



Hair root FMRP expression for screening of fragile X full mutation females

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ABSTRACT

The fragile X syndrome is the most common form of inherited mental retardation in humans, caused by an expansion of the cytosine-guanine-guanine (CGG) repeat in the fragile X mental retardation 1 (FMR1) gene located on the X chromosome. Antibody tests have been developed to identify fragile X patients, based on the presence or absence of fragile mental retardation protein (FMRP) in both lymphocytes and hair roots. The objective of this study was to compare correlations of hair root and lymphocyte FMRP expression with cognitive functioning in female rural area probands carrying the full mutation. Thirty females (normal, premutation, or full mutation) were selected from Indonesian fragile X families and were tested for FMRP expression in lymphocytes and hair roots using the FMRP antibody test. Subject genotype was determined by Southern blot analysis, and IQ equivalent by Raven's Standard Progressive Matrices. Statistical analysis was by Pearson correlation. FMRP expression in blood lymphocytes was relatively higher than that in hair roots, but hair root FMRP expression was strongly correlated with cognitive functioning in female full mutation carriers ($r=0.64$, $p=0.015$), whereas no significant correlation between lymphocyte FMRP and cognitive functioning was found ($r=0.31$, $p=0.281$). Around 14% of subjects had a normal and 7% a borderline IQ level, while 79% had mild mental impairment. In conclusion, hair root FMRP expression may be a useful marker for identification of fragile X full mutation females.

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INTRODUCTION

The fragile X syndrome (FXS) is the most prevalent inherited form of mental retardation (MR) in humans. The prevalence of this disease is approximately 1/4000 for males and 1/6000 for females in Western countries.⁽¹⁾ In

Indonesia the population of FXS is approximately 2% of the MR population, and in Central Java more than 50 patients have thus far been identified.^(2,3) In the same study 53% of institutionalized males and females with fragile X of an isolated village in Central Java could be traced back to a common ancestor.⁽⁴⁾

Another study in a special school for mentally retarded individuals revealed that 3.9% of these were FXS probands.⁽⁵⁾

The causative mutation of FXS resides in the fragile X mental retardation 1 (FMR1) gene, which is located on the long arm tip of the X chromosome. The vast majority of mutations comprises amplification of the Cytosine Guanine Guanine (CGG) repeat in the 5' non-coding region of the FMR1 gene. In normal people the CGG repeats vary from 6 to 54, while premutation carriers have between 55 and 200 repeats, and full mutation subjects have more than 200 CGG repeats in their FMR1 gene⁶. Full mutation expansion causes hypermethylation of the FMR1 promoter and the expanded repeat itself, resulting in absence of FMR1 protein (FMRP) in the neurons, which is responsible for the observed MR in fragile X patients.⁽⁷⁾

Males carrying the full mutation are usually cognitively affected, whereas female full mutation carriers show mild to moderate mental impairment in only approximately 60% of cases. Females carrying the full mutation are characterized by cells with and without FMRP expression, which can be explained by the random inactivation of one of the two X chromosomes (lyonization). It has been suggested that an insufficient number of FMRP-expressing neurons in the brain of affected females causes the learning deficits as a result of the proportion of mutant FMR1 alleles on the active X chromosome.⁽⁸⁾

Willemsen et al. have described alternative diagnostic tests to identify patients with fragile X syndrome on the basis of the absence of FMRP in lymphocytes and hair roots. This antibody test has advantages when compared with the DNA test, including low cost and short (one day) work period; absence of radioactivity; and capability to detect all loss-of-function mutations, including the prevalent CGG repeat amplification.⁽⁹⁾

The ectodermal origin of hair roots may explain the strong correlation existing between lack of FMRP expression in hair roots from full

mutation females and cognitive functioning, compared to FMRP expression in lymphocytes.⁽¹⁰⁾ It is likely that the X-inactivation pattern within the ectoderm during early development give rise to similar X-inactivation patterns in both brain and hair roots. Therefore, hair roots might be of value for predicting the mental capacities of females with a full mutation.

METHODS

Research design

This was an observational analytic research study with cross sectional design, conducted in Senin District, Gunung Kudul Regency, from September 2007 up to August 2008.

