Short Report

Quantitative measurement of FMRP in blood platelets as a new screening test for fragile X syndrome


The fragile X syndrome usually results from CGG repeats expansion and methylation of the FMR1 gene leading to the absence of expression of its encoded protein, fragile X mental retardation protein (FMRP). Therefore, its diagnosis is traditionally based on the detection of these molecular alterations. As an alternative, FMRP-based screening methods have been proposed over the years. Most of them are based on immunohistochemistry analyses applied to a restricted number of lymphocytes (100) or hair roots (10–20) with limited diagnosis potential. In this study, we describe a truly quantitative approach using a new model, the blood platelet, which can be recovered easily with very high purity (99.9%). FMRP levels in platelets were first measured in a control population ($n=124$) and reference values were established. FMRP measurements were also performed in confirmed fragile X subjects. Receiver operating characteristic curve analysis has shown that our test can easily discriminate fragile X males and females from controls (area under curve, $AUC=0.948$). Cognitive functions were also assessed in these individuals using age-specific Wechsler Intelligence Scales for Children and the Vineland Adaptive Behavior Scales. A proportional relationship between FMRP levels, intelligence quotient and adaptive behavior was observed among fragile X individuals, suggesting that our test would be able to detect fragile X cases and may predict cognitive functions.

Conflict of interest
Nothing to declare.

The fragile X syndrome is the most common form of inherited intellectual disability (ID) (1, 2). In most cases, the disease is caused by CGG repeats expansion within the 5′ untranslated region of the fragile X mental retardation 1 (FMR1) gene. More precisely, the full mutation is characterized by more than 200 CGG repeats along with the methylation of a CpG island upstream of the FMR1 gene. This methylation blocks the expression of the gene resulting in the absence (males) or reduction (females) of fragile X mental retardation protein (FMRP) expression (3). The altered level of FMRP is believed to be responsible for the clinical manifestations of the syndrome. However, point mutations or deletions in the FMR1 gene also lead to the same clinical features (4).

Global intellectual functioning is usually assessed by age-specific Wechsler scales that provide the intelligence quotient (IQ). IQ amongst fragile X individuals is highly variable where most affected males have a moderate ID (IQ from 35–40 to 50–55) while only 25–30% of the females are intellectually impaired (IQ < 70) (5, 6). ID may also give significant limitations...
in adaptive behavior that can be measured using the Vineland Adaptive Behavior Scales (VABS) (7, 8). The VABS is a well-standardized test and measures communication, socialization and daily living skills. VABS scores are typically higher in females than in males while being variable within each gender (7). The presence of a normal allele which has the ability to produce FMRP would be responsible for the less severe phenotype observed in females.

Molecular approach using Southern blot and polymerase chain reaction (PCR) analyses, remains the gold standard confirmatory test for fragile X syndrome (9). Because methylation of the *FMR1* gene leads to the absence or reduction in FMRP expression, FMRP-based screening strategies have been proposed (10–14). Most of these methods are based on the detection of FMRP-positive lymphocytes or hair roots following immunohistochemistry with an anti-FMRP antibody. They have shown relationships between FMRP expression and cognitive abilities (10–12). However, these methods have limited sensibility to detect fragile X females and mosaics.

In this study, we developed a new quantitative measurement method for FMRP levels in blood platelet extracts. For the first time, reference values were determined for a control population and showed to be higher as compared to our fragile X group. Additionally, a relationship between FMRP and cognitive functions was shown, suggesting that this method would be a useful diagnostic and prognostic tool.

**Materials and methods**

**Research subjects**

Healthy controls and confirmed fragile X subjects (minors and adults) were recruited according to the research protocol approved by the ethics committee of our institution (08-128).

**Isolation of platelets**

Platelet-rich plasma was obtained from blood collected in ethylenediaminetetraacetic acid (EDTA) tubes following its centrifugation at 350 g for 10 min. Platelets were counted with an automated fluorescent flow cytometer then washed with PBS supplemented with 5 mM EDTA. Whole protein extracts were prepared in 2% sodium dodecyl sulfate (SDS) sample buffer as described previously (15).

