FEASIBILITY OF BLOOD SPOT PCR IN LARGE-SCALE SCREENING OF FRAGILE X SYNDROME IN SOUTHERN TAIWAN

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Background and Purpose: Fragile X syndrome (FXS) is the most common form of hereditary mental retardation. Early diagnosis of the disease may lead to better prognosis for children who participate in early intervention programs. This study attempted to evaluate whether screening newborn boys with simple polymerase chain reaction (PCR) assay could be an effective approach for detection of mutation carriers and FXS, a process that may also facilitate detection of young carrier mothers.

Methods: Filter paper blood spot samples of 4843 newborn boys were collected from five hospitals in southern Taiwan. They were tested with a simple non-radioactive PCR for the presence of FMR1 gene mutation by determining the number of FMR1 CGG repeats. By this method, the examined sample can be reliably classified as normal (<40), intermediate (40–54), and mutant group (> 54), according to the number of CGG repeats.

Results: The FMR1 CGC repeat number of all but four samples was below 54, with 90 samples (1.8%) between 40 and 54 (the intermediate range). Two of the four abnormal samples were carriers of the premutation. The other two failed repeatedly in PCR amplification for the FMR1 gene, but not for the control K-ras gene. Hence, these samples seemed to be candidate carriers of large premutation or even full mutation, indicating the need for confirmation with standard Southern blot analysis.

Conclusions: This study demonstrated that a simple PCR combined with blood spot sampling is effective and feasible for large-scale screening of newborn boys for fragile X carrier status. The relatively low carrier rate in this population suggests that the cost-effectiveness of implementation of such screening on a population-wide basis would be lower than in the Jewish and Caucasian populations.

Key words: Fragile X syndrome; Newborns; Neonatal screen; PCR; Blood

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Fragile X syndrome (FXS) is the leading heritable form of mental retardation. The disorder-causing mutation is the amplification of a CGG repeat in the promoter region of the FMR1 gene located at Xq27.3.1,2 The number of CGG repeats has been used to divide subjects into four groups: common (6–40 repeats, usually 29–30), intermediate (41–53 repeats), premutation (54–200 repeats), and full mutation (> 200–230 repeats).

The normal allele is usually transmitted from parent to child uneventfully. 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 number of the repeats in a female carrier is positively related to the risk of expansion into full mutation in her offspring.3,4 Once an FMR1 gene expands to the full mutation range, its CGG repeat and surrounding CpG islands become hypermethylated and transcriptionally inactive.

Specific treatment for the FXS is not yet feasible; however, this disorder can be prevented. Because the mutant FMR1 genes in all FXS patients are maternally derived, these mothers are carriers of premutation or full mutation genes. It is thus essential to effectively identify carrier women before pregnancy or in early pregnancy, in order that they can take the necessary preventive measures. Screening relatives of FXS...
patients is also an effective approach, although there may be difficulties in obtaining cooperation from relatives at risk, as has been previously reported.5,6 Screening pregnant women with a family history of mental retardation is an alternative approach. However, FXS may often appear in a family with no prior history of mental retardation, because of the dynamics of FMR1 gene mutation.7,8 Population-based screening for fragile X carriers in women before pregnancy or in early pregnancy can theoretically avoid any missed detection.9,10 But the cost of such screening is generally prohibitive, since reliable determination of the presence of mutant FMR1 gene in women is a rather complicated procedure.

Detection of mutant FMR1 gene is relatively simple in men, because there is only one allele. The number of FMR1 CGG repeats could be semi-quantitatively measured with a simple non-radioactive polymerase chain reaction (PCR) assay, and the examined sample can be reliably classified as normal, intermediate, or mutant.5 Men with full mutation or large premutation generally fail in the PCR amplification. Preparation of DNA can also be convenient and inexpensive by use of dried blood spots on filter paper.5 Combination of the above two procedures appears suitable for large-scale screening of newborn boys for the detection of candidate carriers of mutant FMR1 gene. The exact mutation status of these candidates should be further verified with standard diagnostic procedures. Early diagnosis of FXS patients allows substantial benefit from early intervention programs. Identification of boys with premutation (normal transmitting males) is also very important, because it provides a method for determining the carrier status in the mother and other family members.

