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Retraction of a FXS allele to normal size.

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Running title: Retraction of a FXS allele to normal size.

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Abstract

The *FMR1* premutation (PM:55-199 CGG) is associated with Fragile X–associated tremor/ataxia syndrome (FXTAS) and when maternally transmitted is at risk of expansion to a hypermethylated full mutation (FM: ≥ 200 CGG) that causes fragile X syndrome (FXS). We describe a maternally transmitted PM (77 CGG) that was passed to a son (103 CGG), and to a daughter (220 – 1822 CGG), who were affected with FXTAS and FXS, respectively. The male with the PM showed low level mosaicism for normal size of 30 and 37 CGG. This male had two offspring: one female mosaic for PM and FM (56, 157, > 200 CGG) and another with only a 37 CGG allele detected in multiple tissues, neither with a clinical phenotype. The female with the 37 CGG allele showed normal levels of *FMR1* methylation and mRNA and passed this 37 CGG allele to one of her daughters, who was also unaffected. These findings show that post-zygotic paternal retraction can lead to low level mosaicism for normal size alleles, with these normal alleles being functional when passed over 2 generations.

Keywords: fragile X syndrome, *FMR1*, mosaicism, CGG, premutation

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Introduction

Fragile X syndrome (FXS) is the most common genetic cause of intellectual disability, usually resulting from a trinucleotide expansion of ≥ 200 CGG repeats in the 5' untranslated region of the *FMR1* gene, known as full mutation (FM) [Jin and Warren 2000]. FM alleles are associated with abnormally high DNA methylation of the *FMR1* promoter, resulting in silencing of *FMR1* transcription and decrease in its product FMRP [Jin and Warren 2000]. FMRP is essential for normal neurodevelopment, with its loss thought to be the primary cause of FXS [Bagni and Zukin 2019; Devys and others 1993]. Smaller alleles called premutation (PM: 55-199 CGGs), and less common unmethylated FM alleles, express elevated levels of both *FMR1* mRNA and antisense *FMR1* (*ASFMR1*) mRNA, which are postulated to exert physiological effects via gain of function toxicity [Loesch and others 2011; Tassone and others 2000]. This 'toxicity' has been linked to late onset disorders including fragile X-associated tremor /ataxia syndrome (FXTAS) [Hagerman and others 2004].

There is a high propensity for PM alleles to expand upon maternal transmission, causing most cases of FXS. In contrast, nearly all PM alleles in males are passed to their daughters as PM alleles [Nolin and others 2019]. In addition to CGG allele size in the PM range, the presence of interspersed AGG repeats that interrupt the CGG tract is also a risk factor for PM alleles to exapnd in progeny. Specifically, PM alleles that lack AGG interruptions are more likely to expand to FM on maternal transmission, also contribute to repeat instability [Nolin and others 2019]. AGG interruptions may also be relevant for paternal transmissions [Alvarez-Mora and others 2017].

Retractions of the CGG repeat are also observed, predominantly with paternal transmission of PM alleles [Nolin and others 2019], and postulated to result from mitotic instability in the male germ line. To date, all observed paternal contractions of PM alleles have resulted in the generation of smaller PM alleles, and paternal contraction of a PM or FM allele to a normal allele has not been reported, until this study.

Materials and Methods

Participants

The clinical and molecular assessments were completed at the Murdoch Children's Research Institute, and the DNA methylation and *FMR1* mRNA analysis reference data were analysed previously as part of the FREE FX study [Kraan and others 2020], with approval from The Royal - Children's Hospital Human Research Ethics Committee (Reference numbers: HREC 34227, HREC 33066, HREC/13/RCHM/24).

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Molecular Analyses

Buccal epithelial cell (BEC) and saliva samples were collected for DNA extraction using the Master Amp Buccal Swab Brush kit (Epicentre Technologies, Madison, WI, USA), and the Oragene® DNA Self-Collection Kit (DNA Genotek, Global), respectively. Three to 10 mL of blood was collected in ethylene-diaminetetra acetic acid (EDTA)-treated tubes, with: (i) 3 to 5 mL used for peripheral blood mononuclear cells (PBMC) isolated using Ficoll gradient separation as described previously [Loesch and others 2011], and purified using the RNA-easy extraction kit, as per the manufacturer's instructions (Qiagen Inc., Hilden, Germany); (ii) the remaining blood was frozen at −80°C for DNA extractions. DNA was extracted from the BEC, saliva, and blood samples using the QIAsymphony DSP DNA extraction Kit (Qiagen, Hilden, Germany).

