EXOME REPORT: Two novel intragenic variants in the *FMR1* gene in patients with suspect clinical diagnosis of Fragile X Syndrome and no CGG repeat expansion.

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1. Abstract.

The major and most well-studied genetic cause of Fragile-X syndrome (FXS) is expansion of a CGG repeat in the 5'-UTR of the *FMR1* gene. Routine testing for this expansion is performed globally. Overall there is a paucity of intragenic variants explaining FXS, a fact which is being addressed by a more systematic application of whole exome (WES) and whole genome (WGS) sequencing, even in the diagnostic setting. Here we report two families comprising probands with a clinical suspicion of FXS and no CGG repeat expansions. Using WES we identified deleterious variants within the coding region of *FMR1* in both families. In a family from Finland we identified a complex indel c.1021-1028delinsTATTGG in exon 11 of *FMR1* which gives rise to a premature termination codon, p.Asn341Tyrfs*7. Follow-up mRNA and protein studies on a cell line from the proband revealed that although the mRNA levels of *FMR1* were not altered, a complete loss of the Fragile X Mental Retardation 1 Protein (FMRP) was consequent. Additionally, we identified a variant, c.881-1G>T, affecting a canonical acceptor splice site of exon 10 of *FMR1* in an Australian family. Our findings reinforce the importance of intragenic *FMR1* variant testing particularly in cases with clinical features of FXS and no CGG repeat expansions identified.

2. Introduction.

Fragile X Syndrome (FXS) is the leading cause of X-linked intellectual disability / autism spectrum disorder. It is primarily caused by an expansion of the CGG trinucleotide repeat sequence in the 5'-UTR of the *FMR1* gene from 5-40 repeats in healthy individuals, to >200 repeats in affected individuals [Hagerman et al., 2017]. As a result of this expansion, the *FMR1* gene is methylated and its transcription is silenced, resulting in the absence of FMRP. In addition, pre-mutation carriers with CGG repeats between 55-199 have also been observed to experience disease; males most commonly present with Fragile X Associated Tremor and Ataxia Syndrome (FXTAS) and females present with significantly higher rates of Fragile X Primary Ovarian Insufficiency (FXPOI) [Sherman, 2000, Hall and Berry-Kravis, 2018]. Pre-mutation carriers have generally increased *FMR1* mRNA, and decreased FMRP, levels with the molecular pathogenesis still to be fully understood [Boivin et al., 2018].

The global prevalence of FXS is estimated to be around 1:5000 males and around 1:4000-1:8000 females [Hagerman et al., 2017], with females generally experiencing milder cognitive deficits [Bartholomay et al., 2019]. Given the high prevalence of FXS in the general population, diagnostic testing of the CGG repeat expansion is routinely performed in any individual presenting with developmental delay. The contribution of intragenic *FMR1* mutations has not been routinely investigated until recent years, with the availability of next generation sequencing methodologies. While it had been previously reported that intragenic mutations of *FMR1* were unlikely to contribute to ID cases in any amount greater than expected for the gene's size [Quartier et al., 2017], there are nonetheless a growing number of reported cases of *FMR1* intragenic mutations leading to clinical phenotypes that overlap with

classical FXS. Here, we report two novel cases with intragenic *FMR1* mutations in association with an FXS-like phenotype, further reinforcing the notion that *FMR1* coding sequence analysis should routinely be undertaken in cases where CGG repeat expansion testing is negative and a diagnosis of FXS is suspected.

3. Clinical description and Previous Investigations

Family 1, Individuals II:1 and II:3 (Figure 1a) are two male siblings with severe ID. Individual II:1, aged 79 years, has prominent ears (Supplementary Figure 1a) but no macroorchidism. His adult height is 185 cm. His brain CT is normal. At the age of 29 years his mental age was 3 years 6 months. At the age of 50 and again at the age of 79 his mental age was 1 year 9 months. He walks independently and his speech is clear. He is friendly, social and active in communication. He has no signs of dementia. He has cardiac flutter, and is on acetylic salicylic acid and beta-blocker medication. At the age of 68 he had one epileptic seizure, with EEG showing generalized slowing. He is presently medicated for prostate hyperplasia, hypertension and anxiety. He is living in a nursing home for disabled individuals and needs help with hygiene and household tasks. Fragile site testing was normal with the *FMR1* CGG expansion excluded. Array-CGH analysis was also negative. Patient II:3 had a quite similar phenotype. At the age of 24 years his mental age was 2 years 10 months and at the age of 39 years 3 years 1 month. His speech was unclear and he showed mild autistic features. He died suddenly at the age of 50 years. The mother is known to have had epilepsy. Siblings II:2 (aged 70 years), II:4 (aged 66 years) and II:5 (aged 65 years) are healthy and without epilepsy.