In this study, we evaluated whether simple PCR of blood spot samples is feasible for large-scale screening of newborn Taiwanese boys to detect candidate carriers with FMR1 mutation.

**Methods**

A total of 4843 newborn boys referred from five medical centers in three counties of southern Taiwan were included in this screening study. In addition to the blood spots on filter paper collected for routine screening of metabolic disorders, one more blood spot from each newborn boy was also anonymously submitted for detection of FMR1 gene mutation. Therefore, follow-up and confirmation could not be undertaken.

To develop an ideal protocol for DNA preparation from the dried blood spot on filter paper for a large-scale screening procedure, we had to consider not only the DNA quality necessary to make PCR feasible but also the cost efficiency of the procedure. Our previous study demonstrated that the DNA isolated from blood spots by simply washing in water and then incubating in 5% Chelex at 95°C was adequate for PCR analysis of the FMR1 gene.5 To further simplify our protocol with special consideration of possible future automation of the process, we modified the pretreatment procedure before adding the punched spots to Chelex solution. We tested a total of 100 samples by either washing the punched spots twice with water according to our previous protocol, or by adding the spots directly into Chelex solution without any pretreatment.

Detailed components and the program of PCR for amplifying the CGG repeat region of the FMR1 gene have been described previously.5 However, we recently confirmed that using a longer primer set, as described by Levinson et al11 and inclusion of 0.5 M betaine to replace 7′-deaza-2′-dGTP would result in more stable PCR results.5

In each batch of PCR, samples of 52-CGG and 98-CGG were included as positive control to ensure the efficiency of the PCR conditions. A blank punch of filter paper used as negative control was also included in each batch of PCR. The products of PCR were separated on 5% polyacrylamide gel and stained with ethidium bromide. Their relative sizes could be determined by comparing with PCR mixtures of samples of 29-, 40-, and 52-CGG. When within the normal CGG repeat range, a difference of 5-CGG could be distinguished without difficulty.

Samples that repeatedly failed PCR amplification were also placed under PCR amplification for the control K-ras gene, in order to evaluate the possibility of poor DNA quality, according to a previously described protocol.12

**Results**

Comparison of PCR results in all test samples demonstrated that there was no difference in DNA quality when prepared either using the previous protocol or by the modified method described in the present study. As shown in Fig. 1, the PCR products in all samples prepared by either protocol were almost identical in amplification efficiency, and in particular those with CGG repeat within the normal range. Removal of the pretreatment washing step saved more time and labor in the DNA isolation process. Clearly, the fewer steps needed for the procedures, the greater their applicability for the...
development of an automated device for this process in the future.

As shown in the upper panel of Fig. 2, the CGG repeat size of each sample can be semi-quantitatively measured against a control PCR product mixture containing alleles with 29-, 40-, or 52-CGG. The CGG repeat number was below 40 in the vast majority of samples (98.1%), as shown in lanes A (around 29 or 30), B (around 36), and C (around 40). The CGG repeat number was less than 54 in all but four samples, including 90 samples (1.86%) exhibiting repeat in the intermediate range between 40 and 52, as shown in lanes C, D, G, I, and K (Fig. 2, upper panel).

Two of the above four samples had CGG repeat numbers which were clearly larger than 52, as shown in lanes H and J (Fig. 2, upper panel). They were verified to be premutation carriers with 62-CGG and 58-CGG repeats, respectively (data not shown). The PCR amplification of the other two samples failed repeatedly for the CGG-region of the FMR1 gene, but not for the control K-ras gene, as shown in lanes E and F (Fig. 2). Hence, the possibility that these two samples carried a large premutation or even full mutation could not be excluded entirely, indicating the need for further confirmation with standard Southern blot analysis.