Subjects of study

FXS subjects were selected according to the following criteria: female, coming from a rural FXS family, age range 4 to 65 years, and without other disabilities. Purposive non-random sampling was used for recruiting the subjects. Thirty females from seven families, both affected and non-affected, were included in this study. Three mentally retarded persons as non-FXS controls were also taken to find out whether FMRP also influenced their cognitive functioning.

Measurements

After obtaining written informed consent, from each subject we collected 20 to 30 hair roots and a ten mL venous blood sample, from which smears were immediately made. The remaining blood sample was subsequently stored in EDTA tubes for DNA extraction in the laboratory. Twenty to thirty hair roots were plucked smoothly from different areas behind the ears, either manually or by means of a special pincette, and were then coded.

FMRP expression in hair roots

FMRP expression was determined by the Willemsen method: hair roots were fixed in 3%

paraformaldehyde at room temperature (RT) for 10 min. Cells were permeabilized by treatment with 100% methanol at RT for 20 min. Hair roots were washed with PBS⁺ (phosphate-buffered saline with added 0.5% bovine serum albumin and 0.15% glycine), and incubated as whole mount with mouse monoclonal anti-FMRP antibodies at 4 °C overnight. Visualization of antibody-antigen complexes was achieved by an indirect alkaline phosphatase technique, using a m-PowerVision polymeric alkaline phosphatase (poly AP) for 1 hr, followed by incubation in New Fuchsin Substrate System (DAKO) for 10-15 min. Levamisole was added to the substrate solution according to the guidelines of the manufacturer, to block endogenous alkaline phosphatase activity. Immunolabeled hair roots were examined with a stereo zoom microscope at a final magnification of 70x. The number of FMRP-positive hair roots showing red color was expressed as a percentage of the total number of hair roots examined.

FMRP expression in blood smears

Blood smears were fixed in 3% paraformaldehyde for 10 min followed by a permeabilization treatment with 100% methanol for 20 min at RT. After washing in phosphate-buffered saline (PBS) for 5 min, the endogenous peroxidase activity was blocked with PBS-Blocked (100 mL 0.1M PBS, 2 mL 30% H₂O₂, 1 mL 12.5% sodium azide) for 30 min. The smears were washed in PBS⁺ and incubated with mouse monoclonal anti-FMRP antibodies at 4° C overnight. Smears were rinsed in PBS⁺ for 3x5 min followed by biotinylated secondary antibody treatment for 10 min (Zymed-Kit Reagent B). Subsequently, smears were incubated with peroxidase conjugated streptavidin for 10 min after rinsing in PBS⁺ for 3x5 min. Finally, smears were rinsed in PBS⁺ for 4x5 min and PBS for 5 min, respectively. As a final step, smears were incubated with DAB substrate (DAKO liquid DAB substrate-chromogen system) for 2x20

min and 1x10 min followed by Nuclear Fast Red counterstaining. The smears were serially dehydrated in ethanol and xylene, mounted in Entellan (Merck # 1.07960) and dried overnight at 37°C in an incubator. Immunolabeled smears were examined under a light microscope at 1000x final magnification. A brown precipitate in the cytoplasm of lymphocytes signified positive FMRP expression. The percentage of FMRP expression was obtained by counting one hundred lymphocytes in different areas of the smears and scoring them for FMRP expression. The test results were evaluated by three different investigators who were unaware of the identity of the probands and the counting results of each other.

Southern blot analysis using digoxigenin labeled probe pP2

Seven mg of genomic DNA extracted by the Miller method (35µL of 200 ng/µL) was mixed well with 4.75 µL aquadest, 5 µL 10x SuReCut Buffer H (Roche), 3 µL 50 mM spermidine, 1.25 µL Hind III (recognition site = AAGCTT – Roche cat. #10656313001), 1 µL Eag I, a methylation sensitive enzyme (recognition = site CGGCCG - Biolabs cat. #R0505L). The mix was incubated overnight at 37°C to complete digestion. Five µL 10x Ficoll loading buffer was added to 45 µL of each digestion product, which was then electrophoresed on 0.7% agarose gel overnight at 45V. DNA was transferred to a nylon membrane (Hybond N1, Amersham) in Southern blotting apparatus overnight, followed by baking the membrane for 20 min at 120°C. Prehybridization was performed in roller bottles with pre-heated DIG Easy Hyb hybridization solution (Roche cat. #11603558001) for at least 1 hr at 42°C and 10 rpm. The prehybridization solution was replaced with a hybridization solution containing denatured digoxigenin labeled probe pP2 (courtesy Prof Ben Oostra, Erasmus Medical Center) and left at 42°C overnight to

