Validity of Analysis of FMRP Expression in Blood Smears as a Screening Test for Fragile X Syndrome in the Indian Population

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Molecular diagnosis of Fragile X Syndrome (FXS) is carried out by PCR or Southern blot analysis on DNA isolated from leukocytes. These DNA analyses are time consuming and expensive, making it impractical for mass screening programs. We have recently standardized and tested the diagnostic potential of a rapid antibody test on blood smears, based on the presence of FMRP, the protein product of the FMR1 gene, in lymphocytes from normal individuals and the absence of FMRP in lymphocytes in patients with FXS. This test is essentially similar to the one developed at Erasmus University in the Netherlands, with suitable modifications. The diagnostic power of the antibody test is perfect for

males, whereas the results are less specific for females. The cutoff value for affected male individuals, expressed as the percentage of FMRP-positive cells, was 20%. In normal individuals, the cutoff value was 85%. The results of the antibody test correlated well with that of Southern blots. Sensitivity of the test was 100% and specificity was 97.5%. This noninvasive test requires one or two drops of blood and is rapid, simple, and cheap, making it an ideal choice for large screening large groups of male mental retardates and neonates for FXS in developing countries such as India. *J. Clin. Lab. Anal.* 19:120–123, 2005. © 2005 Wiley-Liss, Inc.

Key words: FXS; India; screening; antibody test

INTRODUCTION

Fragile X syndrome (FXS) is the most common specific, monogenic X-linked cause of mental retardation in humans and has a prevalence of 1 in 4,000 among mentally retarded boys and 1 in 6,000 in girls (1). Fragile X males tend to have a long face, with large anteverted ears and mandibular prognathism, macro-orchidism, speech defects along with lack of eye-to-eye contact, and moderate to profound mental retardation. Affected girls mainly present with anxiety and ear defects (2,3). The FMR1 gene contains a CGG repeat at the 5' region that is polymorphic in the normal population and varies from 6 to 54 repeats. Premutation alleles show that the CGG repeats between 52 and 200 repeats while full mutation alleles show a number beyond 200 (4). The knowledge about this expansion of CGG repeats within exon 1 of the FMR1 gene has resulted in the development of reliable diagnostic methods using direct DNA analysis (5). This expansion of CGG repeats leads to hypermethylation, which leads to silencing of the FMR1 gene, thus preventing protein production. Lack

of expression of the FMR1 gene is responsible for the mental retardation. The FMR1 gene is expressed in almost every tissue, with the highest expression in brain and testes.

Since the discovery of the gene defect, the molecular diagnosis of FXS has been carried out mainly by use of Southern blot hybridization or PCR analysis (2,6). These analyses generally take more than 7 days. Here we describe a noninvasive antibody test to identify FXS patients. This test requires only two drops of blood and the results of this test are available within 24 hr.

Female premutation carriers have a high risk of having affected sons and daughters. There is a strong

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correlation between the length of the premutation and the risk for expansion to full mutation in the next generation (7,8). The likelihood of repeat instability increases with the repeat number. There is no expansion of the premutation allele to the full mutation if it is transmitted to the offspring by males. Thus male premutation carriers will never have affected children (9). These men are called normal transmitting males because all their daughters will inherit their father's unchanged expansions and hence they become female premutation carriers, "at risk" of having offspring with the full mutation.

In this study, we report the evaluation of the antibody test by determination of FMRP expression in 185 blood smears from healthy individuals and subjects suspected to have FXS. This simple test involves the use of a monoclonal antibody against the FMR1 protein FMRP in a peripheral blood smear (10). In normal individuals, FMRP is detected as a pink precipitate in the cytoplasm of the lymphocytes, while in lymphocytes of male Fragile X patients, there is no precipitate (since the protein is not expressed).

In blood smears from control individuals and carriers with the premutation, FMRP can be detected in the cytoplasm of lymphocytes, whereas lymphocytes of male FXS patients are devoid of FMRP. But it has been observed that some lymphocytes of affected males expressed FMRP, which may be due to a premutation in the positively labeled cells (10).

MATERIALS AND METHODS

The study was carried out at Kamayani School, a school for the mentally challenged in Pune, India. The antibody test was carried out in 25 normal individuals and 165 children with idiopathic mental retardation, after ruling out all other causes.

The indirect alkaline phosphatase technique employed in this study was essentially similar to the method described in 1995 by Willemson et al. (10), with suitable modifications (9).

Blood smears were made from one or two drops of blood directly after bleeding and were subsequently stored at -20°C for ≤ 1 week before processing. Then the slides were air dried and fixed in 0.1 M phosphate buffer (pH 7.3) containing 3% paraformaldehyde for 15 min at room temperature. Further, the cells were permeabilized by treatment with 100% methanol for 20 min at room temperature. After washing with phosphate buffered saline containing 0.15% glycine and 0.5% bovine serum albumin, the lymphocytes were incubated with mouse monoclonal antibodies against FMR1 protein (Chemicon, Temecula, CA) for 16 hr at 4°C . Visualization of the antibody-antigen complex was

achieved by an indirect alkaline phosphatase technique using goat anti-mouse immunoglobulin conjugated with biotin (Bangalore Genei, India) (60 min) and streptavidin-biotinylated alkaline phosphatase complex (Bangalore Genei; Bangalore, India) (45 min) as secondary and tertiary steps, respectively. The new fuchsin substrate-chromogen system (DAKO, Cytomation, Carpinteria, CA) was used for 30 min in the final staining step utilizing alkaline phosphatase activity. Levamisole (Sigma, St. Louis, MO) was added in the substrate solution (1 g/40 mL) to block endogenous alkaline phosphatase activity. Slides were counterstained with Gill's hematoxylin (Sigma) and mounted with aqua mount (DAKO). Slides were examined under an Olympus (Japan) light microscope.