Routine *FMR1* testing was performed on extracted DNA using PCR-based assessment of CGG repeat size using a validated PCR assay, with an upper limit of detection of 170 CGG repeats in males and 120 CGGs in females [Khaniani and others 2008], and Southern blot analysis, as described in [Francis and others 2000]. Of note, is that the Southern blot method used in this study was not methylation sensitive, and was exclusively used for CGG sizing. CGG sizing was also performed using long-range Triplet-primed PCR analysis with three different triplet primed long-range PCR kits for CGG sizing: X Sense commercial kit (Abbott Molecular, Illinois, U.S.A.), AmplideX screening assay and AmplideX *FMR1* mPCR assay (Asuragen, Texas, USA). PCR products were separated by capillary electrophoresis, as previously described [Aliaga and others 2016; Chen and others 2011; Hantash and others 2010].

The EpiTYPER system was used to measure methylation output ratios for Fragile X Related Epigenetic Element 2 (FREE2) CpG sites, located at the 3' end of the *FMR1* promoter, as previously described [Godler and others 2010]. The sites analysed included CpGs 1 and 2 (located within *FMR1* exon 1) and CpGs 6–12 (located within *FMR1* intron 1). The methylation output data was expressed as either Aggregate FREE2 value that represented mean methylation across CpG 1, 2, 6 to 12, or just CpG 10-12 that was previously related to intellectual functioning in FXS [Arpone and others 2018; Inaba and others 2013; Inaba and others 2014]. Single-nucleotide polymorphism (SNP) linkage analysis was performed using Illumina CytoSNP300K microarray, as per manufacturer's instructions (Illumina, Global) on DNA from the parent II:3 and both of his daughters III:2 and III:4, to confirm inheritance and exclude sex chromosome aneuploidy.

FMR1 mRNA analysis was performed on RNA extracted from PBMCs using the relative standard curve method, with the mean 5'and 3' *FMR1* mRNA levels were normalized to the mean of

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EIF4A2 and *SDHA* mRNA levels used as internal controls, expressed in arbitrary units (a.u.), as described [Kraan and others 2016].

Results and Discussion

This study describes a family identified when mosaicism for expanded *FMR1* alleles was detected in a female undergoing reproductive genetic carrier screening (Fig. 1). Follow-up testing led to diagnoses of FXS and FXTAS in other family members. The expanded allele, with no AGG interruptions, demonstrated unusual instability (Suppl Fig 1). Cascade testing revealed it had been inherited from the proband's father who had a 105 CGG-sized unmethylated PM allele, as well as two normal size alleles of 30 and 37 CGGs (Fig 2). This individual was a healthy professional until the sixth decade when he reported hand tremor at age 57 years and MRI scan showed mild generalized cortical atrophy, marked vermian atrophy and white matter lesions affecting the middle cerebral peduncles, consistent with a diagnosis of FXTAS. By age 65 years he had developed gait ataxia, word-finding difficulties, and slurred speech. His daughter (the proband) was mosaic for four different allele sizes (a smaller PM allele, two larger PM alleles and a FM allele) whilst his other daughter inherited a normal sized allele of 37 CGG. In both daughters, these alleles had no AGG interruptions, consistent with paternal origin, and somatic instability across generations. Both daughters were healthy with no PM-associated phenotypes. The daughter with the 37 CGG allele had three daughters of her own, one of whom inherited the 37 CGG allele that was detected in different tissues (Suppl Fig 2) and had no AGG interruptions (Suppl Fig 1).

Low level mosaicism suggests that a post-zygotic retraction to normal size has occurred.

