Am. J. Hum. Genet. 65:98–103, 1999

Noninvasive Test for Fragile X Syndrome, Using Hair Root Analysis

Rob Willemsen,[1] Burcu Anar,[1] Yolanda De Diego Otero,[1] Bert B. A. de Vries,[1] Yvonne Hilhorst-Hofstee,[2] Arie Smits,[3] Eddy van Looveren,[1] Patrick J. Willems,[1] Hans Galjaard,[1] Ben A. Oostra[1]

[1]MGC Department of Clinical Genetics and Centre for Biomedical Genetics, Erasmus University Rotterdam, Rotterdam; [2] Department of Clinical Genetics, Leiden University Medical Center, Leiden; and [3]Department of Human Genetics, University of Nijmegen, Nijmegen, The Netherlands

Summary

Identification of the *FMR1* gene and the repeat-amplification mechanism causing fragile X syndrome led to development of reliable DNA-based diagnostic methods, including Southern blot hybridization and PCR. Both methods are performed on DNA isolated from peripheral blood cells and measure the repeat size in *FMR1*. Using an immunocytochemical technique on blood smears, we recently developed a novel test for identification of patients with fragile X syndrome. This method, also called "antibody test," uses monoclonal antibodies against the *FMR1* gene product (FMRP) and is based on absence of FMRP in patients' cells. Here we describe a new diagnostic test to identify male patients with fragile X syndrome, on the basis of lack of FMRP in their hair roots. Expression of FMRP in hair roots was studied by use of an FMRP-specific antibody test, and the percentage of FMRP-expressing hair roots in controls and in male fragile X patients was determined. Control individuals showed clear expression of FMRP in nearly every hair root, whereas male fragile X patients lacked expression of FMRP in almost all their hair roots. Mentally retarded female patients with a full mutation showed FMRP expression in only some of their hair roots (<55%), and no overlap with normal female controls was observed. The advantages of this test are (1) plucking of hair follicles does no appreciable harm to the mentally retarded patient, (2) hairs can be sent in a simple envelope to a diagnostic center, and (3) the result of the test is available within 5 h of plucking. In addition, this test enabled us to identify two fragile X patients who did not show the full mutation by analysis of DNA isolated from blood cells.

Received February 12, 1999; accepted for publication May 13, 1999; electronically published June 7, 1999.

Address for correspondence and reprints: Dr. B. A. Oostra, Department of Clinical Genetics, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: oostra@kgen.fgg.eur.nl

0002-9297/99/6501-0014$02.00

Introduction

Fragile X syndrome (MIM 309550), the most common inherited cause of intellectual disability, was one of the first of an increasing number of neurogenetic disorders in which a dynamic mutation of a trinucleotide repeat was identified (Kooij et al. 1998). Expansion of a CGG repeat to a full mutation of >200 repeats within the *FMR1* gene results in hypermethylation of the *FMR1* promoter and subsequent transcriptional silencing of the *FMR1* gene (Oberlé et al. 1991; Verkerk et al. 1991; Sutcliffe et al. 1992). The absence of the *FMR1* gene product (FMRP) is responsible for the mental retardation observed in fragile X syndrome (Devys et al. 1993; Verheij et al. 1993).

The fragile X syndrome affects 1 in 4,000 males and 1 in 6,000 females (Turner et al. 1996; De Vries et al. 1997). In males, the methylated full mutation is always associated with mental retardation, whereas ~65% of females with a full mutation have only mild mental impairment.

The identification of the molecular basis of the fragile X syndrome led to the development of reliable DNA-based diagnostic methods (Rousseau et al. 1991*a;* Oostra et al. 1993). Southern blot hybridization (Rousseau et al. 1991*a*) and PCR (Fu et al. 1991; Brown et al. 1993) are mainly performed on DNA isolated from leukocytes obtained from peripheral blood. Both DNA tests determine the length of the CGG repeat in persons carrying the *FMR1* gene, who can be divided into three categories: (1) normal individuals with 6–53 CGG repeats (normal allele), (2) normal transmitting males and unaffected females with a repeat length of 43–200 (premutation), and (3) patients with a repeat length of >200 CGG repeats (full mutation).

