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Molecular Screening for Fragile X Syndrome in Mentally Handicapped Children in Korea

Fragile X syndrome is one of the most common forms of inherited mental retardation and is caused by the expansion of the CGG trinucleotide repeats in the FMR-1 gene. This study was aimed to facilitate the molecular screening of fragile X syndrome in Korean children with mental retardation of unknown etiology. The subjects were tested by Expand Long Template PCR system in the presence of 7-deaza-dGTP, and then by Southern blot analysis. The PCR method provided rapid and reliable results for the identification of fragile X negative and positive patients. One hundred one mentally retarded children (78 males and 23 females) were screened by PCR amplification, which detected only one abnormal sample. The PCR-positive case was confirmed by the CGG repeat expansion on Southern blot analysis with a positive cytogenetic result. In conclusion, Expand Long Template PCR may be used as the first screening test for detecting the fragile X syndrome.

Key Words : *Fragile X Syndrome; Mental Retardation; Trinucleotide Repeats; Polymerase Chain Reaction*

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INTRODUCTION

Fragile X syndrome or X-linked mental retardation associated with marXq28 is the most common inherited form of familial mental retardation. It is characterized by mental retardation being the most common clinical manifestation, large testes, facial dysmorphism such as a long face, large ears and prominent jaw, etc. This syndrome is caused by the expansion of CGG repeats in the gene called fragile X mental retardation 1 (FMR1) and hypermethylation of its 5' upstream CpG island (1, 2). On rare occasions, this phenotype is associated with deletion of the FMR 1 gene (3-5). The normal number of CGG repeats varies from 6 to about 50. Healthy carriers of both sexes have a premutation consisting of up to about 200 repeats, while affected individuals have full mutations of as many as hundreds to thousands of repeats (1).

DNA analysis played an important part in detecting the mutations of fragile X syndrome in the last decade. Since alleles with expanded CGG repeats are somewhat resistant to amplification by PCR, Southern blot analysis is still the best way to detect the mutations. Southern blot analysis allows us to detect normal, premutated, and full-mutated alleles as well as the methylation status of the gene (6, 7). However, it is expensive, time-consuming, laborious, and requires large quantities of genomic DNA. It is thus not suitable for screening tests. On the other hand, PCR-based technique appears to be a rapid, efficient and reliable tool for screening the mutations of the fragile X syndrome (8-12).

Several screening or diagnostic strategies for fragile X syndrome have evolved from molecular studies, but the experience is still limited in Korea (13-16). In this study, we attempted to screen the mentally retarded or developmentally delayed patients using DNA assay and estimate the frequency of fragile X syndrome among them. The diagnostic scheme was based on screening PCR strategy, so called the Expand Long Template PCR system which enables the amplification of normal, premutated, and full-mutated alleles (17), followed by Southern blot analysis to confirm the diagnosis of fragile X syn-

drome. The data obtained were then compared with the cytogenetic data.

MATERIALS AND METHODS

Patients

Between March 1, 1999 and February 28, 2000, 101 participants (77 males and 24 females) were recruited from the pediatric neurology clinic, Kyungpook National University Hospital, Taegu, Korea. Of these, 63 patients had mental retardation of undetermined cause, 31 had autism spectrum, and 7 had learning disability. Their ages ranged from 10 months to 18 yr (mean, 5.5 yr). Clinical and demographic characteristics of the patients are summarized in Table 1.

Patients were assessed neurologically on entry, and then evaluated by neuropsychological tests when possible. Blood samples taken from 101 patients were analyzed by Expand Long Template PCR, then followed by Southern blot analysis when required. The data were then compared with the cytogenetic data in each case.