complete hybridization. The membrane was washed for 2x5 min with 50 mL 2xSSC/0.1 % SDS at room temperature. Detection was achieved in clean trays with the following procedure: equilibration of membrane in 10 mL washing buffer for 5 min followed by incubation in blocking solution for 60 min. The membrane was put in a piece of SaranWrap. 10 µL Anti-digoxigenin-AP-conjugate was added to the blocking solution and the membrane was incubated in this solution for 30 min. After incubation, the antibody solution was discarded and the membrane washed in washing buffer for 2x15 min and in detection buffer for 5 min. In the meantime 15 CDP star solutions (Roche Cat. no. 1685627) were diluted in 1500 µL detection buffer. After the washing procedure, the membrane was placed between the two sides of a plastic bag and CDP star solution was pipetted on top of the membrane. Bubbles present under the sheets were removed and the membrane was incubated for 5 min. After incubation, liquid excess was removed and the plastic bag was sealed. The membrane was exposed to film for 10 and 30 min. A normal band was characterized by the presence of a 2.8 kb band indicating a normal X chromosome, while a 5.2 kb band reflected a methylated X chromosome.

Determination of IQ equivalents

Raven's standard progressive matrices were used to measure IQ equivalents of the subjects. Testing and analysis were performed by a certified psychologist. Cognitive functioning was measured by the non-verbal Raven test based on non-verbal intervention.⁽¹¹⁾

Statistical analysis

Using SPSS 16.0 for Windows, Pearson correlation analysis was performed to determine a possible correlation of FMRP expression in hair roots and lymphocytes with cognitive functioning ($p < 0.05$).

RESULTS

FMRP expression

FMRP expression was demonstrated in both blood smears and hair roots by using an immunohistochemical test introduced by Willemssen (Figure 1). Positive specimens have a clear red staining in bulb and hair shaft respectively, whereas negative specimens exhibit no red color in either bulb or hair shaft. FMRP expression in lymphocytes is shown by brown staining of the cytoplasm, while no staining indicates a negative result.

The distribution of FMRP expression in all probands is depicted in Table 1. Normal samples and premutation carriers showed high expression of FMRP both in blood smears and hair roots, with an average of 91.78% and 82.89%, respectively. Three samples of mentally retarded people from non-fragile X families also showed a high FMRP expression (over 80%), whereas full mutation subjects showed on average 65.85% expression in blood and 53.98 % in hair roots.

These findings show that in full mutation individuals the average FMRP expression in blood smears was higher than in hair roots. Fifteen samples show a different result for FMRP expression in blood smears and hairs of over 10%, which were mostly in the full mutation group. A comparison of FMRP expression in lymphocytes vs. hair roots is shown in Figure 2.

To determine whether a relationship exists between the percentages of FMRP expression in lymphocytes and hair roots, a correlation test was applied for these samples. The Pearson correlation of total sample was highly significant ($r=0.82$, $p < 0.001$), while in the full mutation group correlation was lower, although still having statistical significance ($r=0.58$, $p=0.028$). This finding shows that in cells of normal individuals and premutation or full mutation carriers, FMRP expression was presumably similar in lymphocytes as well as

