De Novo Large Deletion Leading to Fragile X Syndrome

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Fragile X syndrome (FXS) is the most frequent cause of X-linked inherited intellectual disabilities (ID) and the most frequent monogenic form of autism spectrum disorders. It is caused by an expansion of a CGG trinucleotide repeat located in the 5'UTR of the FMR1 gene, resulting in the absence of the fragile X mental retardation protein, FMRP. Other mechanisms such as deletions or point mutations of the FMR1 gene have been described and account for approximately 1% of individuals with FXS. Here, we report a 7-year-old boy with FXS with a de novo deletion of approximately 1.1 Mb encompassing several genes, including the FMR1 and the ASFMR1 genes, and several miRNAs, whose lack of function could result in the observed proband phenotypes. In addition, we also demonstrate that FMR4 completely overlaps with ASFMR1, and there are no sequencing differences between both transcripts (i.e., ASFMR1/FMR4 throughout the article).

Keywords: fragile X syndrome, FMR1 gene, miRNA, ASFMR1/FMR4 gene, large deletion

INTRODUCTION

Fragile X syndrome (FXS) is the most prevailing cause of X-linked inherited intellectual disability and autism spectrum disorder (Penagarikano et al., 2007; Rogers et al., 2001). It is caused by a CGG trinucleotide repeat expansion within the 5' UTR region of the fragile X mental retardation 1 (FMR1) gene located on chromosome Xq27.3, which spans approximately 38 kb of genomic sequence (Verkerk et al., 1991; Yu et al., 1991; Fu et al., 1991; Bell et al., 1991). The CGG repeats expand in the female germline during transmission. When the CGG expands to more than 200 repeats, the so-called full mutation, the promoter of FMR1 becomes hypermethylated and transcriptionally silent, leading to gene inactivation and consequently diminished or lack of expression of the FMR1 protein, FMRP (Fu et al., 1991; Tassone et al., 2000)(Figure 1A). Individuals with the fragile X premutation (55-200 CGG repeats) are at risk of developing a number of clinical problems falling under the umbrella of FMR1-associated disorders. Male individuals with FXS have moderate to severe intellectual impairment, and a behavioral phenotype characterized by repetitive behaviors, social difficulties, poor eye contact, excessive shyness, anxiety, aggression, tactile defensiveness, hyperarousal response to sensory stimuli, tantrums, attention deficits, hyperactivity, impulsivity, self-injury, stereotypic movements including hand flapping, and perseverative speech. Physical manifestations range from the large forehead, prominent ears, hyperextensible finger joints, and macroorchidism (Hagerman and Hagerman, 2002; Hatton et al., 2002; Reiss and Dant, 2003; Hagerman et al., 2009; Symons et al., 2010; Kidd et al., 2014).
Although the CGG repeat expansion is the main underlying cause of FXS, other mechanisms, including point mutations or deletions, can lead to FXS (Handt et al., 2014; Monaghan et al., 2013; Quartier et al., 2017; Sitzmann et al., 2018) (Figures 1B,C). The deletions in FXS individuals commonly range from hundreds to several millions of base pairs and frequently encompass a portion or the entire sequence of the \textit{FMR1} gene (Coffee et al., 2008). Several reports during the last 3 decades demonstrated \textit{de novo} deletions (Tarleton et al., 1993; Gu et al., 1994; Petek et al., 1999; Luo et al., 2014; Zink et al., 2014; Luo et al., 2014; Zink et al., 2014; Jorge et al., 2018; Myers et al., 2019) spanning the \textit{FMR1} or both the \textit{FMR1} and \textit{FMR2} gene (Clarke et al., 1992; Wolff et al., 1997; Fengler et al., 2002; Probst et al., 2007), resulting in the loss of FMRP and a range of phenotypes including physical, cognitive and behavioral features, intellectual disabilities (ID), seizures, and obesity, similarly observed in individuals with FXS (Coffee et al., 2008).