Normal alleles and premutation alleles are usually transcribed and translated, whereas full mutation alleles are not transcribed and lead to the absence of FMR1 protein (Devys et al. 1993; Verheij et al. 1993). We have described an alternative diagnostic test to identify patients with fragile X syndrome on the basis of the absence of FMRP in lymphocytes. This antibody test can identify persons with fragile X by means of only 1 or 2 drops of blood (Willemsen et al. 1995, 1997). Advantages of

this antibody test, compared with the DNA test, include the following: (1) it can be performed in a single day; (2) it does not require radioactivity; and (3) it detects all loss-of-function mutations, including the prevalent CGG repeat amplification and other mutations. Nevertheless, a minority of fragile X patients still escape detection by DNA tests and antibody tests on blood smears. Some males with fragile X show only a premutation *FMR1* allele and not a full mutation on Southern blots.

This report (1) describes the first results of a novel, noninvasive antibody test on hair roots that can identify individuals with fragile X and (2) describes other advantages of this test in the identification of fragile X patients. In addition, we present two cases in which patients with the premutation showed FMRP expression in hair in the range of fragile X patients with a mosaic pattern.

Patients and Methods

Patients

The first patient is a 4.5-year-old boy with psychomotor delay. He was born after an uneventful pregnancy and delivery, weighing 2,500 g. During his first year, he cried more than normal babies usually do. His development was slow, and he first walked unaided at age 24 mo. At age 4.5 years, he has delayed mastery of language and the speech of a 1.5-year-old. He is a restless boy with difficulties in concentrating and in adapting to new situations—e.g., hospitalizations and outpatient clinic visits. He shows no dysmorphic features except for a slight skull asymmetry. The parents have followed secondary education; the mother is working as a nurse and the father is a skilled technical laborer.

The second patient is an 11-year-old boy with an IQ of 77 (WISC-R). Clinical features include hyperextensible metacarpophalangeal joints and otitis media. Behavioral features include language and concentration problems and shyness. He has a 13-year-old brother who is normal. The parents have followed secondary education.

DNA Analysis

Genomic DNA was isolated from blood by the salting out procedure, as described by Miller et al. (1988), and 5 μg of the genomic DNA was digested to completion with *Hin*dIII. The DNA was electrophoresed on a 0.7% agarose gel, transferred to a nylon membrane (Hybond N^+, Amersham), and hybridized to the pP2 probe, which detects a fragment containing the $(CGG)_n$ and the preceding CpG island (Oostra et al. 1993). After overnight hybridization at 65°C, the filters were washed to 0.3 × SSC/0.1% SDS at 65°C, and the signal was detected by autoradiography. PCR analysis was performed according to Fu et al. (1991).

Hair Root Analysis

Informed consent was given, and hair roots were obtained from the two patients, the control group of 130 normal individuals (males and females), 34 fragile X patients with mental retardation (22 males and 12 females), and 15 mentally retarded individuals without the fragile X syndrome. The fragile X syndrome had been excluded in this last group by DNA analysis. A pair of tweezers was used to pluck ~10–20 hair roots from different areas behind the ear on the scalp. Hair with visible bulbs and sheaths were selected and trimmed to just above the sheath. After fixation within 4 h in 3% paraformaldehyde at room temperature (RT) for 10 min, cells were permeabilized by treatment with 100% methanol at RT for 20 min. After being washed with phosphate-buffered saline, containing 0.15% glycine and 0.5% bovine serum albumin, hair roots were incubated as whole mount with mouse monoclonal antibodies against FMRP at RT for 90 min. Visualization of antibody-antigen complexes was achieved by means of an indirect alkaline phosphatase technique, with goat antimouse immunoglobulins conjugated with biotin (Biogenex) and streptavidin-biotinylated alkaline phosphatase complex (Biogenex) used as second and third steps, respectively. The new fuchsin substrate–chromogen system (DAKO), which utilizes alkaline phosphatase activity, was used for 10–15 min during the final staining step. Levamisole was added in the substrate solution, according to the guidelines of the manufacturer, to block endogenous alkaline phosphatase activit. Immunolabeled hair roots were examined with a stereo zoom microscope at a final magnification of 70×. An investigator who was unaware of the phenotype of the individual randomly numbered and scored all the hair roots for presence or absence of FMRP. The number of FMRP-positive hair roots showing red color was expressed as a percentage of the total number of hair roots examined. Different storage conditions were investigated during the course of the study. Normal hair roots were either stored at RT for several days (maximum 10 d) or directly frozen on dry ice and stored at −80°C until used. In hair roots that are stored at RT, the level of FMRP expression gradually decreases with time. Therefore, the immunoprotocol was adapted for hair roots that were stored at RT, by incubation of the mouse antibodies against FMRP for 16 h at 4°C instead of 90 min at RT. For detailed technical information about the antibody test, please visit our website (http://www.eur.nl/fgg/ch1/fragx/).