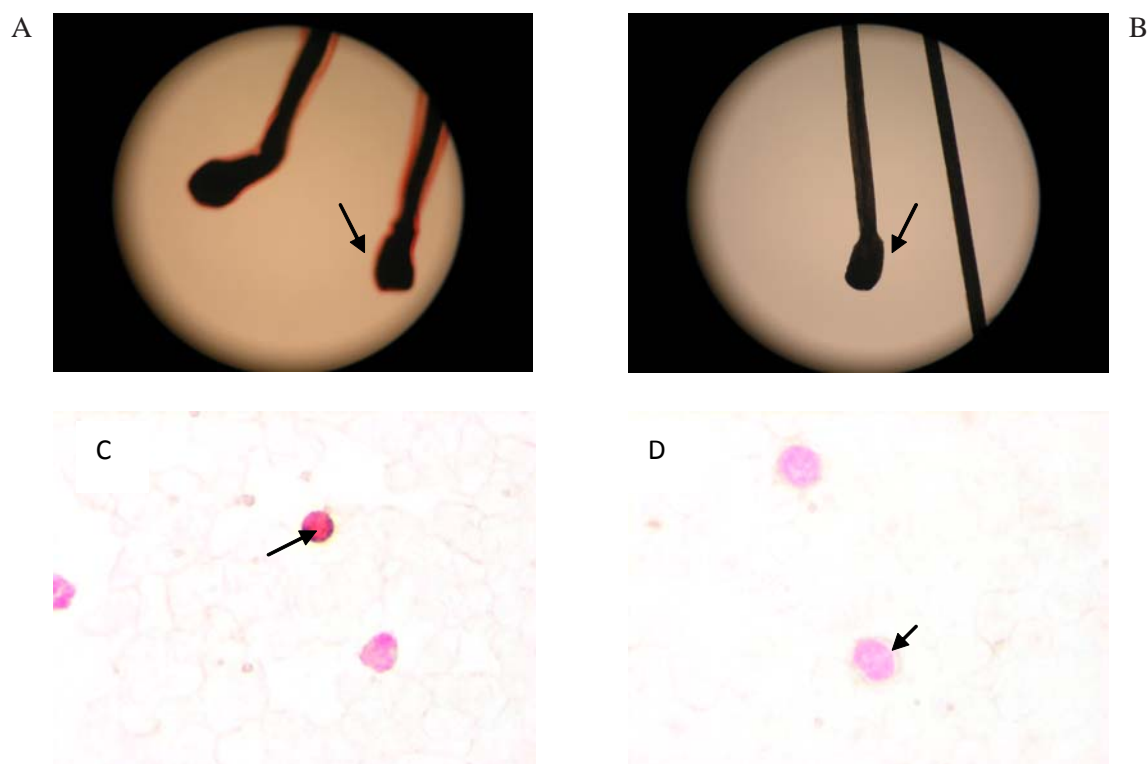


Figure 1. Fragile X Mental Retardation Protein (FMRP) expression in both blood smears and hair roots. A: Positive appearance in follicle hair roots from normal probands, B: Negative appearance in follicle hair roots from full mutation probands, C: Positive appearance in lymphocytes cell from normal probands, D: Negative appearance in lymphocytes cell from full mutation probands

hair roots, although more variation was seen in the full mutation group.

IQ equivalents of probands

Distribution of IQ levels among these groups is depicted in Figure 3. Thirty percent of the sample shows IQ levels of ≥ 80 whereas the IQ levels of the other subjects were below 80. The mean value was 77.88 in the normal group, 83.42 in premutation carriers, and 63.07 in full mutation carriers. In the full mutation group about 14% of subjects was in the normal range (≥ 85), 7% in the borderline range (70-84) and 79% showed mild mental impairment (50-69).

Genotypes of probands

To confirm genotyping of the probands, DNA testing was performed by Southern blot

analysis, using a methylation sensitive enzyme, allowing discrimination between methylated and unmethylated FMR1 alleles. This analysis showed the classification of the alleles, whether normal, premutation, or full mutation alleles. Results are shown in Figure 4.

Correlation between FMRP expression and IQ equivalents of full mutation females

In the full mutation group, Pearson correlation test was carried out to determine whether a relationship exists between FMRP expression and IQ equivalent. The p value of the Pearson correlation test for FMRP in hair roots and IQ equivalent parameter showed high significance ($r=0.635$, $p=0.015$). In contrast, FMRP expression in lymphocytes showed no statistically significant relationship with IQ equivalents ($r=0.31$, $p=0.281$) (Figure 5).

