Tissue-Specific Size and Methylation Analysis in Two Fragile X Families: Contribution to the Clinical Phenotype

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Abstract

Methylation at critical CpG sites on the expanded FMR1 gene is crucial for pathological manifestation of fragile X syndrome and fragile X-related disorders. Methylation status from blood, oral mucosa and root hair was analyzed with the FMR1 mPCR kit (Asuragen). Differential allele expression was studied by TP-PCR. Psychological and neurological explorations were performed in the probands. Patient II-1 of family 1 showed an extremely skewed X-chromosome inactivation of the normal allele in blood, oral mucosa cells and root hair. Analysis of differential expression of both alleles in blood showed the preferential expression of the expanded allele. Similarly, patient II-3 of family 2 showed an extremely skewed X-chromosome inactivation of the normal allele in blood, oral mucosa and root hair. Both females presented clinical features compatible with their skewed methylation toward the normal allele. Methylation analysis at critical CpG sites in the first FMR1 exon may predict clinical manifestations in carriers of premutation or full mutation. Analysis of differential expression of both alleles in women using TP-PCR could contribute to clarify the real impact of skewed methylation on the phenotype.

Keywords: Fragile X syndrome and fragile X-related disorder; Methylation analysis; Epigenetics; Skewed X-chromosome inactivation; Differential allele expression

Introduction

Fragile X syndrome (FXS) was the first pathology in which a dynamic mutation was described as the cause of the disease. The FMR1 gene has a variable number of CGG repeats in the 5'-UTR divided in different categories depending on the number of repeats and its phenotypic correlation: normal from 5 to 44, intermediate from 45 to 54, premutated from 55 to 200 and fully mutated above 200 repeats. For risk assessment of FXS and related disorders the number of repeats and methylation status of the gene should be analysed [1,2]. Alleles with more than 200 repeats are commonly methylated and as a consequence the gene is silenced, which results in the absence of FMRP and the manifestation of the fragile X syndrome, the most common cause of inherited mental disability and autism [3]. Fragile X premutation carriers usually are not mentally impaired but can present specific phenotypes as mild form of fragile X syndrome, fragile X-associated tremor ataxia syndrome (FXTAS) and, in females, fragile X-associated premature ovarian insufficiency (FXPOI) [2]. The pathophysiology of premutation-associated disorders is complex and the elevated expanded FMR1 transcript seems to play a central role either by RNA toxicity, non-canonical AUG translation or other mechanisms [4-6].

In both females that have been extensively analysed the skewed X-chromosome inactivation determines the phenotype. This methylation analysis and the differential expression of both alleles in female carriers may provide a simple protocol for routine diagnosis that may have a predictive value of clinical phenotype.

Methods

Patient samples

Genomic DNA was extracted from different sources (blood, oral mucosa and root hair) by standard procedures and by automatic extraction using the Maxwell extraction kit [11]. RNA was obtained from blood using the Paxgene tube and standard procedures [12]. For SB analysis genomic DNA (gDNA) from blood was doubled digested (See supplementary material).

CUG triplet repeat-primer FMR1 PCR (TP-PCR)

gDNA was analyzed using the Amplidex™ FMR1 PCR kit by Asuragen Inc. (Austin, TX) according to the manufacturer’s instructions. Briefly, 2 different forward primers, one a CUG repeat primer, were used with a fluorescently tagged reverse primer [13]. TP-PCR was also used with CDNA to study the differential expression of both alleles of the FMR1 gene in females (See supplementary material).

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Results

Family 1

The pedigree of the family is shown in Figure 1A. Molecular diagnosis showed that patient II-1 is a female carrier that inherited a 22 CGG repeats allele from her mother and a premutation from her father (I-1) (79 CGG) with a small expansion (94 CGG). She had 3 children, two fragile X boys, one with a mutation above 200 CGG repeats and the other with a low abundant premutation of 69 CGG and a mutation above 200 repeats. The third boy inherited the normal 22 CGG repeats allele from her mother. SB with the StB12.3 showed that patient II-1 (Figure 1B) had a 100% methylation of the normal FMR1 allele and methylation sensitive restriction enzymes at FRAXA, FRAXE and AR loci that were informative in this family indicated an extremely skewed X chromosome inactivation with 100% methylation of the X chromosome that carried the normal FMR1 allele (Figure 1SM).

Tissue-specific sizes showed minor difference from the three tissues analyzed with size of 94 +/-2 CGG (blood), 92 +/- 2 (oral mucosa) and 96 +/-1 (root hair). Methylation analysis showed 4 +/-2% methylation of the expanded allele in blood, 7 +/- 1% in oral mucosa and 12 +/- 1% in root hair (The values are the mean of at least 3 different measurements +/- the standard deviation; a standard record of the methylation analysis is shown in Figure 2SM). The results of allele-specific methylation from others members of the family are summarized in Table 1. Clinical findings of patient II-1 are indicated in Table 2. An extremely skewed X-chromosome inactivation was also

Analysis of X-chromosome inactivation

We analyzed additional loci at FRAXA, androgen receptor (AR) phosphoglycerate kinase (PGK) and FRAXE to confirm the skewed X-chromosome inactivation observed by SB as previously described [14] (See supplementary material).