Results

The two boys with cognitive deficits were tested for fragile X syndrome, and in both cases we identified the presence of a premutation in DNA isolated from white blood cells. The 4.5-year-old boy showed a repeat size of 60 and the 11-year-old boy a repeat size of 190 CGGs. No full mutation could be detected (data not shown). As the presence of a premutation alone could not explain the psychomotor delay in the children, we tried to exclude the presence of a mosaic pattern with a full mutation in other tissues, including the brain. In an effort to test for the mutation in an accessible tissue whose lineage during embryonal development is closer to brain cells, we decided to set up a method to test the expression of the *FMR1* gene in hair roots.

The expression of FMRP in a hair root from a control individual is illustrated in figure 1*a*. The highest expression is observed in the bulb, and the labeling gradually decreases in the direction of the hair shaft. Although the positive reaction—a red color caused by precipitation—can already be seen macroscopically, scoring was performed with a stereo zoom microscope. The detection of FMRP in hair roots that were stored frozen or at RT (for 10 d) was unchanged under these storage conditions. Figure 2 shows the percentage of FMRP-expressing hair roots from normal individuals, mentally retarded individuals not having fragile X syndrome, and male and female patients with fragile X syndrome. Most hair roots from controls showed positive staining (range, 77%–100%), and ~40% of the controls had 10%–20% of hair roots without clear FMRP activity (fig. 2). Unlabeled hair roots in normal individuals probably result from technical occurrences during the procedure (fixation, permeabilization, and immunoincubation).

Most hair roots from male fragile X patients were devoid of FMRP (fig. 1*b*); only in 10 of 22 male patients was a mosaic pattern seen with the presence of $\leqslant$30% normally staining hair roots (fig. 2). This is in line with the percentage of affected males showing a mosaic genotype at the DNA level (Rousseau et al. 1991*a*; Nolin et al. 1994; De Graaff et al. 1995).

Female subjects with a full mutation and some intellectual impairment showed a variable expression (range, 0%–55%) of FMRP in hair roots (fig. 1*c*), with either positive-labeled or completely negative-labeled hair roots. No overlap existed in the percentage of FMRP-expressing hair roots between control individuals and the tested fragile X patients. The expression pattern of FMRP in hair roots from affected individuals without fragile X syndrome showed a percentage within the normal range in all 15 persons. With this newly developed method, the two boys with cognitive deficits and a premutation but no detectable full mutation in their blood cells, along with their mothers, were tested for FMRP expression in hair roots. We found FMRP expression in 83% and 90% of the hair roots of the respective mothers, which is clearly in the normal range (fig. 2). The normal brother in the second case showed an FMRP expression of 100%. On the other hand, the 4.5-year-old boy showed FMRP expression in only 27% of his hair roots, and the 1-year-old boy showed an FMRP expression of 11%, both of which are in the range of fragile X patients with a mosaic pattern (fig 2).