Table 1. Distribution of Percentage of FMRP expression in hair roots and blood smear, IQ equivalent, and Genotypes of individual samples in Indonesia

Number of probans	IQ equivalent	% FMRP in blood smear	% FMRP in hair roots	Genotypes
1	60	98	85	Non F
2	65	97	90	Non F
3	70	95	86	Non F
4	95	97	89	N
5	75	88	85	N
6	75	90	84	N
7	85	87	82	N
8	77	97	70	N
9	75	93	87	N
10	73	92	86	N
11	66	75	76	N
12	80	95	83	N
13	83	80	73	P
14	75	94	85	P
15	77	94	77	P
16	75	97	80	P
17	88	86	90	P
18	101	94	85	P
19	85	95	82	P
20	60	65	50	F
21	65	65	50	F
22	60	44	64	F
23	60	76	40	F
24	63	63	41	F
25	70	55	60	F
26	65	61	54	F
27	50	97	75	F
28	85	88	87	F
29	50	45	31	F
30	50	58	25	F
31	55	62	50	F
32	85	76	69	F
33	65	67	60	F

Note: N = normal; P = premutation; F = full mutation; Non F= MR non fragile X

DISCUSSION

Studies on FMRP expression in hair roots have shown that this may serve as a predictor of cognitive functioning in females carrying the fragile X full mutation,⁽⁹⁾ while FMRP expression in blood smears can be used to identify male fragile X patients. However,

there were overlapping results in the female group (full mutation versus controls).

In this study, all samples from normal and premutation female carriers expressed normal levels of FMRP (75 -100%), both in blood smears and hair roots, whereas full mutation female carriers showed a large variance in percentage of FMRP expression (Figure 2).

