Partially Methylated Alleles, Microdeletion and Tissue Mosaicism in a Fragile X Male with Tremor and Ataxia at 30 Years of Age: A Case Report

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ABSTRACT

CGG repeat expansion >200 within *FMR1,* termed full mutation (FM), has been associated with promoter methylation, consequent silencing of gene expression and fragile X syndrome (FXS) – a common cause of intellectual disability and co-morbid autism. Unmethylated premutation (55 to 199 repeats) and FM alleles have been associated with fragile X related tremor/ataxia syndrome (FXTAS), a late onset neurodegenerative disorder. Here we present a 33 year old male with FXS, with white matter changes and progressive deterioration in gait with cerebellar signs consistent with probable FXTAS; there was no evidence of any other cerebellar pathology. We show that he has tissue mosaicism in blood, saliva and buccal samples for the size and methylation of his expanded alleles and a *de novo*, unmethylated microdeletion. This microdeletion involves a ~80bp sequence in the *FMR1* promoter as well as complete loss of the CGG repeat in a proportion of cells. Despite *FMR1* mRNA levels in blood within the normal range, the methylation and CGG sizing results are consistent with the diagnosis of concurrent FXS and probable FXTAS. The demonstrated presence of unmethylated FM alleles would explain the manifestation of milder than expected cognitive and behavioural impairments and early onset of cerebellar ataxia. Our case suggests that individuals with FXS, who manifest symptoms of FXTAS, may benefit from more detailed laboratory testing.

Keywords: Fragile X Syndrome, fragile X related tremor/ataxia syndrome, tremor, cerebellar ataxia, mental retardation, molecular biology, methylation, mosaicism

INTRODUCTION

Fragile X syndrome (FXS) is the most common heritable form of intellectual disability and autism, found in 1 in 4000 in males and 1 in 8000 in females in the general populaiton, reviewed in [Hagerman et al., 2009]. FXS usually results from CGG repeat expansion > 200, termed 'full mutation' (FM), subsequent methylation of the regulatory regions and silencing of the *FMR1* gene located on the X chromosome [Godler et al., 2010]. Fragile X associated tremor/ataxia syndrome (FXTAS) is a 'late-onset' neurodegenerative disorder affecting carriers of the CGG expansion between 55 and 199 repeats, termed premutation (PM)[Jacquemont et al., 2004]. These PM alleles have estimated frequencies of 1 in 468 males and 1 in 151 females in the general population, and are associated with over-expression of the *FMR1* gene and toxicity from excessive RNA production leading to cell death contributing to FXTAS pathology, reviewed in [Hagerman et al., 2009]. Probable FXTAS has also been described in FM males with an unmethylated *FMR1* promoter region (UFM), resulting in active *FMR1* gene and IQ greater than 70 [Loesch et al., 2012; Santa Maria et al., 2013]. Here we present a case of one FXS male with clinical features consistent with probable FXTAS first manifesting in his early 30s. We show that he has unmethylated as well as methylated FM alleles, tissue mosaicism for the size of his expanded *FMR1* alleles and a *de novo* microdeletion in the *FMR1* promoter involving complete loss of the CGG expansion.

CLINICAL REPORT

Clinical History and Sample Processing

At 5 years of age, this male was diagnosed with FXS based on morphological features and cytogenetic testing showing 40% of cells expressing the Xq27.3 fragile site. At 33 years of age he was referred to the neurology service for assessment of progressive unsteadiness developing over 18 months. There was no accompanying weakness or sensory disturbance.

His physical examination revealed mildly increased tone in all limbs, no tremor, mild symmetrical proximal weakness in the lower limbs and moderate midline ataxia.

20 ml of peripheral blood was collected in EDTA tubes for CGG sizing, methylation and *FMR1* mRNA analyses. Buccal samples were also collected using the Master Amp Buccal Swab Brush kit (Epicentre technologies, USA). DNA was extracted from 15ml of blood and from buccal brush samples using the NucleoSpin®Tissue genomic DNA extraction kit (Machery-Nagel, Germany). 5 ml of blood was used for peripheral blood mononuclear cell (PBMC) isolation using Ficoll gradient separation [Loesch et al., 2011]. RNA was extracted from the isolated PBMCs using RNeasy kit (Qiagen Global) as per the manufacturer's instructions.