Family 2, (Figure 1b) belong to a collection of ID patients that were analysed using whole genome sequencing techniques (Jozef Gecz, personal communication). The index case in Family 2 is a 20 year old male with significant global developmental delay (IQ 42), some autistic features (poor eye contact and avoidant behaviour - not formally diagnosed) and subtle dysmorphic features (Supplementary Figure 1b). When initially examined at age 14 ½ years he was relatively large (90% for all growth parameters), with a long face, large nose, short philtrum, deep set eyes, high arched palate, prominent jaw, large ears (>>97%), mild – moderate hirsutism of the back and prominent foetal finger pads. Testes were reportedly large but formal genital examination was not undertaken. To our knowledge, the mother is healthy. Mother's XCI show significant skewing (1:99). There is no significant FHx. The perinatal history is significant for a long (18 hour) labour following induction at 41 weeks and fetal distress with meconium liquor resulting in an emergency caesarean section. There were no significant neonatal concerns apart from difficulty breastfeeding.

4. Methods.

4.1. Ethical Statement

The study was approved by Ethical Committee of Helsinki University Hospital, and the Women's and Children's Health Network Human Research Ethics Committee in Adelaide. Written informed consent was obtained from the participating families.

4.2. Whole Exome Sequencing (WES) / Whole Genome Sequencing (WGS)

DNA (genomic) from individuals was extracted from whole-blood (QIAamp DNA blood maxi kit; Qiagen, Limburg, Netherlands) and screened by either WES or WGS.

DNA from individual III:1 from Family 1, was Whole Exome Sequenced (WES) (Agilent SureSelect v6) on a HiSeq2500 (Illumina) by the Australian Genome Research Facility. Reads were mapped to the human genome (Hg19) using BWA-MEM [Li and Durbin, 2009] and mapping refined using Genome Analysis Toolkit (GATK) version 3.5 [DePristo et al., 2011]. Mapping achieved a minimum median target coverage depth of 48 reads/sample and covered 87.5% of intended targets with at least 20 reads.

Single nucleotide variants and small insertions and deletions were called by the GATK haplotype caller version 3.5 [DePristo et al., 2011]. Larger copy number and structural variants were analysed by CoNIFER [Krumm et al., 2012]; however, no segregating, rare copy number variants were detected. All variants were annotated for allele frequency, clinical significance, locus identity, and likely pathogenicity using ANNOVAR [Wang et al., 2010]. Novel genotypes shared between affected individuals, but absent from a control set of 5 exomes, were separated using the vcf-contrast module from VCFtools [Danecek et al., 2011].

DNA from the proband of Family 2, II:1, was Whole Genome Sequenced (WGS) on an Illumina HiSeq2500 by the Australian Genome Research Facility. Reads were mapped to the human genome (hg19) using BWA-MEM [Li and Durbin, 2009] and mapping refined using Genome Analysis Toolkit (GATK) version 3.5 [DePristo, et al., 2011]. Mapping achieved a minimum median target coverage depth of 49.5 reads/sample and covered 98% of intended targets with at least 20 reads. Single nucleotide variants and small insertions and deletions were called by the GATK haplotype caller version 3.5 [DePristo, et al., 2011]. All variants were annotated for allele frequency, clinical significance, locus identity, and likely pathogenicity using ANNOVAR [Wang, et al., 2010].

4.3. Variant Validation by Sanger Sequencing

Sanger sequencing using standard methods was used to validate and segregate the identified variants in genomic DNA in both families. Total RNA was isolated using Maxwell RSC simply RNA Cells Kit on a Maxwell RSC Instrument (Promega, Wisconsin, USA), cDNA generation and PCR was carried out as per standard methods, and sequencing was also undertaken to confirm expression and structure of *FMR1* transcripts identified from affected and control (WT) male LCL cDNA in Family 1. Additional transcript analyses on Family 2 were not possible due to the unavailability of patient material. Primer sequences are listed in Supplementary Table 2.