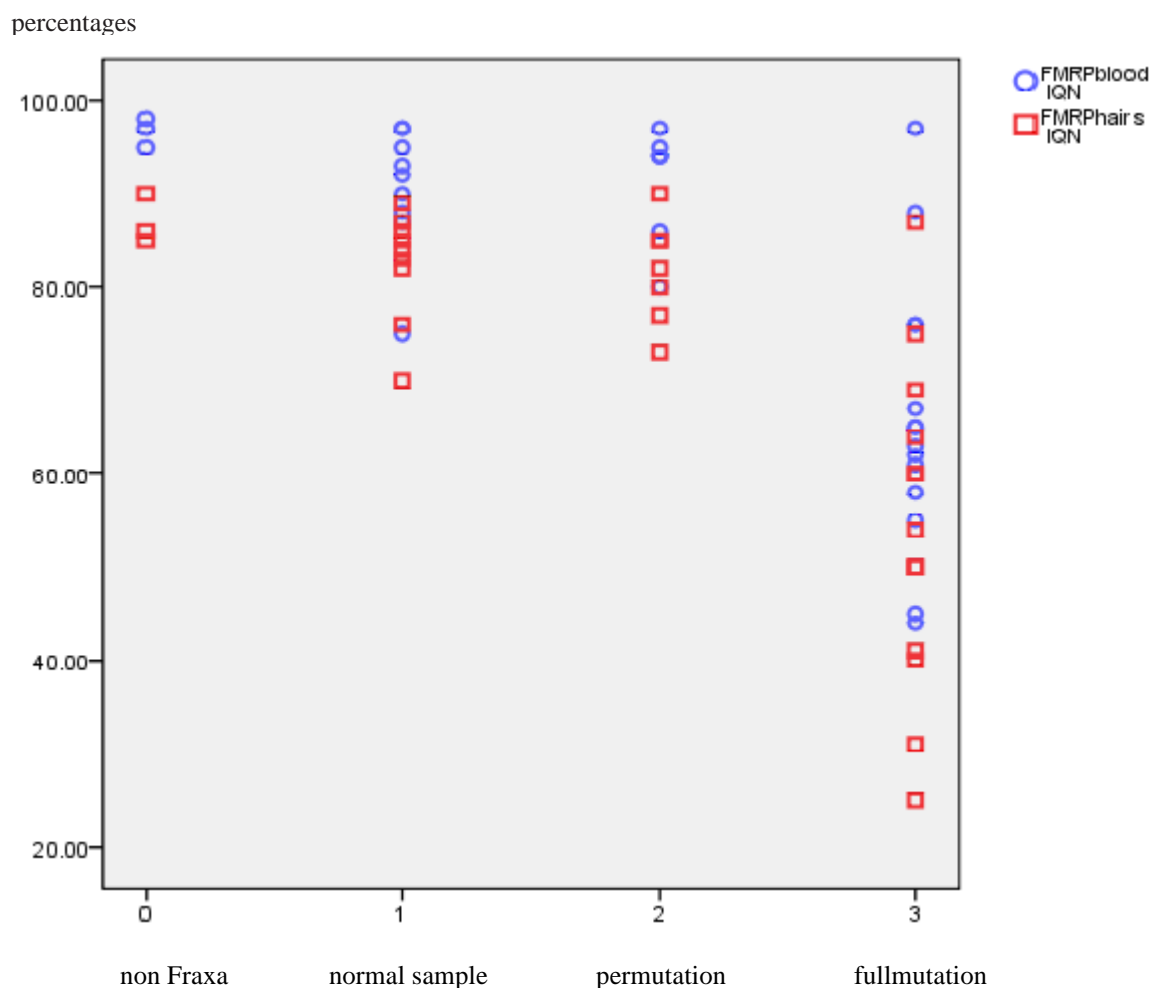


Figure 2. Diagram of percentage of FMRP expression in lymphocytes and hair roots in individual samples. Non-full mutation groups show high expression of above 75% except two samples, whereas the full mutation group varies from 25% to 97%. The hair roots FMRP is lower than in lymphocytes

This finding is in line with the current hypothesis that normal and premutation carriers show a normal translation of the FMR1 mRNA, in contrast to carriers of the full mutation alleles, which usually show a methylation of the FMR1 gene, resulting in lack of FMRP. A random lyonization process in the cells causes the variance of FMRP expression in full mutation females.