FMR1 Methylation, mRNA, CGG and Proximal Region sizing/copy number changes.

CGG repeat sizing in all samples was performed using polymerase chain reaction (PCR) as previously described [Khaniani et al., 2008]. The AmplideX™ *FMR1* PCR Kit was used to perform CGG sizing on and determine mean methylation at two *HpaII* sites, 5' and 3' of the CGG expansion using blood DNA as per manufacturer's instructions (Asuragen, Texas, USA)**.** Methylation sensitive Southern blot was used to determine CGG size and methylation of the *FMR1* CpG island 5' of the CGG expansion. Appropriate normal CGG size, PM and FM DNA samples used in a previous publication [Godler et al., 2010] were included as methylation controls. Seven to 9µg of DNA were digested with either methylation sensitive enzyme combination of *NruI*/*HindIII* or *EagI/ EcoRI* or *PstI* which is not sensitive to methylation changes (Figure 1). P32 labelled *Pfxa3a* probe (PCR Dig Synthesis kit; Roche Diagnostics Global) was used to detect *FMR1* alleles by autoradiography as previously described [Godler et al., 2010], with the PS8 control probe used for the *PstI* digestion.

FREE1 and FREE2 PCR products were synthesised using primers and conditions previously described [Godler et al., 2010], and sized using capillary electrophoresis to rule out presence of the deletion at the 5' epigenetic boundary of the FMR1 promoter and the exon1/intron 1 boundary at the 3'end of the *FMR1* promoter (Figure 1)**.** The Sequenom MALDI-TOF MS EpiTYPER system (Sequenom, USA) was used to analyse mean methylation of the FREE2 region located 3' of the CGG repeat in blood, buccal and saliva samples, as previously described [Godler et al., 2010]. Two and a half million probe based microarray analysis was performed using blood DNA, to rule out large pathogenic copy number changes other than within *FMR1*, as well as to examine copy number changes of SNPs proximal to the CGG expansion, as previously described [Motoike et al., 2014]. FMR1 mRNA was reverse-transcribed in two separate cDNA reactions and each cDNA sample was analysed using real time-PCR reactions, as previously described [Loesch et al., 2011].

Clinical assessments

Blood tests, including thyroid function tests, vitamin B12 and autoimmune antibody panel, were within normal limits. Nerve conduction studies performed at the time did not reveal any evidence of peripheral neuropathy. MRI of his brain and spine revealed normal cerebellum without white matter lesions in the middle cerebellar peduncles and diffuse but non-specific T2 hyperintensities in the supratentorial cerebral white matter (Figure 2) that were stable on repeat imaging 9 months later despite development of spastic paraparesis in the intervening period. These radiological findings and the gait ataxia met the diagnostic criteria for probable FXTAS [Loesch et al., 2005]. He was referred to a clinical geneticist to explore the possible presence of PM and unmethylated FM alleles.

At 35 years of age formal psychological assessment for this male was also performed. The Wechsler Adult Intelligence Scale (WAIS-IV) [D. 2008] showed that his cognitive ability

was in the 'Extremely Low' range with a full scale IQ of 51 (percentile rank 0.1; 95% confidence interval: 48-56). His WAIS-IV indexes scores did not differ significantly and were all within the 'Extremely Low' range. The Autism Diagnostic Observation Schedule – Second Edition (ADOS-2) [Lord 2012] assessment revealed some behavioural features consistent with autism spectrum disorder including deficits in communication skills, reciprocal social interaction and social-emotional reciprocity that appeared to be separate from his impairment in verbal communication and general intellectual function. However, he did not satisfy the diagnostic criteria for autism spectrum disorder as per *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (American Psychiatric Association 2013).

Testing for Causes of Ataxia

Friedreich's ataxia (FA) and Spinocerebellar Ataxias (SCA) repeat regions were examined using PCR and gel electrophoresis, as previously described protocols [Biros et al., 1998; Campuzano et al., 1996]. Furthermore, omni2.5-8 microarray was used to identify no copy number changes associated with whole exon deletions/duplications in the known ataxia or neuropathy genes, based on average probe density [Motoike et al., 2014].