The paternal retraction phenomenon observed in this family could be attributed to the presence of mosaicism in the male with the 105(+/-3) CGG PM (II:3), with normal size alleles (30 and 37 CGG) detected in blood, BEC, and saliva by multiple PCR assays (Fig 2). These normal size alleles were below the analytical sensitivity of Southern blot at approximately 20% [Aliaga and others 2016]. However, the 37 CGG allele was passed on to one of his daughters (III:2) and was also detected in one of her daughters (IV:3).

The origin of this retracted 37 CGG allele showing low level mosaicism has clinical significance. After initial testing showed a normal allele in the daughter of a father who carried a PM, having ruled out non-paternity, the result raised concern that she might also carry a cryptic paternally-inherited PM allele, presenting a risk of expansion in her children. Whilst this allele was in the normal range, knowing it had arisen from an unstable PM allele led to caution about assuming it would be stably transmitted to her offspring. Follow-up testing alleviated these concerns, as no alleles larger

than 37 CGGs were detected in three different tissues with three different assays in III:2 and her offspring (Suppl Fig 2). The detection of the 37 CGG allele in II:3 pointed to the retraction having occurred post-zygotically.

This finding supports the view that PM retractions observed in males occur in mitosis rather than meiosis [Nolin and others 2019], and is the first report of a daughter of a male PM carrier having a normal sized allele. The origin of the FM and the three different PM alleles (76, 136, 157 CGGs) in III:4 is less clear. Since none of these repeat sizes are the same as in her father, it is possible that meiotic instability may have occurred. However, previous reports of males with PM alleles having daughters with a FM have suggested that these expansions have occurred post-zygotically in the daughters [Alvarez-Mora and others 2017; Zeesman and others 2004], providing a possible explanation for the larger PM and FM results in III:4. Another possibility is that II:3 also carries a mosaic FM allele that was not detected because it was either not present in the tissues tested, or was present at a level below the analytical sensitivity of the AmplideX and X Sense assays (Fig 2). These normal size alleles may have originated from a post-zygotic retraction of a FM allele that was undetected in the tissues tested in II:3. This hypothesis also provides a potential explanation for all of the allele sizes detected in III:4, one of the daughters of II:3, who had a mosaic FM allele of low abundance detected in multiple tissues using different techniques (Suppl Fig 3).

FMR1 methylation and mRNA analyses suggested that retracted alleles are fully functional

AmplideX mPCR testing of II:3 revealed that the 105 CGG allele was unmethylated, while methylation status for both retracted normal size alleles of 30 and 37 CGGs could not be ascertained due to low abundance. The 37 CGG allele from the father (II:3) was transmitted through two further generations in an unmethylated state, consistent with methylation being reset at gametogenesis. The *FMR1* mRNA levels were elevated in II:3 compared to controls and FM males (Suppl Fig 4A) and were within the range for PM males [Baker and others 2020; Field and others 2019]. The elevated *FMR1* mRNA level is consistent with the diagnosis of FXTAS in this male. In his daughter III:2, analyses of *FMR1* mRNA in PBMCs and *FMR1* methylation in three different tissues indicate that the paternally transmitted 37 CGG allele is fully functional consistent with her lack of phenotype. In contrast, the female clinically affected with FXS, II:1, had increased methylation of FREE2 (Suppl Fig 4B), as observed in FXS females with ID [Inaba and others 2014].

The benefits of comprehensive testing in unusual fragile X cases

The II:3 case highlights the postzygotic mitotic instability of PM and FM alleles resulting in mosaicism. II:3 carried a very high degree of somatic mosaicism that was not only present in the