4.4. Cell Culture and MG132 assay

LCL was established for individual II:1 from Family 1 (Figure 1a). Peripheral blood lymphocytes were isolated and transformed with Epstein Barr Virus using standard protocols [Neitzel, 1986]. Established cultures were grown in RPMI-1640 medium (Sigma-Aldrich, Missouri, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific), 2 mM L-glutamine (Sigma-Aldrich), and 1 x penicillin streptomycin (Gibco; Thermo Fisher Scientific, Massachusetts, USA). Blood sample for LCL establishment was not available for Family 2.

For MG132 treatment, 8x10⁵ lymphoblastoid cells from Family 1, II:1 and three male WT controls were incubated with either 20 μ M MG132 or DMSO for 5 hours at 37 degrees, 5% CO₂. Cells were then harvested, washed in PBS, and snap frozen in dry ice for subsequent protein extraction and western blotting.

4.5. Western Blotting

Protein extracts were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% NP-40, 5 mM EDTA, 50 mM KCl) supplemented with protease inhibitor cocktail (Roche Applied Sciences, Penzberg, Germany) followed by sonication and centrifugation. 8 μ g protein was resolved on NuPage 4-12% Bis-Tris gels (Invitrogen; Thermo Fisher Scientific) and transferred to nitrocellulose membranes (BioTrace NT, Pall Life Sciences, New York, USA). All washes were performed in TBS-T and blocking was performed with 10% skim milk. Blots were probed with the following antibodies, prepared in 2% skim milk: FMRP mouse mAb 1C3, raised against the N-terminus exons 1-7, 1:1000; FMRP mouse pAb 1R, raised against the C-terminus spanning aa516-632 [Bonaccorso et al., 2015], 1:500; Beta Catenin (610153, BD Biosciences, New Jersey, USA), 1:500; and ACTB (AC-74 Sigma-Aldrich), 1:20000. Secondary antibody was goat anti-mouse immunoglobulins/HRP (P0447, Dako; Agilent technologies, California, USA), with chemiluminescent detection performed by Clarity Western ECL Substrate and imaging on a ChemiDoc MP Imaging System (Bio-Rad, California, USA).

5. Results

WES of proband II:1 (Family 1) revealed a complex insertion_deletion (indel) variant within exon 11 of *FMR1* resulting in a premature termination codon (PTC) 7 bp downstream (hg38: chrX:147937496-147937503delinsTATTGG, *FMR1* c.1021-1028delinsTATTGG; p.Asn341Tyrfs*7). Sanger sequencing confirmed the presence of this variant in the proband and his unaffected sister II:4. The variant was absent in his two unaffected brothers II:2 and II:5 (Supplementary Figure 2a).

FMR1 mRNA analysis of (Family 1) proband II:1-derived LCLs revealed mRNA levels similar to male controls (Figure 1b). Transcripts NM_002024.5 and NM_001185076.1 were also Sanger sequenced to confirm the presence of the indel (Figure 1c and Supplementary Figure 2c).

To investigate if a truncated FMRP protein product was created we performed western blot analysis on LCL lysates from affected male II:1 (Figure 2a). We used two different FMRP antibodies targeting either the FMRP N-terminus (exons 1-7, 1C3) or the C-terminus of the longest isoform (amino acids 516-632, 1R). We failed to detect FMRP using either antibody. To test whether a truncated FMRP was being made, but subsequently degraded, we blocked the proteosomal degradation pathway using MG132. However, we did not recover any detectable amount of the predicted, truncated FMRP protein (Figure 2b). In case deletion of exon 11 might play some role in the splicing or regulation of other *FMR1* exons, perhaps removal of the region corresponding to the epitope for these antibodies, and hence the subsequent absence protein product, we tested exons 1-7 in patient cDNA for aberrant splicing and absence. We observed no difference in transcript size between patient cDNA and normal controls (Supplementary Figure 3).

In proband II:1 (Family 2), we identified a *FMR1* splice mutation, which was also present in his mother. This variant (hg38: chrX:147936503, *FMR1* c.881-1G>T) affects the invariable acceptor splice site of exon 10 (Supplementary Figure 2b). No other family members were available for testing and a cell line for further functional studies was not available.

