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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]. New PCR protocols allow the amplification of fully mutated alleles and, in addition, different strategies are being developed to substitute Southern blot (SB) as the gold standard for molecular diagnosis [7]. A set of works analysing new specific fragile X-related epigenetic elements at the first intron (FREE) using mass spectrometry (MALDI-TOF) have shown a good correlation between methylation and clinical findings but the technique is not yet available for most clinical laboratories [8-10]. Here we report the methylation status of two CpG sites in the first *FMR1* exon using the Amplidex mPCR kit (Asuragen) in normal, pre-mutated and mutated alleles from different tissues in two fragile X families.

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).

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

gDNA was analyzed using the Amplidex™ *FMR1* PCR kit by Asuragen Inc. (Austin, TX) according to the manufacter´s instructions. Briefly, 2 different forward primers, one a CGG 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 en females (See supplementary material).

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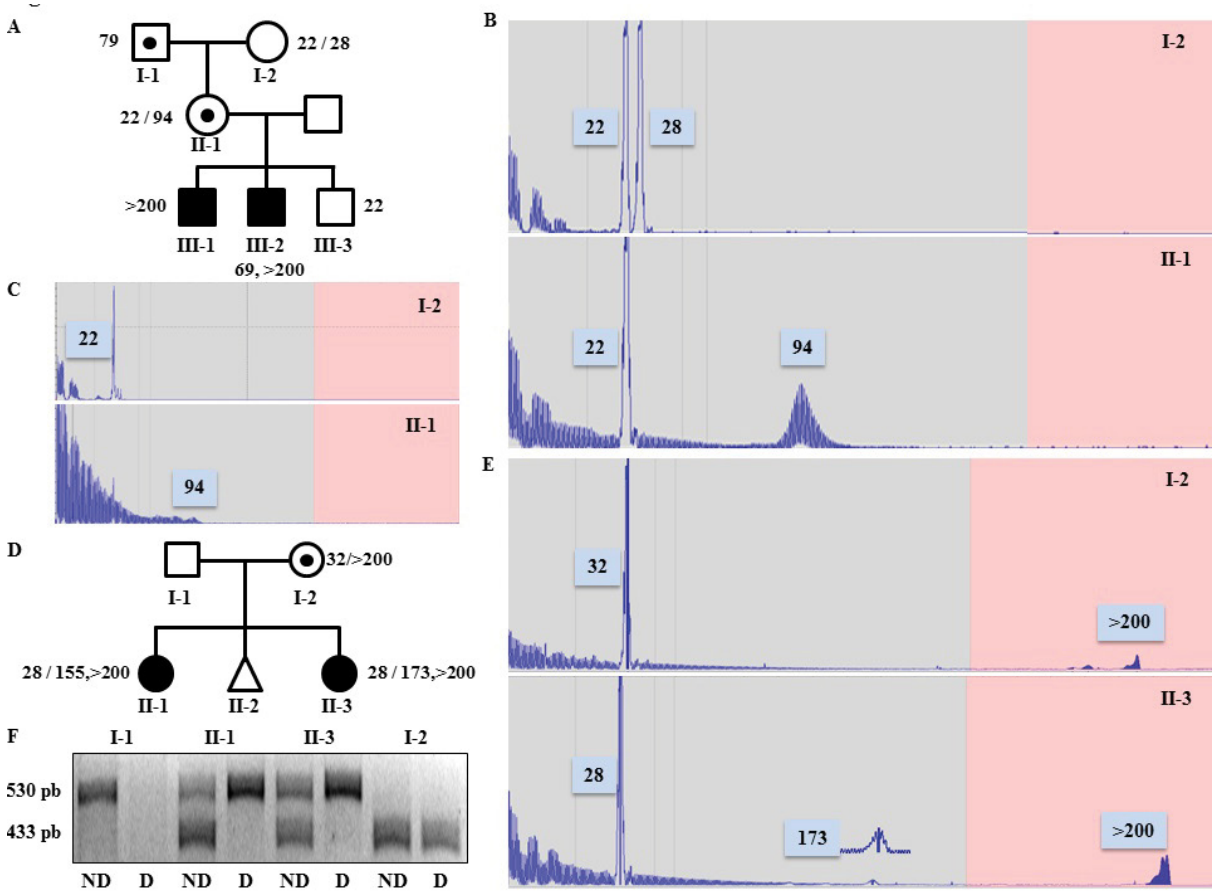

Figure 1: The pedigree of the family.