The human genome is extensively transcribed and gives rise to various long non-coding RNAs (lncRNAs), defined as RNAs longer than 200 nucleotides and not translated into functional proteins (Fang et al., 2018). Approximately 40% of mammalian lncRNAs are expressed in the brain (Briggs et al., 2015) and play an essential role in neuronal differentiation and regeneration (Perry et al., 2018). They regulate significant biological roles in DNA damage response and cellular senescence (Tsai et al., 2010; Hung et al., 2011; Kotake et al., 2011; Pastori et al., 2015) through modulation of gene expression regulation. In addition, several lncRNAs play a role in brain development, synaptic signaling mechanisms, differentiation of neural cell lineage, and formation of mature neuronal connections (Mercer et al., 2010; Qureshi et al., 2010). Altered expression of lncRNAs has been associated with neurodevelopmental disorders like Prader–Willi syndrome (Statello et al., 2021) and neurodegenerative disorders such as Parkinson’s disease, Huntington’s disease, lateral amyotrophic sclerosis, or Alzheimer’s disease (Wei et al., 2018).

A comprehensive analysis of the transcriptional landscape of the human \textit{FMR1} gene, discovered a decade ago, the \textit{ASFMRI} gene, an lncRNA, a unique antisense transcript, overlapping the CGG repeat region at the \textit{FMR1} locus. Its expression is determined by two promoters that are flanked by CTCF-binding sites: the \textit{FMR1} bidirectional promoter and the other one located in the second intron of the \textit{FMR1} gene, which is the major promoter in premutation cells (Ladd et al., 2007). The \textit{ASFMRI} mRNA, similarly to the \textit{FMR1}, is upregulated in lymphoblastoid cells and peripheral blood leukocytes derived from individuals with the premutation, and it is not expressed in subjects with the \textit{FMR1} full mutation (Ladd et al., 2007; Loesch et al., 2007).
et al., 2011). In addition, the ASFMRI1 transcript demonstrates premutation-specific alternative splicing suggesting a potential contribution of bidirectional transcription to the clinical phenotypes of the FMR1-associated disorders (Al Olaby et al., 2018; Vittal et al., 2018; Zafarullah et al., 2020). Additional studies have revealed the presence of several lncRNAs within the FMR1 gene, including, FMR4 (Khalil et al., 2008), FMR5, and FMR6 (Pastori et al., 2014). Like the FMR1 mRNA, FMR4, a primate-specific non-coding RNA transcript (~2.4 kb) residing upstream and sharing a bidirectional promoter with FMR1, is silenced in patients with FXS and upregulated in carriers of an FMR1 premutation allele. It was reported that FMR4 has an antiapoptotic function in HEK293T and HeLa cells but no effect on FMR1 gene regulation, suggesting a potential indirect regulatory transcript for FMR1 (Khalil et al., 2008).

Furthermore, numerous studies have indicated that FMRP acts as a mediator effector of the microRNA (miRNA) pathway by interacting with miRNA and proteins to form RNA-induced silencing complex (RISC) (Caudy et al., 2002; Ishizuka et al., 2002; Caudy et al., 2002; Smalheiser and Lugli, 2009; Dionne and Corbin, 2021). Thus, the lack of FMRP in FXS and its role in the miRNA pathway contribute to the abnormal synaptogenesis in FXS and provide one of the mechanisms underlying the fragile X physiopathology (Edbauer et al., 2010; Gong et al., 2015; DeMarco et al., 2019).

Here, we report an FXS case of a seven-year-old boy with a de novo deletion of chromosome X of approximately 1.1 Mb encompassing several genes, including the FMR1 and ASFMRI1 genes and the miR-506 family of miRNAs (Figure 1B). Their absence is likely related to neurodevelopment and his FXS phenotypes. Interestingly, we also demonstrated that FMR4 completely overlaps with ASFMRI1, and there are no sequencing differences between both transcripts, therefore named in this study as ASFMRI1/FMR4 gene. The characterization of the ASFMRI1 gene, using SMRT sequencing, identified ~6-kb ASFMRI1 transcript that overlaps with FMR4, demonstrating that FMR4 and ASFMRI1 are the same lncRNA (ASFMRI1/FMR4).

