture, including methylated full mutation (FM), premutation of 122-CGG, allele of 52-CGG in the upper normal range, and common alleles of 29- and 30-CGG. A-L: test DNA mixtures.

In a total of five mixtures, their 2.8-Kb bands leveled with the 52-CGG bands in the control mixture, as displayed in lanes H, J, and L of Fig. 1. From these mixtures, a woman with an allele close to 52 CGG-repeats was always identified by re-examining each sample individually with either Southern blot analysis (Fig. 2, right) or

Fig. 1. Southern blot hybridization of DNA mixture from three women. M: control DNA mixture with different FMR1 alleles, including methylated full mutation (FM), premutation of 122-CGG, allele of 52-CGG in the upper normal range, and common alleles of 29- and 30-CGG. A-L: test DNA mixtures.

Chi-square test was used to evaluate the statistical significance of difference between the prevalences of fragile X female carrier reported by other countries and ours.

Fig. 2. Southern blot hybridization of individual samples. M: the control DNA mixture described in Fig. 1. Lanes B1, B2, and B3 are the three component samples of the mixture lane B in Fig. 1, and so on for F1-3, H1-3, and L1-3.

Fig. 3. Non-radioactive PCR assay of individual samples. M: control mixture of PCR products from three males with CGG-repeat of 29, 40, and 52. Lanes B1, B2, and B3 are the three component samples of mixture lane B in Fig. 1, and so on for D1-3, F1-3, H1-3, J1-3, and L1-3.
by simple PCR (Fig. 3, lower panel). Ex act size of the larger allelic CGG-repeat in these five women was measured to be 48 in two and 52 in three using PCR-sequencing gel electrophoresis (data not shown).

The 2.8-Kb bands in all other mixtures were clearly smaller than those of 52-CGG bands in the control mixtures. Sometimes, the 2.8-Kb bands were relatively thick, as shown in lanes B, D, and F of Fig. 1. This is because the size range of allelic CGG-repeat in these mixtures was also wider, as dem on strated in the re sults of ex amining each sample individually using Southern blot hybridization (Fig. 2, left) and PCR (Fig. 3, upper panel).

From the PCR re sults de picted in Fig. 3, it is dem on strated clearly that women with a dif fer ence of more than 5 CGG-repeats between two alleles could reli ably be iden ti fi ed, such as those bands in lanes B3, D1, D2, F1, F3, H1, H3, J2, J3, and L2. Based on this semi-quantitative PCR, such a difference was detected in a total of 326 (32.5%) women. Meanwhile, all PCR results in this study were ac cord ingly com pat i ble with those dis closed by South ern blot hy brid iza tion. This com par i son con firmed that screen ing based on PCR alone could re li ably ex clude these 326 women from the status of fragile X carrier.

DISCUSSION

It is some what un ex pected to learn from the pres ent study that none of the 1002 women screened were car ri ers of an FMR1 gene. In our pre vi ous pi lot study, we ran domly se lected 100 fe males and 100 males from the out pa tient clinic, and found a mu tant allele in a man with 95-CGG. At that time, we be lieved that the preva lence of mu tant FMR1 gene in Tai wa n seemed to be as com mon as those re ported from other coun tries, mostly in Cau casians of northern Eu ro pean descent. The car rier rate was es ti mated to be 1/186 (4/745) in the United States, 11 1/246 (6/1476) in Fin land,7 and 1/259 (41/10,624) in Can ada.12 How ever, ac cord ing to the re sults of the pres ent study screen ing a much larger sample size, we have to cau tuously con sider that the female car rier rate in Tai wa n may not be as high as we be lieved in the past.

Because no car rier was identified in the pres ent study, es ti ma tion of such rate in Tai wa n was thus im pos si ble to make; such an es ti mate will re quire fur ther study screen ing more women. How ever, from the view point of clin ical ap pli ca tion, our re sults clearly in di cated that the fe male frag ile X car rier rate in Tai wa n, more strictly in the Tainan area, is sig ni fi cantly lower than that re ported from Israel (0/1002 vs. 1/113, p = 0.003, Chi-square test),10 and prob a bly also lower than those re ported from the United States (1/186, p = 0.020),11 Fin land (1/246, p = 0.043),7 and Can ada (1/259, p = 0.049).12 In or der to get an idea of how many women would have to be further screen ed in our future study, we think that data from some re lated pub li ca tions might pro vide some clues for us.