Expand Long Template PCR assay of CGG repeats

DNA was extracted from fresh blood lymphocytes. Primer A and Primer B were chosen to flank the CGG repeat region that is to be amplified. The sizes of PCR products were indicative of the approximate number of repeats. We prepared a master mix for DNA amplification with a total volume of 25 μL containing 200 ng of genomic DNA, 300 nM of each primer, Expand Long Template PCR buffer 1 (PCR buffer 10×conc. with 17.5 mM $MgCl_2$), 350 μM of each dNTP and 10% dimethylsulfoxide. dGTP was substituted with 7-deaza-dGTP in a ratio of dGTP (1):7-deaza-dGTP (2) to amplify the premutated and full-mutated alleles. After heating the reaction mixture at about 98℃ for 10 min to separate DNA strands, 3.5 U of Expand Long Template enzyme mix containing *Taq* and *Pwo* DNA polymerases (Boehringer Manheim GmbH, Germany) was added to it. The reaction mixture went through 10 cycles (denaturation at 97℃ for 30 sec, annealing at 56℃ for 45 sec, elongation at 68℃ for 4 min), followed by 20 cycles (97℃, 30 sec; 56℃, 45 sec; 68℃, 4 min and 20 sec). The PCR products were analyzed on about 2% agarose gel containing ethidium bromide by the standard procedure.

Southern blot analysis of the CGG repeat region and cytogenetic analysis

In order to confirm the results of Expand Long Template PCR, DNA samples were analyzed again by Southern blot analysis with StB 12.3 probe after restriction digestion with *Eag* I and *Eco*R I. In cytogenetic study, folate-deficient medium was used for lymphocyte culture.

RESULTS

One hundred one cognitively handicapped children were screened using Expand Long Template PCR system. The PCR products ranged in size from 290 bp to 330 bp, indicating variation of the number of CGG repeats from 29 to 43 in almost all cases. The most common allele ran at about 310 bases (about 36 repeats). There was one case in which the CGG repeat failed to be amplified and no PCR product was found (Fig. 1). This

Table 1. Summary of subjected patients for the screening

Parameters	
Age	5 yr 5 months (2 months-18 yr)
Sex (male/remale)	77/24
Positive family history of mental retardation	6/101(6%)
Clinical diagnosis	
Mental retardation	63/101(62%)
Autism spectrum	31/101(31%)
Learning disability	7/101(7%)
Neuropsychological tests*	
Intelligence quotient (average)	59
Social quotient (average)	70

*Limited to the patients who were able to complete the tests

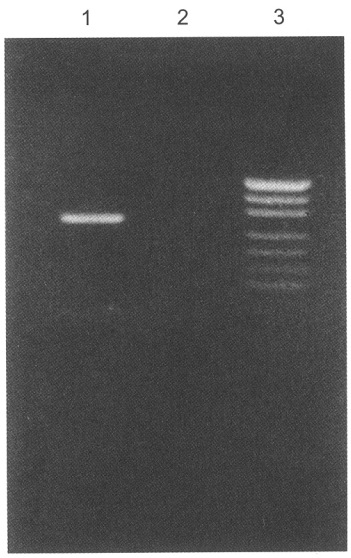

Fig. 1. PCR products on agarose gel. Lane 1: normal control. Lane 2: no PCR products suggesting full-mutated alleles. Lanes 3: DNA marker.

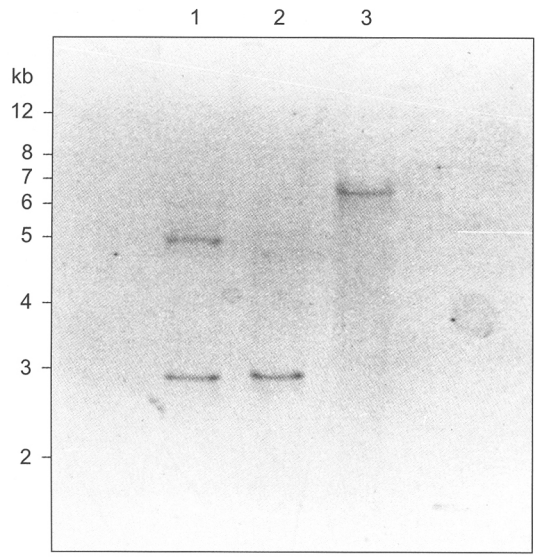

Fig. 2. Southern blot analysis of fragile X syndrome. Lane 1: female normal control. Lane 2: male normal control. Lane 3: the band at molecular weight higher than 5.2 kb suggesting a male with a full mutation.