Normal and premutation subjects in this study show relatively low IQ equivalents. A possible explanation for this observation is that these subjects live in an isolated area where modern facilities are limited. Most of them are

not educated and live in a simple environment. Also the diet may play a role because they eat more cassava than rice, as they live in a relatively arid area. As a consequence their protein intake is low, which may be harmful to the body due to the cyanide content of cassava. This finding represents the condition that they may have low micronutrient intake leading to inhibition of normal brain development. This finding is in line with previous literature mentioning that dietary intake may affect development of cognitive functioning.⁽¹²⁾ Hypothyroidism is also one of the problems in this rural area.⁽¹³⁾ During

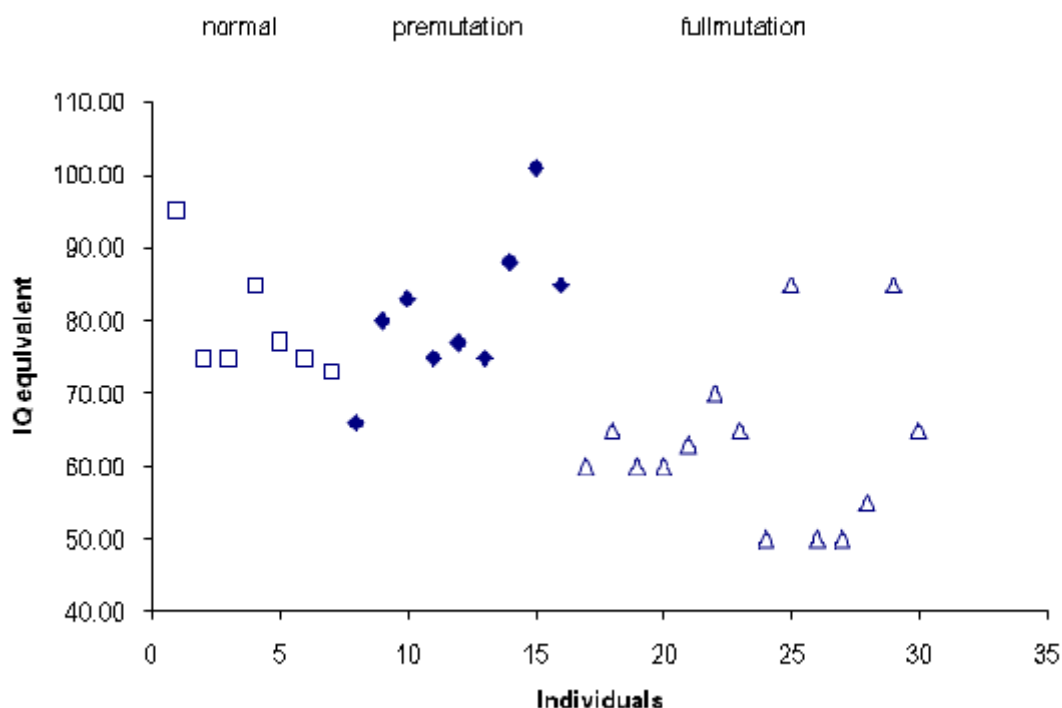
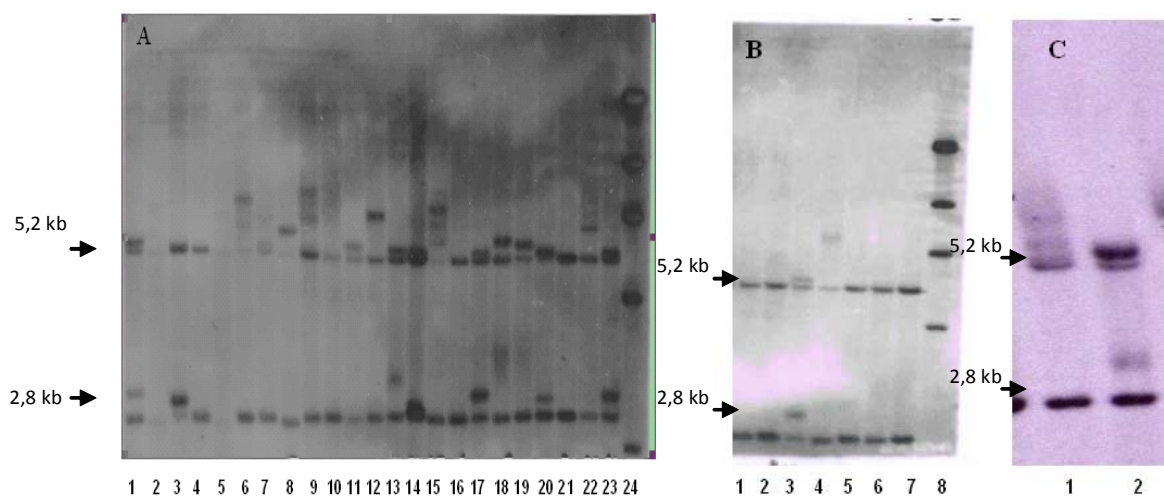


