Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique

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Abstract To date, the identification of patients and carriers of the fragile X syndrome has been carried out by DNA analysis by means of the polymerase chain reaction and Southern blot analysis. This direct DNA analysis allows both the size of the CGG repeat and methylation status of the FMR1 gene to be determined. We have recently presented 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 from patients. Here, we have tested the diagnostic value of this new technique by studying FMRP expression in 173 blood smears from normal individuals and fragile X patients. The diagnostic power of the antibody test is "perfect" for males, whereas the results are less specific for females.

Introduction

Fragile X syndrome is the most frequent form of inherited mental retardation in humans and has a prevalence of approximately 1:4000 males and 1:6000 for females (Turner et al. 1996). The clinical symptoms are moderate to profound mental retardation, macroorchidism and minor dysmorphic features, and specific behavioral characteristics (Hagerman 1996). The identification of the mutational mechanism underlying the fragile X syndrome, an expansion of the CGG repeat within exon 1 of the FMR1 gene, has resulted in the development of a reliable diagnostic method by using direct DNA analysis (Oberle et al. 1991; Oostra et al. 1993; Rousseau et al. 1991; Verkerk et al. 1991). The CGG repeat is polymorphic in the normal population and varies from 6–53 repeats, with a mean of 30 repeats (Fu et al. 1991). In normal transmitting males and unaffected female carriers, a repeat length between 43 and 200 (premutation) is observed, whereas in patients with the fragile X syndrome, an expansion of more than 200 CGG repeats (full mutation) is found. The length of the repeat can be determined by polymerase chain reaction (PCR) analysis of the DNA from cells, e.g., white blood cells. However, only the size of the CGG repeat within the normal and premutation range can be investigated in this way. The reliable detection of a full mutation can only be obtained by Southern blot analysis (Oostra et al. 1993; Rousseau et al. 1991). The gross expansion in repeat number usually coincides with hypermethylation of the repeat and the CpG island proximal to the gene (Hansen et al. 1992; Sutcliffe et al. 1992). Methylation is associated with silencing of the FMR1 gene, thus preventing protein production (Devys et al. 1993; Verheij et al. 1993). The absence of the FMR1 protein is now well established as causing fragile X syndrome.

We have developed a new diagnostic test involving the use of monoclonal antibodies against the protein product of the FMR1 gene, FMRP. This rapid test is performed on blood smears and thus needs only one drop of blood (Willemsen et al. 1995). Recently, a similar test with which we have been able to identify the lack of FMRP in fetuses with a full mutation has been described for chorionic villi (Willemsen et al. 1996). In blood smears from control individuals and carriers with a premutation, FMRP can be detected in the cytoplasm of lymphocytes, whereas the lymphocytes of male fragile X patients are devoid of FMRP, because of lack of transcription caused by the hypermethylation of the promoter region of the FMR1 gene. Interestingly, we have frequently observed FMRP expression in some lymphocytes from affected males, suggesting the presence of a premutation in these positively labeled cells. This phenomenon can be expected because of
the mosaic DNA pattern found in over 50% of affected males (De Graaff et al. 1995; Rousseau et al. 1991). In our initial report, we have also described a female carrier who had a full FMR1 mutation and who showed expression of FMRP in approximately 50% of her lymphocytes. Apparently, random inactivation of the X-chromosome had occurred in the lymphocytes of this carrier, because only the transcription of the FMR1 gene on the active “normal” X-chromosome would result in expression of FMRP. However, these results were based on a limited number of patients and control individuals. In this study, we report the evaluation of the antibody test by determination of FMRP expression in 173 blood smears from healthy individuals and fragile X patients with a full mutation.

Materials and methods

Mouse monoclonal antibodies against FMRP were generated in a so-called Tecno-mouse system by using specific hybridoma clone 1C3-1a. This antibody or the commercially available crude ascites fluid (1:1600; Euromedex, France) was used.

Blood smears were made within 6 h after sampling of the blood, air-dried, sealed in Saran Wrap, and stored at −80°C. Alternatively, smears were stored at room temperature in a slide box. Immuno-incubation of the smears was performed as described (Willemsen et al. 1995) with an indirect alkaline phosphatase technique (see Fig. 1). Smears were counterstained with hematoxylin and examined with a Zeiss Axioskop 20. Detailed information concerning this immunocytochemical technique can be found on the Internet at http://www.eur.nl/FGG/CH1/fragx/.

Results

Immuno-incubation and analysis of the blood smears was performed in two different laboratories. Good results were recently obtained by making smears immediately after blood sampling and storage at room temperature for a maximum period of three weeks, making this storage protocol preferable to that of frozen smears. However, for standardization of the technique in both laboratories, the results in this report are based on blood smears that were stored at −80°C. In every blood smear, 100 lymphocytes were examined and scored for the presence of reaction product, viz., FMRP. The numbers of lymphocytes labeled for FMRP were expressed as a percentage of the total lymphocytes examined. The mean and SD of the percentages of FMRP expression in the different groups are given in Table 1.