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 AmplideX *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).

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

Family 1								Family 2						
Sample		Peak 1	Met	Peak	Met	Peak	Met	Sample	Peak 1	Met	Peak	Met (%)	Peak	Met
			(%)	2	(%)	3	(%)			(%)	2		3	(%)
	I-1	79	5	—	—	—	—	01-Feb		15	>200	90	—	—
	01-Feb	22	8	28	80	—	—	II-1		100	155	100	>200	100
food	11-Jan	22	77	94	4	—	—	11-Mar	CO (-1	90	173	90	>200	95
	III-1	>200	100	>200	100	—	—							
	III-2	69	10	>200	100	>200	100							
	11-Jan	24	95	92	7	—	—	01-Feb	32	25	>200	100	—	—
vol.7 ni	T11-1	>200	100	>200	100	—	—	11-Jan	29	100	>200	100	—	—
	III-2	70	14	>200	100	—	—	11-Mar	29	83	169	97	>200	100
l flair	11-Jan	23	72	96	12	—	—	01-Feb	32	65	>200	95		
	III-1	>200	100	>200	83	>200	100	11-Jan	28	100	151	100	>200	100
	I11-2	71	14	>200	93	>200	100	11-Mar	29	90	>200	93	—	—
Tissues Analyzed: Blood, oral mucosa, root hair; Peak: COG triplets; Met: Methylation in %														

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

Patient of Family 1		Patient II-3 of Family 2	
Ovarian fare	At age 34	Forehead	Wide
Tremor	Mild intentional tremor	Zygomatic arch	Increased
Parlcinsonism	Absent	Ears	Low-set
Piramydal signs	Absent	Joint hypermobility	Present
Cerebellar signs	Frequent falls without cerebellar involvement	Atencional system	Mild/moderate deficit. Disfunction in the inhibitory system
Cognitive impairment	Memory problems without cognitive decline	Praxico system	Ideomotor dyspraxia, Imitation contralateral
Psychiatric disorders	Anxiety and depression	Expressive language	Morphosyntactic impairment Echolalia
Autonomic dysfunction	Absent	Comprehensive language	Deficit pragmatic and abstract understanding
Cranial MRI	Generalized mild cortico-subcortical atrophy	Working memory	Moderate/severe deficit
I(123)-FP-CTT SPECT	Possible decreased binding at bilateral putamen	Social cognition	Normal

Table 2: Clinical findings of patients.

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 m*FMR1* compared 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, using corionic villi was done at a different laboratory, and the report indicated that she was a premutated female foetus suggesting a possible mosaicism (knowing that her mother carried a full mutation). The parents decided to continue the pregnancy and a girl was born at term after a normal pregnancy. However, at the age of 9 months she was sent to the paediatricians for development delays. Molecular test showed a mosaic genotype with sizes in the premutated and mutated range (28, very low abundant 173 and above 200 CGG repeats) (Figure 1E). We had previously reported in this family that the patient´s oldest sister (II-1) had a complete methylation (100%) of the normal allele compatible with her severe fragile X phenotype [14]. In addition, we also reported the delayed abortion of a 13 weeks' male foetus (II-2) that could be due to abnormal methylation of his unique X chromosome [15]. Analysing the PGK locus that was informative in this family patient II-3 also showed a 100% methylation of the allele inherited from her father similar to her sister (Figure 1F). We revised the CGG sizes and the *FMR1* methylation pattern in this family with the new protocol and the results are summarized in Table 1. In addition to the full mutation observed by SB, patient II-1 had a low abundant premutated allele of 155 CGG repeats in blood and roots hair with 100 % methylation. 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 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

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* messenger. Patient II-3 of family 2 is a mosaic female that inherited the full mutation from her mother (I-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 *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 mosaicism 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.