Discussion

In the 1970s, human hair roots were frequently used for biomedical research, especially for diagnosis of inborn errors of metabolism. In particular, the detection of female carriers of X-linked disorders, such as Lesch-Nyhan syndrome, glucose-6-phosphate dehydrogenase deficiency, and Fabry disease, benefited from biochemical analysis of hair roots (Gartler et al. 1969, 1971; Vermorken et al. 1978).

In the present study, FMRP expression in hair follicles was examined in an effort to develop a noninvasive test for detection of patients with fragile X syndrome. In control individuals, both male and female, we were able to detect FMRP expression in most hair roots, whereas in male fragile X patients, we observed no expression in the majority of hair roots. The observed mosaic pattern in some male fragile X patients with expression of FMRP in a minority of their roots is compatible with the finding of a mosaic DNA pattern in peripheral leukocytes from $\leqslant$40% of the affected males (Nolin et al. 1994). However, there is no overlap between control individuals and affected fragile X males with a mosaic pattern in the percentages of hair roots expressing FMRP. In our study, the highest percentage found in the group of fragile X males was 33%, whereas for normal individuals the lowest percentage was 77%. Therefore, this novel test on hair roots enables reliable identification of male fragile X patients.

For reliability, the number of hair roots analyzed was 10 for males and 20 for females. All individuals in our study had a positive opinion about the invasive character of hair plucking—subjects experienced hair plucking as less invasive than drawing blood. The advantages of the use of hair roots for diagnostic purposes rather than blood are obvious. First, hair roots can be plucked simply and without appreciable harm to the patient—considerable advantages in sampling mentally retarded patients. Second, afterbleeding and infections, which are possible complications of blood sampling, are avoided. Third, qualified medical personnel are not needed to perform hair plucking. And last, large quantities of hair follicles from many individuals can be sent by routine