RESULTS AND DISCUSSION

CGG sizing and detection of microdeletion

Southern blot *FMR1* CpG island methylation analysis in blood showed unmethylated fragments between 2.5 and 4.5 Kb for *NruI/HindIII* digestion and 2.6 to 4.4 Kb for *EagI/EcoRI* digestion (Figure 1A). There were also methylated fragments between 4.7 and 6.5 Kb for *NruI/HindIII* digestion and 5.2 and 6.1 Kb for *EagI/EcoRI* digestion. For the *PstI* digestion (that was not methylation sensitive) the expanded alleles ranged in size from 1.63kb to 2.83Kb. Together the two methylation sensitive digestions, *NruI/HindIII* and *EagI/EcoRI*, suggested presence of unmethylated and methylated FM alleles ranging in size from 463 to

846 CGG repeats (Figure 1 A and B). The presence of FM alleles within this size range was further confirmed by the *PstI* digestion based Southern blot in buccal DNA (Figure 1C).

All three digests followed by Southern blot also detected a fragment that was ~0.2Kb smaller than that in normal controls. This smaller allele was exclusively unmethylated and absent from the patient's mother's blood, the mother herself being a mosaic of PM and FM alleles (Figure 1C). The absence of the smaller allele in the mother suggested that the pro-band is mosaic for a *de novo* microdeletion and FM expansion. As the same smaller band was detected in all 3 digests, a microdeletion would have to be located within *PstI/PstI* fragment and also within the overlapping *NruI/HindIII* and *EagI/EcoRI* fragments (Figure 1 A). Within this *PstI* fragment, the microdeletion is unlikely to occur within the exon 1 or intron 1 of *FMR1* as microarray SNP copy number proximal to the repeat within intron 1 and PCR amplicon sizing of the FREE2 region on the exon1/intron 1 boundary were both completely normal (Figure 1 A and D). In this context, it is important to note that microarray SNP analysis did not identify any pathogenic variants elsewhere in the genome and the amplicon size of FREE1 located at the 5'end of the FMR1 promoter was also completely normal (Figure 1 D). This microdeletion must therefore involve complete loss of the CGG expansion together with a proximal binding site of one of the Amplidex PCR primers as the microdeletion was not detected using AmplideX PCR (Figure 1 E). Furthermore, this microdeletion must include loss of the Amplidex primer binding site located within the CpG island located ~87 bp 5'of the CGG repeat, as in a previous case [Schmucker et al., 1996].

Discordance between Southern blot and AmplideX PCR CGG sizing.

Unmethylated PM alleles of ~70 repeats were detected by Southern blot following *NruI/HindIII* and *EagI/EcoRI* digests, as indicated by the lower points of smears of ~2.9Kb and 3.0Kb in size, respectively (Fig 1B). However, PM alleles were not detected using

AmplideX PCR or standard sizing PCR [Khaniani et al., 2008], the latter having primer binding sites proximal to those of AmplideX. Only partially methylated FM size alleles were detected using AmplideX PCR. This discordance is unlikely to be due to the sizing imprecision of our Southern blot analyses (+/-30 CGG repeats) as an extended Southern blot smear is still within the PM range. An alternative explanation could be microdeletion involving both a portion of CGG repeat and the Amplidex primer binding site, similar to the the smaller ~2.5kb Southern blot band that was also undetected using AmplideX PCR.

Discordance between Southern blot and Amplidex PCR methylation analysis.