**Discussion**

The screening procedure using dried blood spots on filter papers described in this study is not only convenient for sample collection and transportation, but also utilizes a simple and inexpensive DNA preparation and mutation detection technique based on non-radioactive PCR. It can reliably identify the small fraction of suspicious samples among carrier candidates, who require further examination by the more expensive method of Southern blot analysis. Among those proven carriers or FXS patients, the carrier status of their mothers, which would be unknown prior to the procedure, could also be indirectly confirmed. This information is very important for future pregnancy references, as well as assessment of other family members. Furthermore, confirmed diagnosis of some FXS patients could also be made at very young ages. Although specific treatment is not yet available, such young FXS patients can have the opportunity to benefit from early intervention programs, as well as from treatments that may be developed in the future.

In our previous pilot study, a normal transmitting male from a random selection of 100 women and 100 men was incidentally identified from the leftover
blood samples randomly selected from a hematology laboratory. This result suggested that mutant FMR1 allele might be present in around 1 out of 300 X chromosomes. However, in this relatively large series of screening of Taiwanese newborn boys, we found the estimated prevalence of FMR1 mutation carrier to be less than 1:1211 (4 or less in 4843). Such rates have not yet been reported in many other populations but can be estimated indirectly from similar screening studies in women. A previous study found that the prevalence of female fragile X carriers including premutation and full mutation was 1:113 in Israel, which may indirectly imply a rate of 1:226 in men because of the presence of only one X chromosome. The female fragile X carrier rates reported in the United States, Finland and Canada ranged between 1:186 and 1:259, which indirectly suggests a rate of 1:372–518 in the male population. Analysis of the data using chi square and Fisher’s exact tests indicated that the prevalence of fragile X male carrier in southern Taiwan is significantly lower than in these countries (p < 0.001). A similarly low prevalence of FXS mutation in Taiwan was also recently reported by Chiang et al based on the screening of 1000 newborn boys.

Theoretically, the prevalence of carriers should correlate with the overall incidence of FXS in a population. These studies provided an indirect estimate from the relative frequency of FXS in the mentally retarded subjects. In our previous screening study of mentally retarded patients, excluding Down syndrome and cerebral palsy patients, four cases of FXS were diagnosed among 206 boys (1.9%) and one from 115 girls (0.9%). The incidence of FXS reported from studies in Asian countries has varied considerably, from 0.25% to 6.8%. It is difficult to compare our findings with previous reports because of the differences in the criteria applied for sample selection. However, in a Finnish study, Arvio et al also screened mentally retarded adult males with all Down syndrome and cerebral palsy patients excluded, and found 26 (6.4%) had FXS. This comparison suggests that the prevalence of FXS in Finland is three times greater than in Taiwan.

The frequency of intermediate allele status in a population may also provide a clue to the overall prevalence of FXS. Based on the identification of fragile X founder chromosome, Morton and MacPherson first proposed a concept of multi-step change of the FMR1 allele. They suggested that the frequency of transition increased from one mutational stage to the next (N-to-S < S-to-Z < Z-to-L). In this scheme, “N” represents normal and stable alleles, with CGG repeat less than 40; “S” represents alleles of high end normal or predisposed alleles, with repeat between 41 and 60; “Z” represents premutation and unstable alleles; and “L” represents expanded, full mutation alleles. This concept has also been supported by many other studies. Our results suggest that the frequency of FMR1 allele with intermediate CGG repeat size (40–53) was 1.8% (90/4843) in newborn boys from southern Taiwan. Indirectly, this suggests that approximately 3.6% of Taiwanese women may have FMR1 allele with intermediate repeat size, a lower frequency than that reported in US women. Brown et al found that the frequencies of different repeat ranges were 1.5% (≥ 50), 4% (≥ 45) and 10% (≥ 40). Results from the present study combined with previous studies, as well as the results of Chiang et al strongly suggest that the overall prevalence of FXS in the Taiwanese population is lower than the 1:4000 detected in most Caucasian populations.

In conclusion, this study demonstrated that a simple PCR combined with blood spot sampling is effective and feasible for large-scale screening of newborn boys for fragile X carrier status. Compared indirectly to the carrier rate reported in women of Jewish (1:113, equal to 1:226 in men) and Caucasian populations (1:186–259; equal to 1:372–518 in men), the prevalence of fragile X carrier status in newborn boys of southern Taiwan (1:1211) is significantly lower (p < 0.001). Therefore, the cost-effectiveness of implementation of such screening on a population-basis would be lower than in these western populations.

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References