Fig. 3. Cytogenetic analysis of the case being positive on PCR and Southern blot analysis. The fragile site was noted near the end of the long arm of X chromosome (arrow).

result suggested that this patient might have premutated or full-mutated alleles. As expected, the band occurred at a molecular weight higher than 5.2 kb on Southern blot analysis in this case (Fig. 2). This result was also supported by cytogenetic analysis. The fragile site was noted at the end of the long arm of X chromosome, Xq27 (Fig. 3). According to the results of this study, the frequency of fragile X syndrome in mentally handicapped children was about 1%.

DISCUSSION

Fragile X syndrome is one of the most common genetic causes of mental retardation. The expression of the fragile X syndrome is variable, but the major phenotype is the mental retardation (18). The condition may present with autistic features (19). With a discovery of molecular basis for fragile X syndrome, several diagnostic strategies using DNA analysis have been developed. For the present, Southern blot analysis is still the key diagnostic tool to detect the mutations of the fragile X syndrome, but is laborious, time-consuming and expensive. It is thus not suitable for population screening. PCR-based technique is proven to be a rapid, efficient, and reliable tool for screening the mutations of the syndrome (8-12).

We attempted to screen 101 intellectually handicapped or autistic children for the detection of the mutations of the fragile X syndrome using Expand Long Template PCR.

Expand Long Template PCR is composed of an enzyme mix containing *Taq* and *Pwo* DNA polymerases. It produces greater quantities of large repeats to detect normal, premutated, and full-mutated alleles (17). In our study, PCR products ranged in size from 290 bp to 330 bp, indicative of repeats numbering from 29 to 43 in 100 out of 101 cases. Based on the results, they are likely to have normal alleles. The PCR product of 310 bp (36 repeats) was the most common allele, which is similar to those reported in other studies (1, 20). No PCR product was detected in one case as shown in Fig. 1. As previously reported, the PCR result indicated that he might have full mutations (16, 21). Southern blot analysis was carried out to exclude possibility of technical error and confirm the diagnosis. As expected, the band was found at a molecular weight higher than 5.2 kb in this case. In spite of the advances in the molecular understanding of this syndrome, the cytogenetic analysis still has an honorable role to play in making a diagnosis. It may be the most efficient and cost-effective methodology, particularly for virtually all males (22). For this reason, we made a chromosomal assay on most of the participants when possible. In above case, the fragile site was noted near the end of the long arm of X chromosome, Xq 27. Interestingly, there were two other males with classic clinical features of fragile X syndrome, but they were negative on PCR screening, Southern blot analysis, and cytogenetic analysis. It was suggested that these two might represent allelic variants of fragile X syndrome (23, 24).

A few studies have been done to estimate the frequency of the fragile X syndrome in mentally retarded children in Korea. One of these has been estimated the frequency to be as high as 6.4%, but this figure was based on the cytogenetic study that might be less specific (15). On the basis of molecular genetic assay, recent studies suggested that it may be 1.7% to 2.8% (13, 14, 16). As it is a little premature to say, the frequency of the fragile X syndrome in mentally handicapped children was about 1% in our study, which seems to be a little lower than those previously reported. Considering the number of study subjects and the selection bias of sampling, we think the further studies are needed to estimate the accurate frequency.

In conclusion, Expand Long Template PCR system has proven to be convenient and reliable. We propose that our Expand Long Template PCR be used as the first screening test for detecting the mutations of the fragile X syndrome.

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