MATERIALS AND METHODS

DNA Testing: CGG Sizing

The genomic DNA of the proband, mother, and sisters was isolated from peripheral blood leukocytes (3-5 ml of whole blood) using standard methods (Puregene Kit; Gentra Inc, Minneapolis, MN). PCR and Southern blot analysis were performed to determine the CGG sizing and methylation status. For Southern blot analysis, 5-10 μg of DNA digested with EcoRI and NruI fixed on a nylon membrane was hybridized with the FMR1 genomic probe StBl2.3, labeled with Dig-11-dUTP by PCR (PCR Dig Synthesis Kit; Roche Diagnostics) following the protocol as previously described (Tassone et al., 2008; Filipovic-Sadic et al., 2010).

Chromosomal Microarray

CMA was carried out on the proband using GeneChip auto scan 750 K (Affymetrix Santa Clara, United States) following the manufacturer’s instructions. A chromosomal analysis was performed using Chromosome Analysis Suite (ChAS®) software (Affymetrix, Santa Clara, United States). The CNVs found in the patient were analyzed in comparison with public databases, including Database of Genomic Variants (DGV), Database of Chromosomal Imbalance and Phenotype in Humans using Ensemble Resources (DECIPHER), and CytoScanHD™ Array Database. Furthermore, CNVs were classified according to their nature, based on (Miller et al., 2010; Battaglia et al., 2013). After the initial Array-CGH result was acquired, informed written consent was obtained from the patient’s mother to draw blood on all available family members for additional investigation. The blood samples were obtained from the patient, his mother, his sisters, and his maternal uncle for further analysis.

Sequencing of the ASFMRI1 mRNA

The total RNA was isolated from postmortem brains derived from three normal individuals in a clean and RNA designated area using TRIzol reagent (Invitrogen, Carlsbad, CA), followed by quantification via a Qubit fluorometer (Invitrogen, Waltham, MA, United States) and quality control by measurements of the RNA integrity number utilizing an Agilent 2100 Bioanalyzer system.

The genomic sequence data of 2198-bp FMR4 at locus FJ887036 and 2942-bp ASFMRI1 at locus EU048204 were obtained from the UCSC website (http://genome.ucsc.edu/). The ASFMRI1 transcripts were identified by PacBio iso-sequencing from isolated total RNA from three brain samples. Then 1 μg of total RNA was reverse transcribed using the Clontech SMARTer cDNA synthesis kit and six samples specific barcoded oligo dT (with PacBio 16mer barcode sequences). Three reverse transcription (RT) reactions were processed in parallel for each sample. PCR optimization was used to determine the optimal amplification cycle number for the downstream large-scale PCRs. A single primer (primer IIA from the Clontech SMARTer kit 5′- AAG CAG TGG TAT CAA CGC -3′) was used for all PCRs post-RT. Large-scale PCR products were purified separately with 1X AMPure PB beads, and the bioanalyzer was used for QC. An equimolar two pools of 3-plex barcoded cDNA library were input into the probe-based capture with a custom-designed ASFMRI1 gene panel. A SMRTBell library was constructed using captured and reamplified cDNA. One SMRT Cell 1M (20 h movie) was sequenced on the PacBio Sequel platform using 2.0 chemistry. The isofrom sequencing analysis was performed using the IsoSeq3 application in the PacBio SMRT Analysis v6.0.0.