Figure 3. IQ equivalents (converted from Raven test) distribution in individual samples. Normals and premutation carriers have an IQ range between 66 and 101, whereas full mutation carriers have IQ levels between 50 and 85. The average IQ value in Normals and Premutation carriers is 77.88 and 83.42 respectively, whereas full mutation carriers have 63.07. The highest IQ value is 101 in a premutation carrier and the lowest value is 50 in a full mutation carrier



A: Lanes 4,14,21 were normal samples. Lanes 1,3,13,20,23 correspondent to premutation samples whereas full mutation samples were shown in lanes 2,5,7,8,9,10,11,12,15,18,19, and 22. Lanes 6 and 17 indicated full mutation and premutation control, respectively. B: Lanes 1,2,5,6,7 were normal samples. Lane 3 correspondents to premutation sample and line 4 were full mutation sample. DNA ladder was indicated in lane 8. C: Lane 1 was full mutation and lane 2 was premutation

Figure 4. Figure of Southern blotting test from individual samples using double digestion with *HindIII* and *EagI*.

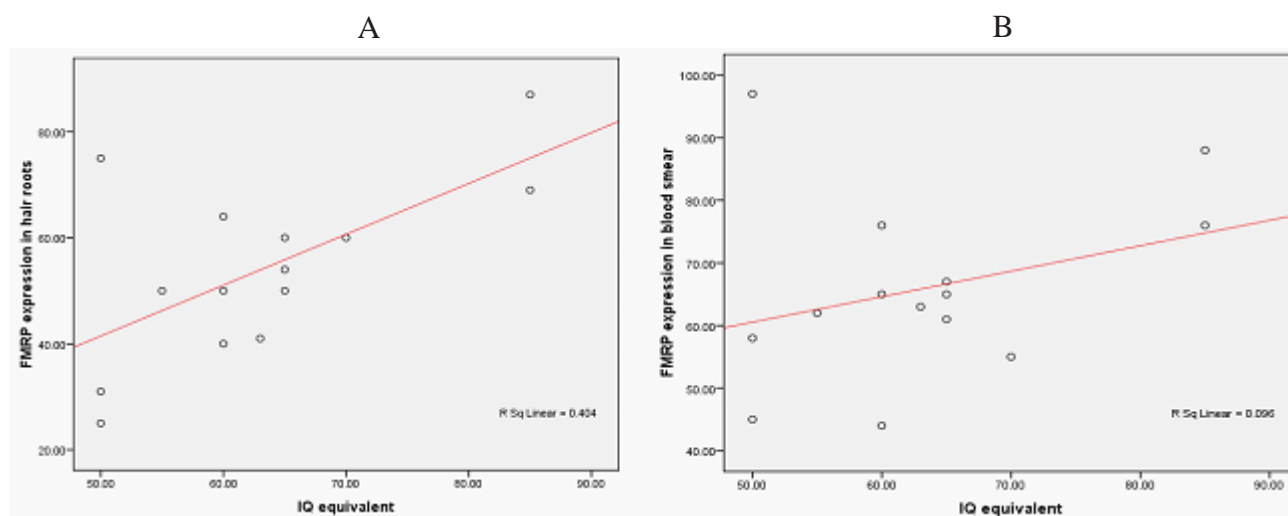


Figure 5. Correlation between FMRP expression and IQ equivalents in blood smear (A) and hair roots (B) in full mutation carriers. The chart indicated a significant correlation between percentage of FMRP expression in hair roots and IQ equivalents in our group of full mutation carriers

application of the Raven test; we had to explain the test to the probands more frequently, as they seem did not care about it. In contrast, they finished their test very fast. This case relates with a previous study that mentioned an alternative hypothesis to general ability (g factor) as to the reasons why non-Western populations score lower than Western populations, such as being test-wise, less interested, more anxious, working less efficiently, or giving up sooner on items they find difficult.⁽¹⁴⁾

Distributions of IQ equivalents in the full mutation group were as follows: about 14% in the normal range (≥ 85), 7% in the borderline range (70-84%) and 79% showing mild mental impairment (50-69). These percentages are higher than those found in previous research studies,⁽¹⁵⁾ namely 60% with mild mental retardation. However, in our study the number of subjects was limited and should be increased in the near future to allow drawing of definite/final conclusions. The differences in IQ equivalents of the full mutation, premutation, and normal groups are shown in Figure 3.

In the full mutation group we found subjects with relatively high IQ levels compared to other groups showing higher FMRP expression in their cells (compared with other samples). This may be due to the fact that females carrying a full mutation are characterized by cells with FMRP expression as well as those without, which can be explained by the presence in females of two X-chromosomes and random inactivation of one X chromosome (lyonization). This finding also noticed that although human intelligence is influenced by many genetic factors, a single mutation in the FMR1 gene resulting in lack of FMRP significantly reduces cognitive functioning in female full mutation carriers.^(16,17)

FMRP expression in blood smears and hair roots are also correlated with cognition, although FMRP expression in blood smears seemed to be higher than in hair roots. This may be explained by differences in origin of the tissues. Blood originates from mesoderm, whereas both hair roots and brain tissue are derived from ectoderm. Lymphocytes originate from a common lymphoid progenitor cell before

differentiating into their distinct lymphocyte types. The formation of lymphocytes, known as lymphopoiesis, has a daily turnover and the lifespan of lymphocytes ranges from several weeks to several years.^(18,19)

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.^(20,21) This may explain why there seems to be a better correlation between lack of FMRP expression in hair roots and mental retardation. In addition, lymphocytes may poorly reflect the conditions in brain tissue, because the rapid turnover may lead to high variation in clones that either do or do not carry the FMR1 mutation on the active X chromosome. Earlier studies using the FMRP test on lymphocytes did show a weak statistical correlation; however, the significance was not high enough to use this method as a reliable diagnostic or predictive test.

The benefit of hair roots as a diagnostic tool is shown in this study because we found, although in a small-sized study, a highly significant correlation between the percentage of FMRP expression in hair roots and IQ equivalent in female full mutation carriers. The statistical analysis showed that level of cognitive functioning in female full mutation carriers typically reflected by FMRP in hair roots rather than in blood smears. The origin of the tissue is important, but methodological aspects may play a role as well and cannot be excluded from this research. Some of the limitation aspects were the limited number of full mutation carriers tested, problems with sample delivery by courier, and climatic differences.

CONCLUSION

FMRP expression in hair roots is probably more useful to predict cognitive functioning in female full mutation carriers than is FMRP expression in blood lymphocytes.

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