Two prominent FM allele types were detected in blood, buccal and saliva DNA using Amplidex PCR (Figure 2E). These alleles were also identified by Southern blot after *NruI/HindIII*, *EagI/EcoRI* and *PstI* digests. However, there was significant discordance between the methylation percentage estimated by Southern blot following *NruI/HindIII* and *EagI/EcoRI* digestion and that by AmplideX analysis of two *HpaII* sites located either side of the expansion (Figure 1). The mean Amplidex *HpaII* methylation estimate was ~60% in blood and saliva and 94% in buccal DNA (Figure 1E). In contrast, *NruI* and *EagI* methylation values were only 11% and 12%, respectively for Southern blot (100 X density of all methylated bands/total of unmethylated and methylated bands) (Figure 1B). If the density of the ~2.5Kb microdeletion band is not taken out of this calculation (as AmplideX does not detect it), *NruI* and *EagI* methylation values increase to 28% and 26%, respectively. This discordance could be because sizing and methylation analysis of all alleles that also have the microdeletion with the AmlideX primer binding sites (exclusively unmethylated by southern blot) being missed, leading to over-estimation of methylation by AmplideX for this case.

FREE2 Methylation, FMR1 mRNA Analyses and Testing for Other Genetic Causes of Ataxia

Increase in FREE2 methylation has been previously linked to *FMR1* protein (FMRP) deficiency and the severity of cognitive impairment in FM males and females with FXS [Godler et al., 2012]. In this male, blood and saliva showed mean FREE2 methylation output ratio (MOR) of ~0.4, while buccal samples showed MOR of ~0.45 (Figure 2C). Interestingly the methylation values remained un-changed between blood and saliva samples collected 1.5 years apart. All MOR values were above those of normal allele and PM methylation ranges in males but were below the FREE2 methylation range for typical FXS males carrying only FM alleles. These MOR values suggest that approximately 40% of leukocytes and ~45% of epithelial (buccal) cells have methylated alleles. This is consistent with the patient's milder cognitive and behavioural impairments compared to those of 'typical FXS' males carrying only highly methylated FM alleles. While unmethylated FM alleles have been associated with overexpression of *FMR1* mRNA and increased risk of FXTAS [Loesch et al., 2012], it is not clear if the PM alleles with the proposed microdeletion could also contribute to the probable FXTAS phenotype observed in this patient (Figure 1).

Analysis of *FMR1* transcription in PBMCs revealed an mRNA level of 0.97 arbitrary units which is within the normal male expression range, above the levels observed for FM only and PM/FM mosaic males with the typical FXS phenotype (Figure 2D). This is consistent with FREE2 methylation results where more than half of all cells would have an unmethylated promoter, overexpress *FMR1* at twice the normal level, as previously described [Loesch et al., 2011; Loesch et al., 2012], but overall would show normal FMR1 mRNA levels because the remaining cells would have a completely methylated FMR1 promoter with no FMR1 mRNA expression. Testing for other causes of ataxia excluded Friedrich's ataxia, spinocerebellar ataxias type 1, 2, 3, 6 and 7 by PCR; and copy number

changes associated with common peripheral neuropathies such as *CMT1A* and *HNPP* by the genome-wide array testing.

CONCLUSION

This is the first reported case of an FXS male showing white matter changes and progressive deterioration in gait with cerebellar signs, meeting the diagnostic criteria of probable FXTAS at 30 years of age. Our patient has cerebellar dysfunction with no evidence of any other cerebellar pathology and has molecular features of mosaicism for CGG repeat size, a *de-novo* microduplication within the *FMR1* promoter for a proportion of cells, methylation mosaicism amongst different CpG sites and tissue types and UFM alleles that are consistent with FXTAS clinically manifesting concurrently with FXS. Our case adds to the expanding and evolving clinical and genetic profile of FXTAS and FXS, suggests that assessment for FXTAS may need to be broadened to detect males with partially methylated FM alleles and highlights the need for comprehensive testing to fully describe the range of underlying molecular pathologies.

CONSENT

Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

COMPETING INTERESTS

DEG holds patents related to the technology described in this article. The other authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

YH, CR, MH and RH clinically assessed the patient. MA and LB performed the psychological tests mentioned in the paper. DEG, DF, XL, AS and HS performed the molecular analyses

related to FXS and FXTAS including Asuragen PCR assay. BC performed the tests for other causes of inherited ataxias. YH and DEG prepared the drafts of the paper. All authors have reviewed, provided corrections and agree with the submitted version of the paper.