Two groups of males and females were identified based on their mutational and mental status. The mentally retarded individuals that were studied in this report had previously been diagnosed for fragile X syndrome by DNA analysis, and all showed a full mutation. Briefly, no significant differences were found between the means of normal males (n = 33) and normal females (n = 27), which were 89% and 80%, respectively, whereas mentally retarded males with a full mutation (n = 69) and affected females with a full mutation (n = 44) showed significantly lower means of 7% and 39%, respectively. The antibody test for diagnosing fragile X syndrome is meant to give an accurate positive or negative test result. Such dichotomization is inherent with respect to clinical practice of the fragile X syndrome. In the blood smears, we observed variability in the number of FMRP-expressing lymphocytes in the various control individuals and fragile X patients. We plotted the distribution of the percentage of FMRP expression in normal and retarded male and female individuals (Fig. 2). For males, the diagnostic power of the test is “perfect”, because there is no overlap between the values of normal males and retarded males with a full mutation (Fig. 2A). For females, there is some over-

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Fig. 2 FMRP distribution in males (A) and females (B). In every blood smear, 100 lymphocytes were examined. The percentage of FMRP expression is depicted in categories (X-axis), whereas the Y-axis illustrates the percentage of patients for each category.

lap of the percentages in the two different groups (Fig. 2B), although this is not an indication that this antibody test is unsuitable for diagnostic purpose.

In clinical practice, a cut-off point is used for diagnostic tests. Below the cut-off point, the individual is suspected as being affected with the fragile X syndrome. In all other cases, the individual is excluded from having a full mutation. Sensitivity should be 100% for a reliable diagnostic test. Therefore, we have chosen the maximum FMRP expression found in fragile X patients as the cut-off point. Because of the accuracy of the detection FMRP expression, the upper limit of the 95% confidence interval for this maximum FMRP expression, based on 100 lymphocytes, has been taken. For males, the cut-off point has been determined to be 42% and for females 83%. By using these cut-off points, the sensitivity is 100% for both males and females. The specificity is 100% for males and 41% for females.

A second question that should be addressed is the number of lymphocytes that should be examined to establish FMRP expression sufficiently accurately for diagnostic use. Less than a 0.1% misclassification of the full mutation has been used as a criterion. The following procedure has been employed to calculate the percentage misclassified when using $n$ lymphocytes. For a carrier, the probability of being misclassified can be calculated by using a binomial distribution with parameters $n$ and probability $P$, by taking the FMRP expression in the 100 investigated lymphocytes. The percentage misclassified is the sum of these probabilities, summing over all full mutation carriers. By using this best cut-off point for males (42%), a reliable test result can be obtained by counting 80 lymphocytes; for females (83%), 50 lymphocytes should be examined.

**Discussion**

To date, the identification of patients with the fragile X syndrome has been carried out by using a combination of a direct DNA test by PCR analysis and Southern blotting. For screening large numbers of individuals, this method is inconvenient, because the technique is time-consuming. The recent development of a rapid antibody test for diagnosing fragile X syndrome enables the detection of fragile X patients in large screening programs. However, a validation of this new test should be performed before its implementation in clinical practice. Here, we present an evaluation of this antibody test on blood smears of a large group of individuals ($n = 173$), including fragile X patients ($n = 113$), as carried out in two independent laboratories.

In males, statistical analysis of the data confirms the high sensitivity of the FMRP test, as previously suggested.
in the original report in which a limited number of patients was investigated (Willemsen et al. 1995). DNA analysis by Southern analysis of white blood cells from affected males previously revealed the presence of a full mutation and premutation in some patients (n = 5; mosaic). This has been confirmed by the antibody test, which shows FMRP expression in some of their lymphocytes. No overlap should exist between patients and controls. The data from affected males show a high specificity for all patients studied. This implies that the FMRP test can discriminate between affected and normal males on the basis of FMRP expression in lymphocytes. Nevertheless, carriers with a premutation cannot be detected with this antibody test, because they express normal amounts of FMRP in their lymphocytes (Feng et al. 1995). However, premutations can be detected by PCR analysis and Southern blot analysis. In females, the results of the antibody test in blood smears are less specific because of an overlap of the values in this patient group and the controls.

The values of FMRP-expressing lymphocytes in blood smears from males show a higher discriminating power than those from females. The best cut-off point for males is 42% positive labeled lymphocytes and for females 83% positive labeled lymphocytes. Thus, no female with the fragile X syndrome will be missed, but approximately than those from females. The best cut-off point for males is 42% positive labeled lymphocytes and for females 83% positive labeled lymphocytes. Thus, no female with the fragile X syndrome will be missed, but approximately 45% of the smears from healthy females with a normal repeat length will also show a value below 83%, giving in a large number of possible fragile X patients (false-positive test results). It is essential to confirm a positive antibody test result in the identified patients via a DNA test. Although this seems to make the test less suitable for females, the majority of the samples from females can be excluded before DNA testing.

In addition, the storage of smears at room temperature for a maximum period of three weeks is an important step toward routine application of the antibody test for diagnosing fragile X syndrome. This enables preparation of the blood smears and subsequent transport of the slides by regular mail. Finally, all blood samples have been independently tested in a second laboratory. Although the results of the two laboratories are similar, this has taken some time to achieve. The main cause for this problem has been the use, in one laboratory, of the commercial antibody, which gives higher backgrounds than the Technoantibody used in the other laboratory, making the interpretation of the test more difficult. However, a new dilution scheme of 1:1600 of the commercial antibody now gives reliable results. Furthermore, recognition of the different types of white blood cells by microscopy initially proved problematic.

In conclusion, the rapid antibody test enables normal and affected males to be discriminated, making this test extremely suitable for screening males in, for example, institutes for the mentally handicapped or schools for children with learning disabilities. 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.

In theory, the antibody test may also be used for screening every newborn male for fragile X syndrome. Although no technical obstacles for neonatal screening are present, ethical issues remain to be resolved before such a screening program can be started. Furthermore, after identifying patients in this way, DNA testing and adequate genetic counseling facilities will again be necessary to identify carriers of the fragile X mutation in these families.

References