Methylation analysis of the FMR1 gene

Allele-specific methylation status was analysed using the Asuragen AmpliseX FMR1 mPCR kit (Austin, TX) following the instructions of the manufactures. gDNA was extracted from blood, oral mucosa and root hair. Briefly, pre-treatment of DNA with methylation-sensitive restriction enzyme was followed by PCR amplification using dye-tagged primers. PCR from the digested reaction was performed with HEX-labelled FMR1 primers, while for the control reaction FAM-labelled FMR1 primers were used. A CGG DNA control provides a reference (REF) peak and is co-amplified with each sample. PCR products were analysed as described for TP-PCR.

Clinical exploration

Neuropsychological and clinical assessment of patient II-1 from family 1 was performed at the age of 47 and neurological examination at the age of 54. Supplementary tests were carried out including cranial Magnetic Resonance Imaging (MRI) and I(123)-FP-CIT-SPECT. (See supplementary material). Clinical and psychological examination of patient II-3 from family 2 was performed periodically from 9 months to 6 years and 5 months of age (See supplementary material).
observed at the FRAXA, FRAXE and AR loci (Figure 1SM). For specific allele expression we used the TP-PCR protocol with cDNA instead of gDNA and the results are shown in Figure 1C. According with the methylation status observed in both females, patient II-1 expressed almost exclusively the FMR1 expanded allele and her mother (that we used as a control) expressed only the 22 CGG repeats allele that was predominately unmethylated. Real time PCR (See supplementary material) showed that patient II-1 had 2.7 times mFMR1 expression with her non-fragile X mother (I-2) (Figure 3 SM).

**Family 2**

The pedigree of the family is shown in Figure 1D. Molecular diagnosis was previously reported except from individual II-3 that was born after the study was performed [14]. Prenatal diagnosis, obtained at 6 years and 5 months old are summarized in Table 1 (See supplementary material). The proband (II-3) showed a 90 +/- 5% methylation of the normal allele in blood, 83 +/- 2% in oral mucosa and 90 +/- 2 % in roots hair (The values are the mean of at least 3 different measurements +/- the standard deviation). As observed in her sister the 173 CGG premutated allele in II-3 was almost completely methylated. Clinical and psychological examination of patient II-3 was done at different period and the result obtained at 6 years and 5 months old are summarized in Table 1 (See also supplementary material).

**Discussion**

This work reports the methylation analysis of specific CpG sites in different tissues from two fragile X families and their correlation with the phenotype. New technologies are currently incorporated for clinical laboratory to implement the exact number of the CGGs and the methylation status of the FMR1 gene that would help to predict clinical manifestations and could substitute SB as the gold standard. Phenotypes in women are highly variable and although it is generally accepted that skewed X chromosome inactivation (XCI) should have a predominant role no clear correlation has been observed between the methylation status of the FMR1 gene that would help to predict clinical manifestations and could substitute SB as the gold standard. Phenotypes in women are highly variable and although it is generally accepted that skewed X chromosome inactivation (XCI) should have a predominant role no clear correlation has been observed between the methylation status of the FMR1 gene that would help to predict clinical manifestations and could substitute SB as the gold standard. Phenotypes in women are highly variable and although it is generally accepted that skewed X chromosome inactivation (XCI) should have a predominant role no clear correlation has been observed between clinical findings and the methylation analysis obtained by SB [15-18]. In addition, the use of different methylation sensitive restriction enzymes in SB may give different results [14], indicating that not all

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**Table 1: Tissue specific size and methylation of FMR1 alleles.**

| Tissues Analyzed: Blood, oral mucosa, root hair; Peak: CGG triplets; Met: Methylation in % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | Patient of Family 1 | Met | % | Met | % | Met | % | Sample | Patient II-3 of Family 2 | Met | % | Met | % | Met | % |
| Blood | 11-Jan | 22 | 77 | 94 | 4 | — | — | 11-Mar | CO | 90 | 173 | 90 | >200 | 95 |
| Blood | III-1 | >200 | 100 | >200 | 100 | — | — | 11-Jan | 100 | 155 | 100 | >200 | 100 |
| Blood | III-2 | 69 | 10 | >200 | 100 | >200 | 100 | 01-Feb | 32 | 25 | >200 | 100 | — | — |
| Blood | 11-Jan | 24 | 95 | 92 | 7 | — | — | 01-Feb | 32 | 25 | >200 | 100 | — | — |
| Blood | vol.7 ni | T11-1 | >200 | 100 | >200 | 100 | — | — | 11-Jan | 29 | 100 | >200 | 100 | — | — |
| Blood | II-3 | 70 | 14 | >200 | 100 | 83 | >200 | III-1 | 28 | 100 | 151 | 100 | >200 | 100 |
| Blood | II-3 | 70 | 14 | >200 | 100 | 83 | >200 | III-2 | 29 | 90 | >200 | 93 | — | — |