analysed tissues (PBMCs, saliva, and buccal epithelial cells), but must also involve the germline since different alleles are present in his two daughters. Unfortunately, the sperm of this male, could not be collected to directly confirm this mosaicism in germline. Previously, paternally transmitted PM alleles have been reported to increase by small increments during meiosis, but there has been no report of a paternal PM expanding to FM alleles [Nolin and others 2015]. In contrast, maternally transmitted PM alleles frequently expand to FM size, but can also be subject to large retractions to normal or GZ allele size [Prawer and others 2018; Stark and others 2015; Tabolacci and others 2020]. This retraction phenomenon is not fully understood, but may result from excision of the expanded alleles in the early post-zygotic period, resulting in somatic mosaicism. Mosaicism may be variable between tissues, and may not be detected if only one tissue is analysed [Stark and others 2015] and/or if the proportion of the mosaic alleles is below the detection limit of the assay used [Aliaga and others 2016; Hensel and others 2019]. In this study this was observed for III:4, where the mosaic FM that was initially missed by Southern blot and AmplideX mPCR, but was identified as part of the follow-up testing using AmplideX TP-PCR and X Sense assays, different tissues (Suppl Fig 3). This study thus emphasises the value of using different assays and testing different cell types to understand the molecular mechanisms behind unusual cases and challenges the notion that males with PM will always have daughters with similar PM alleles. The study also highlights the importance of considering the possibility of post-zygotic retraction of paternal PM alleles down to normal size or expansion to FM and mosaicism of *FMR1* alleles when providing genetic counselling.

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Conflict of Interest

David E. Godler is named as an inventor on patent applications (PCT/AU2010/000169 and PCT/AU2014/00004) related to the technology described in this article. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

EB, AA, DJA and DEG contributed to the conception and design of the study, data acquisition, data analysis and interpretation of data. EB, AA, DF, LL, RT, GC, AP, MBD, EB, DJA, MB, EKB, CH, KS, MS, GH, KF, BB and DEG conducted data analysis and interpretation of the data. DEG, EB, DJA, AA and MD all contributed to patient recruitment/acquisition of data. All authors have been involved in the drafting of the manuscript and/or revising it critically for important intellectual content and have read and approved the final manuscript. All authors had complete access to the study data that support the publication.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figure Legends

Figure 1. (A) Pedigree of the studied family. CGG sizes are included in square brackets, with expanded alleles in bold. The proband is highlighted by an arrow. **(B)** Southern blot analysis of saliva (SL) and buccal epithelial cell (BEC) DNA from II:3 PM male affected with FXTAS and II:1 FM female affected with FXS. Note: There was insufficient amount of DNA available form blood to perform Southern blot analysis for I:2, II:1 and II:3. **(C)** Southern blot analysis of SL, BEC and blood (BL) DNA from III:2 with a 37 CGG allele resulting from retraction of a 102 CGGs in II:3; III:4 PM carrier female with a 157 CGG allele resulting from expansion of a 102 CGGs in II:3 in all tissues; and a FM allele only in saliva. Note: MC: male control (30 CGGs); numbers next to each blot indicate CGG sizes of corresponding bands.

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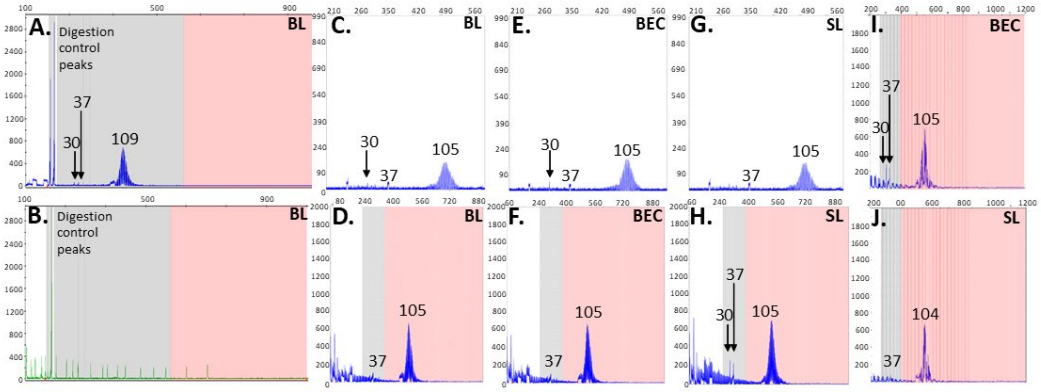
Figure 2. Amplidex mPCR, standard CGG sizing PCR, AmplideX and X Sense CGG screening assay profiles from blood, buccal epithelial cell (BEC) and saliva DNA for PM male II:3. AmplideX mPCR in blood (BL) DNA targeting methylation of two *HpaII* sites, on either side of the CGG expansion **(A)** HEX and **(B)** FAM channels from capillary electrophoresis. Note: pink background indicates region of >200 CGG repeats where presence of positive FM alleles with methylated *HpaII* sites and the control digestion (*HpaII* methylation independent) was detected. Number in **(A)** indicate CGG sizes, while numbers in **(B)** indication % methylation (based on *HpaII* digestion) of the corresponding 30, 37 and 109 CGG alleles. **(C)** and **(D)** standard CGG sizing PCR and AmplideX screening assay capillary electrophoresis profiles in blood DNA with numbers indicating CGG sizes of the 3 alleles. **(E)** and **(F)** standard CGG sizing PCR and AmplideX screening assay capillary electrophoresis profiles in buccal epithelial cell (BEC) DNA with numbers indicating CGG sizes of the 3 alleles. **(G)** and **(H)** standard CGG sizing PCR and AmplideX screening assay capillary electrophoresis profiles in sa) X Sense TP-PCR assay capillary electrophoresis profiles in BEC and saliva DNA with numbers indicating CGG sizes of the 3 alleles. Note: Y axis Fluorescence Units (FU); X axis fragment size (bp). It is important to note that the mPCR results used in this study were from the AmplideX mPCR assay, utilizing a commercial kit, with all quality control procedures performed as per manufacturer's instructions (Asuragen, Inc., Austin, TX, U.S.A.). Specifically, while through visual inspection II:3 in Fig2 A and B has low height peaks for 30 and 37 CGG alleles relative to the 109 CGG allele; all peaks called had >50 fluorescence units (above background levels), with all digestion control peaks for all samples analysed were present, showing >90% digestion.