**FMRP quantification**

His-tagged purified FMRP isoform 1 (provided by Edouard W Khandjian) was quantified and used as FMRP standards for quantification purposes. Protein extracts corresponding to 5 × 10⁶ platelets for each subject along with known amounts of FMRP standards ranging from 0.032 to 0.32 ng were loaded on a 4–12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following protein transfer, Western blot was carried out using the anti-FMRP antibody IC3 (Chemicon, Temecula, CA) along with an infrared fluorescence-labeled (goat anti-mouse Alexa® Fluor 680, Invitrogen, Eugene, OR) secondary antibody. In some experiments, Western blot analyses were performed with an anti-tubulin antibody and a goat anti-rabbit Alexa® Fluor 680 secondary antibody. The Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) was used to detect FMRP fluorescence signals from subjects and standards. Using a linear standard curve, as calculated by the included software, quantitative FMRP levels were determined for each subject. The obtained value represents an average amount of FMRP in picograms per million of platelets (pg/10⁶ platelets).

**Cognitive evaluations**

Cognitive evaluations were performed on 15 males and 9 females with the fragile X syndrome by a neuropsychologist. French Canadians versions of the Wechsler Intelligence Scale for Children fourth edition (WISC-IV) was administrated to five fragile X children and the Wechsler Adult Intelligence Scale third edition (WAIS-III) to 19 adults (minimal score 40). A French Canadian version (CHU Ste-Justine) of the VABS was administered to caregivers. For each cognitive evaluation, full scale IQ as well as the Vineland Adaptive Behavior Composite (VABC) and all their respective subscales were considered (minimal score 20).

**Data analysis**

The data were analyzed with SPSS (version 17.0). MedCalc version 11.3.8.0 was used to produce the receiver-operating characteristic (ROC) analysis. Data from inter-group were compared with Student’s *t*-test (normal distribution) and Mann–Whitney’s test (abnormal distribution). Relationship between FMRP and cognitive functions was analyzed with Spearman’s *ρ* test. The data are presented with 95% confidence intervals.

**Results**

**Normal distribution of platelet FMRP levels in control subjects**

Western blot analysis for FMRP was performed on whole cell extracts from platelets of a confirmed fragile X male and a control individual (Fig. 1a). FMRP in control platelets shows a classic protein pattern with a major band at 80 kDa, as a tight doublet, and 5–6 minor bands as shown in other cells or tissues (16). These bands were not detected in a fragile X individual, thus confirming the specificity of the obtained signal (Fig. 1a).

FMRP levels were determined for the first time in control individuals. As shown in Fig. 1b, data followed a normal distribution allowing parametric...
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Fig. 1. Fragile X mental retardation protein (FMRP) levels from a control population follow a normal distribution. (a) FMRP in platelets has a classic protein pattern which is not detected in a fragile X male. Western blot analyses were performed on platelet extract from a control and fragile X (FX) male using an anti-FMRP antibody and an anti-tubulin antibody as a loading control. (b) FMRP levels were measured in a control population in order to establish reference values. The control group was composed of 124 subjects (61 females and 63 males) evenly distributed between 4 and 50 years of age without intellectual disability.

statistical analysis. A mean value of 29.6 ± 7.5 pg/10⁶ platelets was obtained that was not affected by age or gender (data not shown). Considering two standard deviations, the reference values would range from 14.6 to 44.6 pg/10⁶ platelets. Within-run and between-run precision was less than 5% and 10%, respectively.