The o retically, preva lence of fe male car rier would be corre lated well with the over all in ci dence of FXS in a pop u la tion. Re cent re ports were mostly in di rectly es ti mated from the re la tive fre quency of FXS in all MR sub jects.14,16 In our pre vi ous screen ing study of MR pa tients ex cluding Down syn drome and ce re bral palsy, we di ag nosed four FXS from 206 boys (1.9%) and one from 115 girls (0.9%).8 On the other hand, in a very sim i lar study re ported from Finland, Arvio et al. screened a total of 409 MR adult males, also with Down syn drome and ce re bral palsy ex cluded, and detected 26 (6.4%) FXS.17 The above com par i son sug gested that FXS in Fin land ap peared to be three times more com mon than that in Tai wa n, which im plies that the Finn ish fe male car rier rate is prob a bly also three times higher than that in Tai wa n. Be cause the Finnish rate was 1/246 (6/1476, 95% CI: 1/1221 to 1/137), we as sumed that the max im um sam ple size of women to be screened for reach ing an es ti mate will re quire fur ther study.

Sim ilarly im por tant is to know how large is the risk of hav ing FXS fe tus in car rier moth ers iden ti fi ed through such a screen ing of gen er al pop u la tion. In a study from Israel, 127 car rier moth ers were iden ti fi ed through a screen ing of 14223 women.10 Dur ing the 8-years pe riod of study, a total of 177 pre na tal diagnoses were per formed, from which 5 FXS fe tus (2.8%) were di ag nosed. An other Finnish study screen ed 1474 preg nant women and iden ti fi ed six car rier moth ers. From these moth ers, one FXS fe tus (16.6%) was proved.7 The above two stud ies clearly point out that such a risk may vary rather
widely between different populations, which relates to factors mainly including CGG-repeat size, distribution pattern of AGG in the CGG-repeat tract, and pattern of CGG-repeat size, distribution pattern of AGG in the CGG-repeat tract, and pattern of haplotype. These risk-related factors, combined with female carrier rate in a population, will form the basis for understanding what will be the benefits obtained from a wide screening program.

From the viewpoint of expenses involved in a wide screening program, molecular screening tests are obviously the key components. Therefore, availability of reliable, efficient, as well as inexpensive tests is also essential in determining the overall cost-efficiency of a program. PCR seems to be a good tool for such an application. However, some intrinsic problems specifically relating to the FMR1 gene cannot be overcome easily, including the amplification problem of CG-rich DNA, preferential amplification between heterozygous alleles, and homozygous status in 15% to 30% of women. Recently, some reports demonstrated that these problems can be resolved by using fluorescence PCR combined with automated DNA sequencer. However, the required expenses are high, and agents will limit its applicability for wide screening. On the contrary, our PCR test was not only simple and inexpensive, but also could reliably prove one-third of women in our population were not fragile X carriers.

For the conventional Southern blot hybridization, many disadvantages, such as being labor-intensive and time-consuming, will obviously limit its applicability in screening large amount of samples within a short period. Pooling sample prior to hybridization is an alternative that substantially reduces such disadvantages. Rousseau et al. first applied this approach to screen a huge number of women. They pooled 40 μL of blood from five women, followed by DNA extraction, restriction digestion, and Southern blot analysis. However, the number of white blood cells from these women may vary widely, which could lead to missed detection. Therefore, in our previous and current studies, we had to confirm each individual’s DNA to be equal in both digestion quality and quantity to be screened with others.

Obviously, the more samples in a DNA mixture, the less the workload of the above mentioned Southern blot assay, whereas its sensitivity and resolution should still be able to detect all mutant alleles in mixtures, including light smear of methylated full mutation and small premutation close to the upper normal range. In our procedure, the DNA was pooled from three women, in stead of five as done by Rousseau et al. This was because the distribution pattern of the FMR1 CGG-repeat in Chinese is different than that of Caucasians. Next to the most common alleles of 29- and 30-CGG discerned similarly in both populations, the allele with 36 CGG-repeats accounted for approximately 10% of alleles in Chinese, but was rare in Caucasians. Difference between alleles of 36-CGG and 54-CGG is only 18 repeats (54 bps), which is often indistinguishable using Southern blot hybridization. Reduction of sample size in DNA mixtures can prevent the 2.8-Kb bands from being very thick, so that distinction between alleles in the upper normal range and small premutation would not likely be a problem.

In conclusion, approximately one third of women in the present study could likely be excluded from a status of fragile X carrier with simple PCR. High frequency of 36-CGG in our population may be the major contributing factor. Southern blot High-resolution hybridization combined with pooled sample was an other effective test for the rest. This strategy was reliable and efficient, and suitable for wide screening, especially in Chinese and other Asian countries in which the allele of 36-CGG is also common. However, we doubt that the cost-efficiency of such wide screening program in Taiwan is acceptable. This was because both the male carrier rate and the risk of having FXS fetuses in carrier mothers identified from such screening were as assumed to be not reasonable high. However, this effective screening strategy proved in this study would be very helpful for women with family history of MR of undetermined etiology.

ACKNOWLEDGEMENTS

This study was supported by a grant (8908) from Chi-Mei Foundation Hospital, Tainan, Taiwan. We are grateful to Mr. Wei-Chen Chao for his excellent technical support.

Kuo-Feng Huang et al. Journal of the Chinese Medical Association Vol. 66, No. 4
REFERENCES