Retraction of a FXS allele to normal size.

Fig 2.



Retraction of a FXS allele to normal size.

Fig 1.

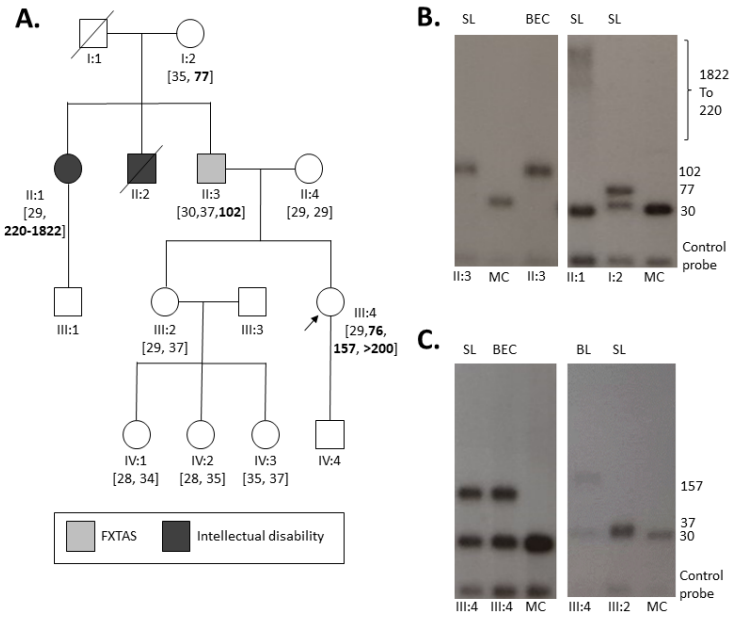