RESULTS

FMRP expression is seen in lymphocytes of normal subjects, whereas no or very low expression is seen in lymphocytes of fragile X patients. In every blood smear, 100 lymphocytes were examined and scored for the presence of FMRP. The number of lymphocytes labeled for FMRP was expressed as a percentage of the total lymphocytes screened. The mean and standard deviation (SD) of the percentage of FMRP expression in normal males and affected males was calculated.

The mentally retarded children suspected to have FXS by antibody test were subjected to direct analysis and Southern blot, which showed the full mutation. The mentally retarded males with full mutation showed 5–20% of lymphocytes positive for FMRP while normal males showed 85–95% cells positive. Expression of FMRP in normal and retarded males shown in the scattergram showed a clear-cut demarcation (Fig. 1). A total of 20 samples negative for FXS by antibody test were also sent for DNA analysis and gave negative results. In clinical practice, a cutoff point is used for diagnostic tests. Below the cutoff point, the individual is suspected to be suffering from FXS. The cutoff point has been determined to be 20% for affected males and 85% for normal males. The antibody test showed 100% sensitivity, 97.5% specificity and accuracy (Table 1) when used only for males.

DISCUSSION

To date, the identification of patients with FXS have been carried out by direct DNA analysis such as PCR analysis and Southern blots. For screening a large number of patients, these methods are time-consuming and inconvenient. The antibody test is an efficient and reliable method to detect FXS in screening programs. In males, the statistical analysis of data confirms the high sensitivity of the FMRP test. Data from affected males

Fig. 1. Scattergram showing expression of FMRP in normal and retarded males.

TABLE 1. Summary of results of FXS status on the basis of antibody test and southern blot

	Diagnosis by Ab test	
	Normal	FXS
Diagnosed by Southern blot		
Normal	153	4
FXS	—	8
	Sensitivity = 8/8	100%
	Specificity = 153/157	97.5%
	Accuracy = 161/165	97.6%
	Predictive value for normal 153/153 = 100%	
	Predictive value of FXS 8/12 = 66.6%	

show high specificity, as there is no overlap between patients and controls. This implies that the FMRP test can discriminate between affected and normal males on the basis of FMRP expression in lymphocytes. However, permutation carriers cannot be detected with this test as the percentage of expression is between 50 and 60%.

From this antibody test, we identified male patients showing either no or a very low expression of FMRP positive lymphocytes. All of the 20 negative controls were positive for FMRP in their lymphocytes. The test is easy to carry out, inexpensive, and can be used on several samples in a short time and the result can be obtained in 1 day. However, since this test is not a quantitative Enzyme Linked ImmunoSorbent Assay (ELISA), microscopic evaluation of lymphocytes is needed because of endogenous alkaline phosphatase activity.

The mentally challenged individuals who were studied in this report were not tested for FXS earlier by DNA analysis. All the samples in which the FMRP expression was <70% were studied by Southern blot analysis for confirmation. A maximum of 8% FMRP expression was observed in lymphocytes of the affected group.

Not many reports are available from India revealing the frequency of this disorder among the institutionalized mentally retarded population, except for very few studies in Delhi, where Dr. B.K. Thelma and group reported it to be around 7.7%, and another study at the All India Institute of Medical Sciences, where they obtained a frequency of approximately 2.2% (11). In our study, the frequency was approximately 4.8%. This variation in frequency may be due to the small sample sizes in each group. The combined results of studies from England, the United States, Australia, and the Netherlands, irrespective of methodological differences, showed in a total of 4,670 mentally retarded subjects, 30 (0.6%) newly-diagnosed FXS.

The rapid antibody test enables discrimination of normal from affected, making it a suitable test for screening of mental retardates in special schools for children with learning disabilities. This test may also be used for screening neonates, enabling counseling for parents and children.

FX carrier screening studies in Israel involving 14,334 women showed that the cost of the test was much cheaper than the cost of lifetime care for a mentally challenged person, due to the high prevalence of the condition in the general population (12).

The antibody test employed in this study is a very efficient, rapid, simple, and cost effective method for screening a large number of cases. This could be introduced into the school health program in developing countries, to identify affected individuals and subsequently carriers of the FX mutation. All identified patients have to be referred to a clinical genetics center to allow genetic counseling of the family involved. Further evaluation will automatically include DNA analysis for carrier detection. For patients and families, a correct diagnosis is essential and leads to the availability of adequate support for the behavioral and physical problems. Furthermore, female carriers can benefit from genetic counseling about the risk to their offspring, with an option for prenatal diagnosis, and for themselves regarding premature ovarian failure, with the option of completing their families early.

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