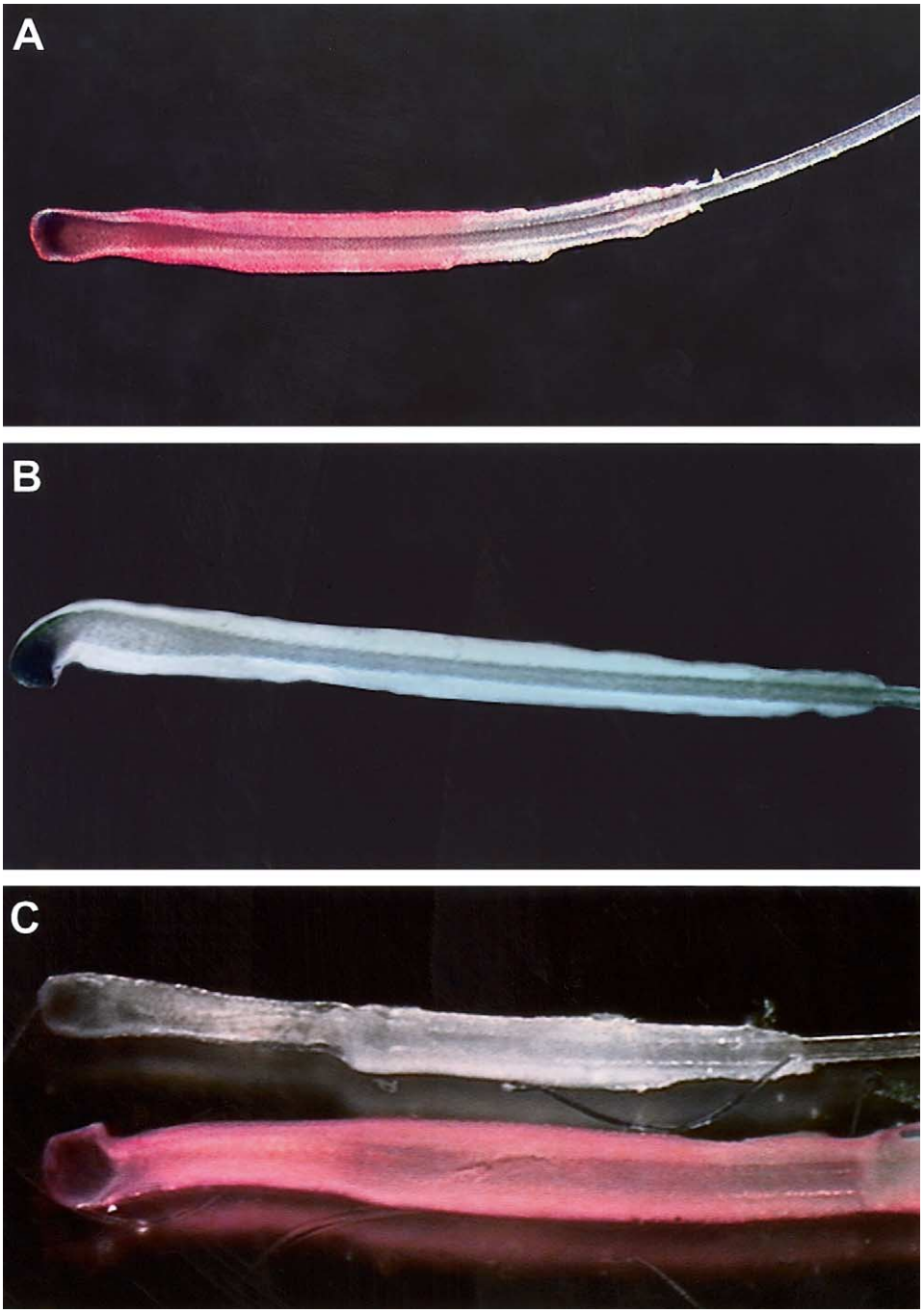

Figure 1 Microscopic localization, by means of the indirect alkaline phosphatase immunolabeling technique, of FMRP in hair roots from a control individual (*a*), a male patient with fragile X syndrome (*b*), and a mentally retarded female patient with a full mutation (*c*). Expression of FMRP is seen as a red precipitate. In control individuals, the red color of the precipitate is clearly visible in the root, and the high expression seen in the bulb gradually decreases upward to the shaft (*a*). Hair roots from affected males are devoid of FMRP labeling (*b*). In affected females with a full mutation, hair roots are either positive or totally negatively labeled (*c*).

postal service to a diagnostic laboratory for analysis within 1 d.

Routine diagnosis of fragile X syndrome is mainly performed by use of DNA isolated from white blood cells. Recently, we described an antibody test to identify fragile X patients by means of an immunocytochemical technique using blood smears (Willemsen et al. 1995). This test, which uses monoclonal antibodies against FMRP, is based on the absence of FMRP in peripheral lymphocytes. We have validated this method and have

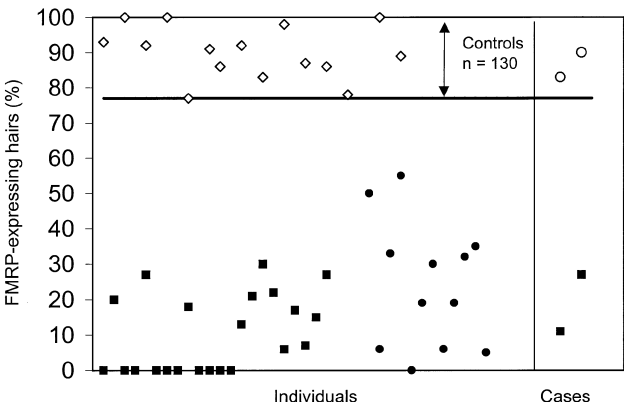

Figure 2 Distribution of the percentage of FMRP-expressing hair roots from affected males ($n = 22$) (blackened squares), affected females with a full mutation ($n = 12$) (blackened circles), and affected individuals without fragile X syndrome ($n = 15$) (unblackened diamonds). Arrowheads indicate the control range (77%–100%; $n = 130$). On the right, two affected males (blackened squares), with FMRP expression in only a minority of hair roots, and their mothers (unblackened circles), with FMRP expression in the range of normal individuals.

shown a high reliability and specificity for detecting male patients (Willemsen et al. 1997). Unfortunately, this method uses blood cells, too, as a source for protein analysis, with disadvantages similar to those of DNA analysis, including blood sampling, possible complications, and origin of blood cells that come from a mesodermal lineage. Both antibody tests on blood smears and hair root analysis have the advantage of having the test results within 1 d.