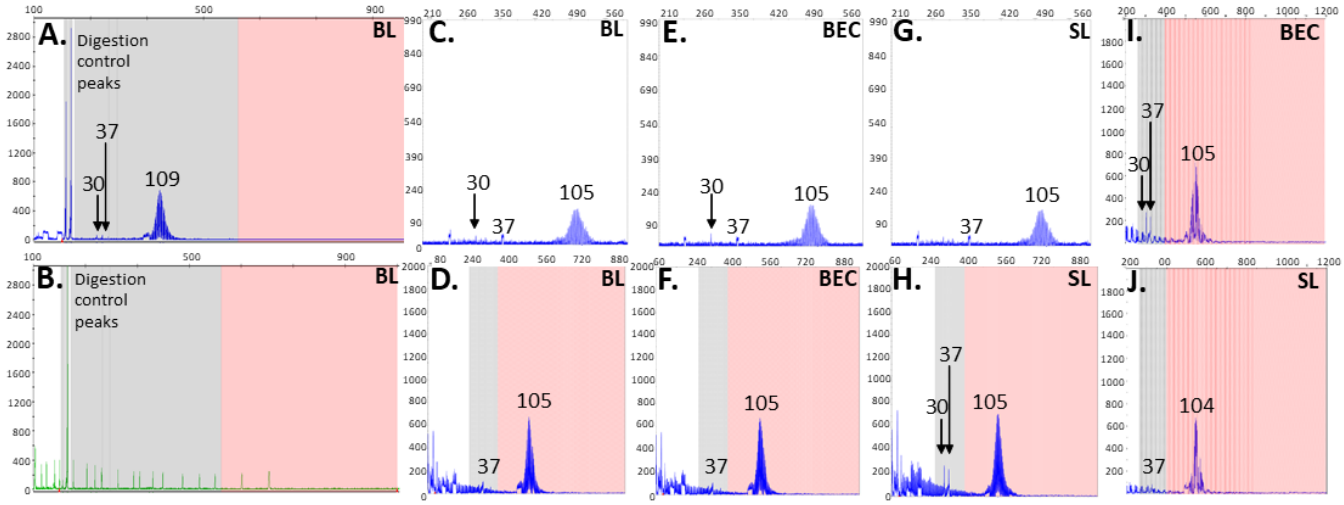
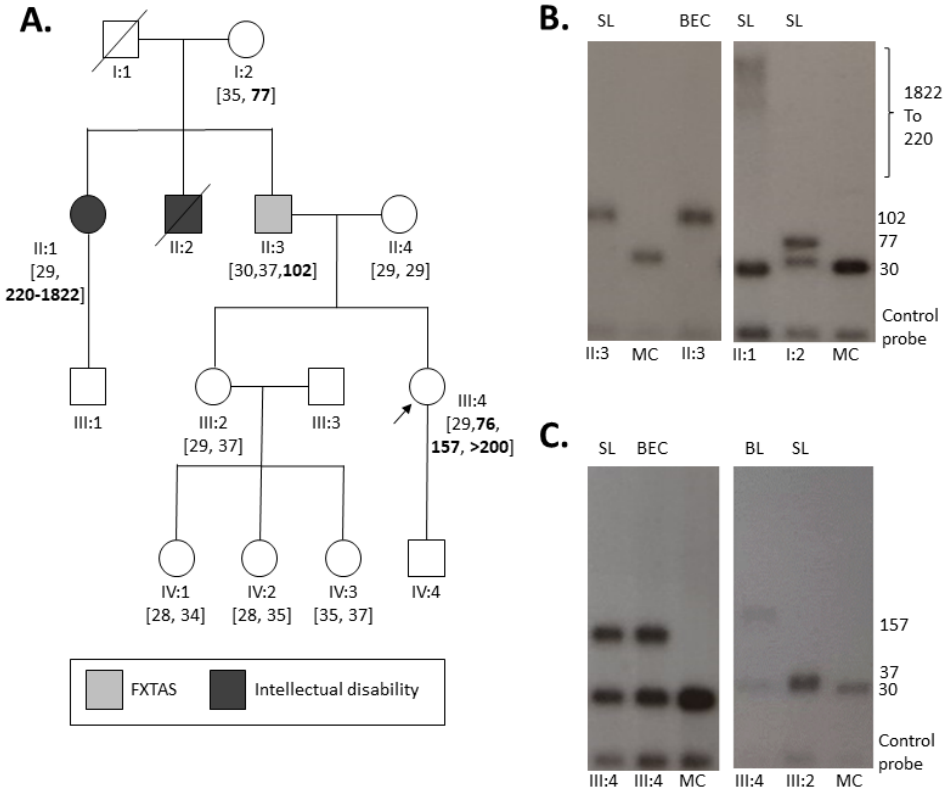


Fig 2.



AJMGA_62500_Fig 2.TIF

Fig 1.



AJMGA_62500_Slide1.TIF