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**Table 2: Clinical findings of patients.**

<table>
<thead>
<tr>
<th>Patient of Family 1</th>
<th>Ovarian fare</th>
<th>At age 34</th>
<th>Forehead</th>
<th>Wide</th>
<th>Patient II-3 of Family 2</th>
<th>Ovarian fare</th>
<th>At age 34</th>
<th>Forehead</th>
<th>Wide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tremor</td>
<td>Mild intentional tremor</td>
<td>Zygomatic arch</td>
<td>Increased</td>
<td>Ears</td>
<td>Low-set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinsonism</td>
<td>Absent</td>
<td>Joint hypermobility</td>
<td>Present</td>
<td>Premature</td>
<td>Psychiatric disorders</td>
<td>Anxiety and depression</td>
<td>Expressive language</td>
<td>Deficit pragmatic and abstract understanding</td>
<td></td>
</tr>
<tr>
<td>Cerebellar signs</td>
<td>Frequent falls without cerebellar involvement</td>
<td>Atentional system</td>
<td>Mild/moderate deficit. Dysfunction in the inhibitory system</td>
<td>Praxico system</td>
<td>Ideomotor dyspraxia, Limition cranial</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cognitive impairment</td>
<td>Memory problems without cognitive decline</td>
<td>Praxico system</td>
<td>Ideomotor dyspraxia, Limition cranial</td>
<td>Motor control</td>
<td>Echotypia</td>
<td></td>
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</tr>
<tr>
<td>Psychiatric disorders</td>
<td>Absent</td>
<td>Comprehensie language</td>
<td>Deficit pragmatic and abstract understanding</td>
<td>Expressive language</td>
<td>Echotypia</td>
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</tr>
<tr>
<td>Autonomic dysfunction</td>
<td>Absent</td>
<td>Comprehensive language</td>
<td>Deficit pragmatic and abstract understanding</td>
<td>Expressive language</td>
<td>Echotypia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranial MRI</td>
<td>Generalized mild cortico-subcortical atrophy</td>
<td>Working memory</td>
<td>Moderate/severe deficit</td>
<td>Social cognition</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I(123)-FP-CTT SPECT</td>
<td>Possible decreased binding at bilateral putamen</td>
<td>Social cognition</td>
<td>Normal</td>
<td>Social cognition</td>
<td>Normal</td>
<td></td>
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</table>
neighbouring CpG sites have the same relevance for clinical outcome. In this report we have used the AmplideX FMR1 mPCR kit to analyse the methylation of two critical CpG sites in the first exon of the FMR1 in two fragile X families and studied its correlation with clinical findings. Patient II-1 of family 1 (AR of 0.23 to 0 in blood) expressed preferentially the premutated allele and she presented a more severe phenotype than expected for a woman of her age (FXPOI at the age of 34 and neurological symptoms started at the age of 47) that could be related to the toxic effect of the expanded FMR1 mRNA. Patient II-3 of family 2 is a mosaic female that inherited the full mutation from her mother (1-2) and an additional contracted premutated allele. Clinical findings are in concordance with her preferential methylation (AR=0.1 to 0 in blood) of the normal allele inherited from her father. It has been suggested that correlation between methylation and phenotype can be obscured because of differences in X-inactivation patterns in blood and brain for some individuals [16]. In these families we found similar methylation pattern from the different embryological origin tested: blood, oral mucosa cells and root hair that can support the good correlation between methylation in the blood sample and phenotype. Several reports using mass spectrometry (MALDI-TOF) and specific quantitative melt analysis (MS-QMA) have shown the relevance of novel fragile X-related epigenetic markers for molecular diagnosis and prediction of clinical involvement [8-10,19, 20]. However, the role of the multiple potential methylation sites on expression is not absolutely defined. In conclusion, we show that methylation of the CpG sites in the first exon strongly determines the FMR1 expression. Methylation analysis with the available AmplideX FMR1 mPCR kit (Asuragen) is an easily feasible technique for a regular clinical laboratory that may be applied to a large sample. Methylation heteromiaxism at different tissues can be also explored. This protocol may be useful to predict the clinical involvement in fragile X and fragile X-related disorders.

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Contributors

MER, RM and EP recruited the samples and performed the molecular analysis. SJ and PM investigated the phenotypes of Family 1. IR and AMG investigated the phenotypes of family 2. EP wrote the manuscript and contributed funding. MER performed the figures and tables of the manuscript. All authors commented on and approved the manuscript.

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Patient Consent

Obtained.

Ethics Approval

This study was conducted with the approval of the University Hospital Macarena of Seville.

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