A summary of intragenic pathogenic variants, either classified as pathogenic in Clinvar (<https://www.ncbi.nlm.nih.gov/clinvar/>) or by published functional evidence, can be seen in Figure 4. Each of these lead to premature termination of FMRP either by nonsense or frameshift variation, or by the process aberrant splicing contained within *FMR1* coding exons.

6. Discussion.

We report two families with novel intragenic *FMR1* variants. The indel variant of Family 1, which affects exon 11 of the canonical isoform of *FMR1*, led to the introduction of a PTC within exon 11 of *FMR1*. Unexpectedly, the *FMR1* mRNA levels were not affected (Figure 1b and Figure1c) despite the PTC in exon 11 being more than 55 bp away from the next exon 11/exon 12 junction. This is one of the rare examples of an escape from nonsense mediated mRNA decay (NMD) degradation of such PTC mRNA. However, despite the *FMR1* PTC mRNA stability, no full length or truncated FMRP protein was detected (Figure 2a) or recovered when blocking proteasome degradation (Figure 2b).

The canonical acceptor splice site variant of exon 10 of *FMR1*, while novel, is similar in its predicted effect (skipping of exon 10) to previously described intragenic variants. Both Wang et al (1997) and Quartier et al (2017) have reported individuals with splicing mutations involving exon 10, both resulting in exon 10 skipping. These affected individuals also presented with a clinical diagnosis of FXS.

Two recent reports reviewed the current knowledge of intragenic *FMR1* disease-causing variants [Sitzmann et al., 2018, Quartier et al., 2017]. Our two cases, together with those presented so far amount to only six reported

protein truncating *FMR1* variants in males with a phenotype consistent with FXS [Gronskov et al, 2011, Okray et al., 2015, Quartier et al., 2017]. These intragenic variants also raise interesting questions as to the fundamental regulation of *FMR1* mRNA and protein. The indel of our Family 1 as well as at least some other variants, for example c.373delA p.Thr125Leufs*35 [Lugenbeel et al., 1995], c.990+1G>A p.Lys295Asnfs*11 and c.420-8A>G p.Met140Ilefs*3 [Quartier et al., 2017], all escape NMD of their respective PTC-containing *FMR1* mRNAs [Lugenbeel et al., 1995, Quartier et al., 2017]. Furthermore, in our case as well as in the case of Lugenbeel et al. no FMRP was identified despite the persistence of mRNA. Inhibition of proteosomal degradation by the addition of MG132 suggests that if any truncated FMRP is made, it is unlikely to be degraded by the proteosomal degradation pathway as was expected based on the previous studies [Choi et al., 2014, Hou et al., 2006].

Summary of intragenic pathogenic variants, (Figure 4) illustrates that nonsense and frameshift variants spans the length of the protein with all variants shown to lead to premature truncation of FMRP. Although we were unable to further study of the outcome of the acceptor splice site variant (hg38: chrX:147936503, *FMR1* c.881-1G>T) in Family 2 due to unavailability of patient material, this variant clusters with two other published variants (Figure 4) both of which have been shown to affect splicing of exon 10 [Wang et al., 1997, Quartier et al., 2017], and we predict that this variant will also affect the splicing of exon 10 and result in subsequent truncation of FMRP.

The growing number of clinically diagnosed or suspect FXS cases with intragenic *FMR1* disease-causing variation, now amounting to at least 8, does highlight the need for a more systematic *FMR1* sequence analysis in cases where the CGG repeat expansion testing is negative.

7. Facultative sections.

7.1. Acknowledgements.

Thank you to Saskia van der Velde-Visser and the Nijmegen group for establishing the LCL culture from affected male II:1, and thank you also to Dr. Barbara Bardoni for the gifted FMRP antibodies. Our thanks go to the families reported here for participating in this study.

7.2. Conflict of Interest statement.

Authors do not have any conflict of interest.

7.3. Appendix A. Supplementary data

Supplementary data related to this article can be found below.