Platelet’s FMRP levels can discriminate fragile X syndrome subjects from controls

These measured levels of FMRP in the control population were compared with those found among fragile X syndrome subjects. As expected, levels of FMRP among males with fragile X syndrome were significantly lower than controls (p < 0.001) (Fig. 2a). In fact, no signal was obtained for 12 out of 16 males whereas the highest level for the remaining four fragile X individuals was 8.6 pg/10⁶ platelets. Two of these males were known to have repeat size mosaicism, explaining why FMRP expression was detected. As expected, fragile X females showed significantly higher expression levels (from 11.1 to 27.3 pg/10⁶ platelets) than fragile X males, while still being inferior to those in controls (p < 0.001) (Fig. 2a).

In order to determine the overall ability of our test to discriminate controls from fragile X individuals, a ROC curve analysis was carried out using data from controls

Fig. 2. Fragile X mental retardation protein (FMRP) levels can discriminate fragile X syndrome subjects from healthy controls. (a) Box-Plot analysis showing lower levels of FMRP in fragile X syndrome subjects as compared to control individuals. The fragile X group (n = 26) was composed of 10 females between 17 and 42 years of age and 16 males between 7 and 39 years. Values for males (white) and females (gray) were calculated separately. There was no statistical difference between control males and control females. Using the Mann–Whitney U test, a statistical difference was observed between control and fragile X group (p < 0.001), between fragile X syndrome and control males (p < 0.001) and between control and fragile X females (p < 0.001). Also, a statistical difference was noticed between females and males with fragile X (p < 0.001). (b) Receiver-Operating Characteristic (ROC) curve analysis illustrating the great diagnostic performance of FMRP levels measurement for the fragile X syndrome. The area under the ROC curve gave a value of 0.948. A threshold value of 27.3 pg/10⁶ platelets gives a sensitivity of 100% and a specificity of 61.3%.
and all fragile X individuals. Our test yielded an area under the ROC curve of 0.948 (Fig. 2b). For screening purposes with a sensitivity of 100%, the FMRP threshold value would be 27.3 pg/10^6 platelets, which has a corresponding specificity of 61.3% for both genders.

Relationship between FMRP levels and IQ among fragile X syndrome individuals

IQ determination in our fragile X population showed, as expected, a significantly higher IQ for females than for males and a low dispersion of IQs among subjects of each gender (Table 1b). When male and female data were combined, a significant relationship was observed between FMRP levels and full scale IQ (r = 0.91, p < 0.01; Fig. 3a). A similar relationship was also observed between FMRP and each subscale (data not shown).

Relationship between FMRP levels and adaptive behavior among fragile X syndrome individuals

Similar to the IQ scale, VABC scores were significantly higher for females than for males (Table 1b). However, we observed for each gender a higher dispersion in adaptive behavior than the IQ (Table 1b). The relationship between FMRP quantification and adaptive behavior was also established. When male and female data were combined, a significant direct proportional relationship was observed between FMRP levels, VABC, communication, daily living skills and socialization domains (Fig. 3b). Considering only the male group, no significant relationship remained. For females, relationships between FMRP and VABC (r = 0.86, p = 0.03), daily living skills (r = 0.93, p < 0.01), socialization remain significant (r = 0.82, p < 0.01). Therefore our results suggest that the FMRP levels quantification may predict adaptive behavior of fragile X subjects and particularly in females.

**Discussion**

Our proposed quantitative method, based on the detection of FMRP in platelets, brings many innovations to FMRP-based screening strategy for the fragile X syndrome. Platelets offer many advantages over lymphocytes since they can be recovered easily with an unmatched purity of 99.9%. They are less prone to physiological and pathological variations than lymphocytes. In contrast, lymphocytes preparation are significantly contaminated by platelets that would significantly affect total FMRP quantification by Western blot (14) or enzyme-linked immunosorbent assay (13).

One cubic centimeter of whole blood can yield the five million platelets from which FMRP quantification is performed. This huge number of platelets is certainly more representative of its overall expression as compared to 100 lymphocytes (12) or 10–20 hair roots (11). Moreover, our method does not require visual judgment of staining (positive/negative) eliminating possible interpretation bias from the operator (10–12).