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Figure 1. Sizing of the CGG repeat and proximal regions using Southern blot and PCR. (A) Organization of the *FMR1* 5' region including the CGG expansion (sequence numbering from GenBank L29074 L38501) in relation to *FMR1* and *ASFMR1* transcription start sites, Fragile X Related Epigenetic Elements 1 and 2 (FREE1 and FREE2), the *FMR1* CpG island and methylation sensitive restriction sites and corresponding fragments analysed, as well as the *Pfxa3 probe* used in routine fragile X Southern blot testing, two *HpaII* sites targeted by Amplidex methylation PCR (red arrows indicate primer binding sites). Purple arrows indicate standard CGG PCR binding sites as previously described [Khaniani et al., 2008]. Locations of probes targeting two single nucleotide polymorphisms from the whole genome 2.5 million probe microarray analysis [Motoike et al., 2014] are indicated by black arrows labelled as OmniSNP#3 and SNP#5. **(B)** Methylation sensitive Southern blot analysis of the *NruI* and *EagI* restriction sites within *FMR1* CpG island. The blood DNA sample in question from the FXS male suspected of FXTAS is in lanes 3 and 6 with broken blue lines indicating positive bands in these samples. Comparator DNA, sized using standard CGG sizing PCR, from healthy control males is in lanes 2 and 5, and from an affected FM female in lanes 1 and 4. The 5.1Kb band size represents ~46 CGG repeat methylated alleles in the *NruI/HindIII* digest. The 4.9Kb band size represents ~30 CGG repeat methylated alleles in the *EagI/EcoRI* digest. All larger bands are methylated and labelled M. All smaller bands are unmethylated, and labelled UM. **(C)** Southern blot analysis of the *PstI* restriction sites (not methylation sensitive). The buccal DNA sample in question from the FXS male suspected of FXTAS is in lane 1; his mother's blood DNA is in lane 2; broken blue lines indicate positive bands in these samples. Comparator DNA sized using standard CGG sizing PCR from two premutation females (90 and 101 CGG repeats by PCR) are in lanes 3 and 4. Male and female healthy control DNA are in lanes 5 and 6. **(D)** Capillary electrophoresis sizing of FREE1 and FREE2 PCR

products synthesised using primers and conditions previously described [Godler et al., 2010]. **(E)** AmplideX PCR targeting methylation of two *HpaII* sites from capillary electrophoresis of the DNA from the blood, buccal and saliva of the male case in question. Note: Presence of positive FM alleles with methylated *HpaII* sites and the control digestion (*HpaII* methylation independent) are indicated capillary electrophoresis signatures in green and blue, respectively.

Figure 2. Magnetic Resonance Imaging of the Brain, FMR1 mRNA expression and FREE2 methylation in peripheral tissues. (A) T2 weighted image of the brain. Note white matter changes bilaterally but more pronounced in the deep white matter of right hemisphere. These changes would not be expected to be seen in a normal 33 year old. **(B)** *FMR1* mRNA assessed using real-time PCR relative standard curve method. Note: the error bars for reference ranges represent 2 standard deviations from the *FMR1* mRNA levels in RNA from peripheral blood mononuclear cells (PBMCs) of 19 male controls (CGG<44) co-run with the RNA from the male case in question. RNA from PBMCs of two FXS affected males, FXS#1 PM/FM mosaic (71, 213-480 CGGs; developmental delay; no formal cognitive testing) and FXS#2 FM only (543-633 CGGs; FSIQ 44; VIQ 50; PIQ 54) were also included. **(C)** Mean methylation output ratio of CpG sites located within the FREE2 region assessed using MALDI-TOF MS. Case's blood and buccal samples were collected at two time points 1.5 years apart. Note: the error bars for reference ranges represent 2 standard deviations from the mean FREE2 methylation in blood of 17 male controls (CGG<44); 39 PM males (56-170 CGGs); 65 FM males with typical FXS (213-2000 CGGs) and 4 atypical 'high functioning' FM males unmethylated by Southern blot (UFM) (CGG 200-637 CGG). The reference samples

were co-run with DNA from FXS male case suspected of FXTAS from blood, buccal and saliva samples in question.