8. Figure Headings

Figure 1. (a) Pedigree of a Finnish family (Family 1) with indel and Australian family (Family 2) with splice variant in the *FMR1* gene. Arrow indicates proband. *FMR1* genotype is indicated where DNA analysis was carried out. **(b)** RT-PCR of *FMR1* transcripts from Family 1 patient II.1 and normal (WT) male LCL cDNA controls. Primers were located in exons 10 and 12 of canonical isoform (NM_001185076.1), spanning exon 11 which contained the indel. **(c)** Sequencing chromatograms of *FMR1* canonical isoform transcript for WT male and patient II.1, showing location of indel and altered amino acid sequence resulting in PTC.

Figure 2. Western blot analyses of FMRP in Family 1. **(a)** FMRP was not detected in patient LCL lysates (black square) using two FMRP antibodies 1C3 and 1R, consistent with results observed in three full mutation FXS males (shaded squares). **(b)** Treatment with MG132 (addition indicated by + / -) did not recover any truncated FMRP protein in patient II.1 LCL lysate (black square), expression was not altered in control lysates (white squares). Beta-Catenin control protein (regulated by proteasome) increases upon treatment. Beta-Actin housekeeping gene included as loading control.

Figure 3. Schematic summary of pathogenic intragenic variation in FMRP. Variants span the length of the FMR protein and are all predicted or shown to lead to premature truncation of FMRP. Variants reported in this study can be seen in red text. Schematic is not to scale. Functional domain position from Specchia et al. [2019]. TD=Tudor/Agenet domain. NLS=Nuclear Localisation Signal. KH=RNA binding domain. NES=Nuclear export signal. RGG=RNA binding domain.

Supplementary Figure 1. Patient photographs of (a) proband from Finnish Family 1 and (b) proband from Australian Family 2.

Supplementary Figure 2. *FMR1* Sanger sequencing from family members and wild-type control (WT) in (a) Family 1 with indel c.1021-1028delinsTATTGG; p.Asn341Tyrfs*7. (b) Family 2 with splice variant c.881-1G>T. Arrow indicates nucleotide variant site. (c) Family 1 patient II.1 and male control (WT) LCL cDNA, indicates persistent wild type splicing for alternative isoform ENST00000621453.4, which does not contain the exon harbouring the indel.

Supplementary Figure 3. Agarose gel image showing PCR to amplify *FMR1* cDNA from exons 1-7, FMRP antibody containing region, to check for aberrant splicing. No difference in transcript size was observed between patient cDNA (black square) and normal control (white square). *ESTD* = Esterase D housekeeping gene included as quality control.

9. Tables

Supplementary Table 1: Oligo sequences

Oligo	Sequence (5'-3')
FMR1_gDNA F	GGGTAGAGAGGTGAGGATGC
FMR1_gDNA R	ACCAATTGTGACATCTGAGACA
FMR1_exon 10 F	TGGACAAGTCAGGAGTTGTGAG
FMR1_exon 12 R	AGTGGCATTAGCGATGCTGT
FMR1.ex10splice-F	GGCTGTGCTTACTGCTTTGAG
FMR1.ex10splice-R	GCCACAAAATATTCGCACTGT
FMR1 ex1-F	CGCTAGCAGGGCTGAAGAGAA
FMR1 ex7-R	ATGTGCTCGCTTTGAGGTGA
EstDF	GGAGCTTCCCCAACTCATAAATGCC
EstDR	GCATGATGTCTGATGTGGTCAGTAA

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(a)

(b)

Nonsense/
Frameshift
Leading to
Premature
Termination

Exonic
Leading to
Premature
Termination

(a)

(b)

(a)

(b)

(c)

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SUOSTUMUS KUVAMATERIAALIN KÄYTÖSTÄ TIETEELLISESSÄ ARTIKKELISSA

Annan suostumukseni siihen, että ^{valokuvaani} lapseni tai edunvalvonnassani olevan

Nimi Raimo Ovaska

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valokuvaa saa käyttää tieteellisessä artikkelissa ilman henkilötietoja.

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RAIMO OVASKA

Suostumuksen antajan allekirjoitus ja nimen selvennös

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29.4.2019

Lahti

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Kirsä Töyrölä

suostumus kuvata nimen käyttöä tieteellisessä
artikkelissa

Antan suostumuseni siihen, että lapseni tai edustajani nimen olevan

Nimi Raimo Ovaska

henkilötunnus 230670-505V

valokuva saa käyttää tieteellisessä artikkelissa ilman henkilötietoja.

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1. As a record for the genetics chart. ☒
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3. To be used for educational purposes. ☒
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