The power of the technique was further illustrated by the identification of two fragile X patients who could not be diagnosed by DNA analysis. Southern blot analysis had shown a premutation without a detectable full mutation; premutation alleles are unmethylated and result in normal FMRP expression. Testing hair roots affords the opportunity of testing a different tissue. Lymphocytes originate from connective tissue, which arises from mesoderm. In contrast, both neurons and skin develop from the ectoderm. It has also been suggested that there might be a selection against cells with a full mutation in dividing lymphocytes or that there is a bias toward inactivation of the X chromosome in women during aging (Rousseau et al. 1991*b*). This may explain why there might be a better correlation between (the lack of) FMRP expression in hair roots and mental retardation.

In summary, this novel test on hair roots is noninvasive, rapid, and inexpensive. In addition, the test allows identification of male and female patients and, in exceptional cases, patients who could not be diagnosed by routine DNA analysis. For these reasons, this test might be ideal to screen populations of intellectually disabled individuals for the fragile X syndrome. In only a limited number of cases is the cause of mental handicap known. Furthermore, the fragile X syndrome is considered to be an underdiagnosed disorder, and the number of fragile X patients in The Netherlands who are not yet diagnosed is estimated to be 65% (De Vries et al. 1997). It might be expected that this percentage is similar in other countries with comparable widespread access to genetic services and even higher in countries without such access. Therefore, the development of a noninvasive test might be valuable. Identification of patients with the fragile X syndrome is important because (1) it allows early intervention with behavioral management strategies and education and (2) it allows genetic counseling for family members at risk. Recently, several approaches to screening for the fragile X syndrome have been introduced. These can be divided into two strategies—case finding, with cascade screening of family members at risk, and population screening (Wildhagen et al. 1998). This antibody test on hair roots has great potential for case finding in screening programs for detection of fragile X patients in institutions for the mentally handicapped or in schools for children with learning disabilities, followed by cascade screening of family members at risk. The experience obtained with such programs can later be applied to large population-screening programs.

In affected females, the situation is more complex because of the presence of two X chromosomes and the random inactivation of one of them. Thus, either the normal or the mutated X chromosome is active. Since human hair roots are of clonal origin (Gartler et al. 1969; Dancis et al. 1981), females with a full mutation will have hair roots that are either completely normal (the mutant *FMR1* allele has been inactivated by the lyonization process) or completely deficient (the wild-type *FMR1* allele has been inactivated by the lyonization process), without FMRP expression. In this study, we included only females with a full mutation who were intellectually disabled in varying degrees. Our results show no overlap in FMRP expression between control individuals and this group of affected females, suggesting a discriminating power of the test for affected women versus controls. Further studies should be performed to show whether this method will enable discrimination between affected and unaffected females with a full mutation. With DNA analysis or an antibody test on blood smears, we have not been able to discriminate between affected and normal females with a full mutation (De Vries et al. 1996; Willemsen et al. 1997). Apparently, the X-inactivation pattern in blood is not similar to the X-inactivation pattern in neurons from the brain. Hair roots might be of value for predicting the mental capacities of females with a full mutation, because they originate, like brain tissue, during embryonic development from the ectoderm. It is likely that the X-inacti-

vation pattern within the ectoderm during early development will give rise to similar X-inactivation patterns in the brain and in hair roots. Studies to evaluate this hypothesis are in progress.

Acknowledgments

We thank the patients and the volunteers for their cooperation. This work was supported, in part, by a grant from the Fragile X Foundation (to B.A.), BIOMED II PL951663 (to B.A.O. and P.J.W.), and ERB-4001-GT-97-2924 (to Y.D.). T. de Vries-Lentsch and R. Koppenol are acknowledged for photography and preparation of illustrations. We are grateful to Dr. L. A. Sandkuijl for help with statistics. The help of Dr. Rasenberg in collecting hairs is greatly appreciated.

Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for fragile X syndrome [MIM 309550])

References

Brown WT, Houck GE, Jeziorowska A, Levinson FN, Ding X, Dobkin C, Zhong N, et al (1993) Rapid fragile-X carrier screening and prenatal diagnosis using a nonradioactive PCR test. JAMA 270:1569–1575

Dancis J, Silvers DN, Balis ME, Cox RP, Schwartz MS (1981) Evidence for the derivation of individual hair roots from three progenitor cells. Hum Genet 58:414–416

De Graaff E, Willemsen R, Zhong N, De Die-Smulders CEM, Brown WT, Freling G, Oostra B (1995) Instability of the CGG repeat and expression of the *FMR1* protein in a male fragile X patient with a lung tumor. Am J Hum Genet 57: 609–618

De Vries BBA, van den Ouweland AMW, Mohkamsing S, Duivenvoorden HJ, Mol E, Gelsema K, van Rijn M, et al (1997) Screening and diagnosis for the fragile X syndrome among the mentally retarded: an epidemiological and psychological survey. Am J Hum Genet 61:660–667

De Vries BBA, Wiegers AM, Smits APT, Mohkamsing S, Duivenvoorden HJ, Fryns J-P, Curfs LMG, et al (1996) Mental status of females with an *FMR1* gene full mutation. Am J Hum Genet 58:1025–1032

Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL (1993) The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4:335–340

Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, et al (1991) Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67:1047–1058

Gartler SM, Gandini E, Angioni G, Argiolas N (1969) Glucose-6 phosphate dehydrogenase mosaicism: utilization as a tracer in the study of the development of hair root cells. Ann Hum Genet 33:171–176

Gartler SM, Scott RC, Goldstein JL, Campbell B (1971) Lesh-Nyhan syndrome: rapid detection of heterozygotes by use of hair follicles. Science 172:572–574

Kooij RF, Oostra BA, Willems PJ (1998) The fragile X syndrome and other fragile site disorders. In: Oostra BA (ed) Trinucleotide diseases and instability. Springer, Heidelberg, pp 1–46

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1214

Nolin SL, Glicksman A, Houck GE, Brown WT, Dobkin CS (1994) Mosaicism in fragile X affected males. Am J Med Genet 51:509–512

Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, et al (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252:1097–1102

Oostra BA, Jacky PB, Brown WT, Rousseau F (1993) Guidelines for the diagnosis of fragile X syndrome. J Med Genet 30:410–413

Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boue J, Tommerup N, et al (1991*a*) Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 325:1673–1681

Rousseau F, Heitz D, Oberlé I, Mandel JL (1991*b*) Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. J Med Genet 28: 830–836

Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, Warren ST (1992) DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1: 397–400

Turner G, Webb T, Wake S, Robinson H (1996) Prevalence of fragile X syndrome. Am J Med Genet 64:196–197

Verheij C, Bakker CE, de Graaff E, Keulemans J, Willemsen R, Verkerk AJ, Galjaard H, et al (1993) Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363:722–724

Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, et al (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905–914

Vermorken AJ, Weterings PJ, Spierebnburg GT, van Bennekom CA, Wirtz P, de Bruyn CH, Oei TL (1978) Fabry's disease: biochemical and histochemical studies on hair roots for carrier detection. Br J Dermatol 98:191–196

Wildhagen MF, van Os TAM, Poldr JJ, ten Kate LP, Habbema JDF (1998) Exploratory study costs, effects and savings for female fragile X premutation and full mutation carriers in the general population. Community Genet 1:36–47

Willemsen R, Mohkamsing S, De Vries B, Devys D, Van den Ouweland A, Mandel JL, Galjaard H, et al (1995) Rapid antibody test for fragile X syndrome. Lancet 345: 1147–1148

Willemsen R, Smits A, Mohkamsing S, Vanbeerendonk H, Dehaan A, Devries B, vandenOuweland A, et al (1997) Rapid antibody test for diagnosing fragile X syndrome: a validation of the technique. Hum Genet 99:308–311