Denaturation of protein extracts with SDS sample buffer is mandatory to preserve FMRP solubility and stability. In fact, no sign of protein degradation has been observed over 6 months following the storage of extracts at −80°C (data not shown). Western blot analyses based on infrared fluorescence detection are increasingly preferred over enzyme approaches particularly for quantitative purposes since they have shown greater linearity and reproducibility (17, 18). Moreover, this approach will detect other mutations (4) within the FMR1 gene substantially affecting FMRP expression of FMRP length (> 2 kDa) and its migration on SDS-PAGE gels.

To our knowledge, this is the first study where FMRP reference values are established from a control population. Our test showed a great overall diagnostic performance (AUC = 0.948), suggesting a high potential for discriminating fragile X males and even females from healthy controls. By selecting the best threshold at 27.3 pg/10^6 platelets for screening purposes (100% of sensitivity), a remarkable specificity of 61.3% can still be reached. By comparison, the proposed threshold for the lymphocytes immunohistochemistry method has

**Table 1.** Subjects characteristics: (a) mean age, SD and age range in the control and the fragile X group and (b) cognitive measures statistics of fragile X syndrome subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls (n = 124)</th>
<th>Fragile X (n = 26)</th>
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<tbody>
<tr>
<td></td>
<td>Males (n = 63)</td>
<td>Females (n = 61)</td>
</tr>
<tr>
<td>Age</td>
<td>Mean</td>
<td>26.1</td>
</tr>
<tr>
<td>SD</td>
<td>13.8</td>
<td>13.3</td>
</tr>
<tr>
<td>Range</td>
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<td>4–48</td>
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<tr>
<td>Cognitive measures</td>
<td>Mean</td>
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<tr>
<td>IQ</td>
<td>SD</td>
<td>4.1</td>
</tr>
<tr>
<td>Range</td>
<td>40–51</td>
<td>62–80</td>
</tr>
<tr>
<td>VABC</td>
<td>Mean</td>
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<tr>
<td>Communication</td>
<td>SD</td>
<td>11.5</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;20–59</td>
<td>61–105</td>
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<tr>
<td>Daily living skills</td>
<td>Mean</td>
<td>32</td>
</tr>
<tr>
<td>Socialization</td>
<td>SD</td>
<td>16.3</td>
</tr>
<tr>
<td>Range</td>
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<tr>
<td>Socialization</td>
<td>Mean</td>
<td>37</td>
</tr>
<tr>
<td>SD</td>
<td>17.8</td>
<td>15.8</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;20–79</td>
<td>63–111</td>
</tr>
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IQ, intelligence quotient; VABC, Vineland Adaptive Behavior Composite; SD, standard deviation.
a specificity of 41% with a corresponding sensitivity of 41% (19) limiting significantly the method’s usefulness in females. Intriguingly, our method detected the presence of FMRP in four fragile X males, where only two were confirmed as mosaic. All four patients had FMRP levels below 10 pg/10⁶ platelets indicating a very low percentage of mosaicity. We propose that these two individuals would have a partially methylated full mutation that was not detected by Southern blot. As both, platelets and lymphocytes are derived from hematopoietic stem cells, we should not expect differences in mosaicity between these cell types.

Our analysis supports a positive relationship between FMRP and cognitive evaluations (full scale IQ and VABS) when male and female data are combined. However, most of these relationships vanished when groups are analyzed separately. Interestingly, this relationship remained between FMRP levels and adaptive behavior in females. This correlation was not observed by other groups who used immunohistochemistry with lymphocytes (7, 8), which might indicate that our quantitative method is superior in many aspects to the previously reported methods, although still requiring additional validation in a larger population. Technically simple to perform, our method would also predict cognitive functions and could be particularly useful for genetic counseling and for optimizing the care of early diagnosed patients.

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References
New screening test for fragile X syndrome