References

1. Jacquemont S, Hagerman RJ, Hagerman PJ, Leehey MA (2007) Fragile X-syndrome and fragile X-associated tremor/ataxia syndrome: two faces of FMR1. Lancet Neurol 6: 45-55.
2. Hagerman PJ, Hagerman RJ (2004) The fragile-X prenutation: a maturing perspective. Am J Hum Genet 74: 805-816.
3. McLennan Y, Polussa J, Tassone F, Hagerman R (2011) Fragile X syndrome. Curr Genomics 12: 216-224.
4. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE, et al. (2000) Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. Am J Hum Genet 66: 6-15.
5. Buijsen RA, Visser JA, Kramer P, Severijnen EA, Gearing M, et al. (2016) Presence of inclusions positive for polyglycine containing protein, FMRpolyG, indicates that repeat-associated non-AUG translation plays a role in fragile X-associated primary ovarian insufficiency. Hum Reprod 31: 158-168.
6. Hall DA, Birch RC, Anheim M, Jonch AE, Pintado E, et al. (2014) Emerging topics in FXTAS. J Neurodev Dis 6: 31- 41.
7. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, et al. (2010) A novel *FMR1* PCR method that reproducibly amplifies fragile X full mutations in concordance with Southern blotting and reliably detects low abundance expanded alleles. Clin Chem 56: 399-408.
8. Godler DE, Tassone F, Loesch DZ, Taylor AK, Gehling F, et al. (2010) Methylation of novel markers of fragile X alleles is inversely correlated with FMRP expression and *FMR1* activation ratio. Hum Mol Genet 19: 1618-1632.
9. Godler DE, Slater HR, Bui QM, Ono M, Gehling F, et al. (2011) *FMR1* intron 1 methylation predicts FMRP expression in blood of female carriers of expanded *FMR1* alleles. J Mol Diag 13: 528-536.
10. Godler DE, Slater HR, Bui QM, Storey E, Ono MY, et al. (2012) Fragile X mental retardation 1 (*FMR1*) intron 1 methylation in blood predicts verbal cognitive impairment in females carriers of expanded FMR1 alleles: evidence from a pilot study. Clinical Chemistry 58: 590-598.
11. Costa A, Gao I, Carrillo F, Cáceres-Redondo MT, Carballo M, et al. (2011) Intermediate alleles at the *FRAXA* and *FRAXE* loci in Parkinson's disease. Parkins Rel Dis 17: 281-284.
12. Conde V, Palomar FJ, Lama MJ, Martínez R, Carrillo F, et al. (2013) Abnormal GABA-mediated and cerebellar inhibition in women with fragile X premutation. J Neurophysiol 109: 1315-1322.
13. Alfaro MP, Cohen M, Vnencak-Jones CL (2013) Maternal FMR1 premutation allele expansion and contraction in fraternal twins. Am J Med Genet Part A 161A: 2620-2625.
14. Martínez R, Bonilla-Henao V, Jímenez A, Lucas M, Vega C, et al. (2005) Skewed X inactivation of the normal allele in fully mutated female carriers determines the levels of FMRP in blood and the fragile X phenoptype. Mol Diag 9: 157-162.
15. Martínez R, Bonilla-Henao V, Ramos I, Sobrino F, Lucas M, et al. (2008) May anomalous X-chromosome methylation be rsponsible for the spontaneous abortion of a male fetus ? J Genet 87: 1-4.
16. Hagerman RJ, Leavitt BR, Farzin F, Jacquemont S, Greco CM, et al. (2004) Fragile-X-associated tremor/ataxia syndrome (FXTAS) in females with the *FMR1* premutation. Am J Hum Genet 74: 1051-1056.
17. Berry-Kravis E, Potanos K, Weinberg D, Zhon L, Gotz CG (2005) Fragile X-associated tremor/ataxia syndrome in sisters related to X-inactivation. Am Neurol 57: 144- 147.
18. Rodríguez-Revenga L, Madrigal I, Badenas C, Xunclá M, Jiménez L, et al. (2009) Premature ovarian failure and fragile X female permutation carriers: no evidence for skewed X-chromosome inactivation pattern. Menopause 16: 944-949.
19. Grasso M, Boon EMJ, Filipovic-Sadic S, Van Bunderen PA, Gennaro E, et al. (2014) A novel methylation PCR that offers standardized determination of FMR1 methylation and CGG repeats length without Southern blot analysis. J Mol Diag 16: 23-31.
20. Godler DE, Inaba Y, Schwartz ChE, Bui QM, Shi EZ, et al. (2015) Detection of skewed X-chromosome inactivation in fragile X syndrome and X chromosome aneuploidy using quantitative melt analysis. Exp Rev Mol Med 17: 1-9.