RESULTS

Clinical History

The patient we present in this study is a 7-year-old boy with moderate intellectual disability (IQ = 41, WISC-V), attention deficit, and hyperactivity. He was a full-term male infant and third child of nonconsanguineous parents, born after a normal pregnancy. His birth weight was 4,500 g, and there were no
perinatal problems. His developmental milestones included sitting at 6 months, walking at 21 months, using words at 17 months, and phrases by 3.5 years of age.

On physical examination at age 7 years, his weight was 29 kg (92nd percentile), height was 125 cm (45th percentile), and head circumference was 52.5 cm (64th percentile). The HEENT (head, eyes, ears, nose, and throat) examination was normal. The neurological examination findings were normal. Other characteristics included a long face, prominent forehead, large and protruding ears, normal palate, long palpebral fissures, epicanthal folds, flat nasal bridge, pectus excavatum, macroorchidism, and no joint hypermobility. There are no cardiovascular abnormalities, and echocardiography showed normal results. Overall, the patient is physically a well-developed boy with no significant anomalies.

He hears normally and follows simple commands. He has an acceptable motor delay and deficits in visual analyses and synthesis. He can eat independently regarding daily living skills and uses the toilet but cannot get dressed without help. The child exhibits hand biting and hand flapping when excited, his mood is generally good, and he shows no aggressive behavior toward others but sometimes he is very anxious. The parents noted excessive shyness, and he consistently avoids eye contact by covering his eyes with his hands, although he is exceptionally social and can interact with others. His early behavior included ADHD. He is frequently irritable and hyperactive at home, exhibits concentration problems, short attention span, distractibility, and impulsiveness. He has learning problems, especially difficulty with mathematics.

The proband has a healthy mother and healthy 16 and 14-years old sisters; the maternal uncle has mild to moderate intellectual disability, while the maternal aunt has depressive disorders and ovarian cysts (Figure 2).

**Molecular Testing: CGH Array and FXS Diagnostic Testing**

At the age of 7 years, the proband underwent array-CGH on DNA extracted from peripheral blood cells (244 K kit, Agilent Technologies, Santa Clara, CA, United States), which revealed a deletion of approximately 1.1 Mb located on Xq27.3 (NC_000023.10: g 145,877,075-147,047,871) encompassing several genes and miRNAs, including the entire FMR1 and ASFMR1/FMR4 genes and microRNAs (miRNAs) such miR506, miR508, miR509-1, miR509-3, and miR510. Molecular DNA testing for FXS, carried out by PCR and Southern blot analysis, showed no PCR amplification and lack of hybridization with the FMR1 specific probe in the proband, indicating the presence of a deletion of the FMR1 gene (Supplementary Figure S1). The mother was not a carrier of the FMR1 premutation as determined by Southern Blot and PCR analysis.

**The lncRNA, ASFMR1, and FMR4 Are the Same Transcripts**

The sequence identity of ASFMR1/FMR4 using the Integrative Genomic Viewer (IGV) was obtained by blasting the transcriptomic sequences reported in the UCSC human genome database. Interestingly, we identify the ASFMR1 transcript in our library data set, and by sequencing comparison, we confirmed that no genetic sequence...
differences exist between the two transcripts (Figure 3A), as one of the hypotheses, proposed by Khalil et al. (2008). The complete sequence overlaps between the two genes (ASFMRI/FMR4) are shown in Figure 3B.

DISCUSSION

We report a case of FXS caused by a de novo deletion spanning a 1.1 Mb of DNA comprising the FMR1 and the ASEMR1 genes, and the miR-506 family of miRNAs. Importantly, we verified the overlap of the ASFMRI/FMR4 genes, previously identified which are therefore the same transcripts.

In 2007, 1 year prior to the discovery of the FMR4 lncRNA, Ladd (Ladd et al., 2007) identified the novel ASFMRI (antisense fragile X mental retardation 1) gene, highly expressed in the brain and kidney but hardly noticeable in the heart. Similarly, to the FMR1 gene, the novel ASFMRI transcript was upregulated in premutation alleles but repressed in full mutations and exhibited premutation-specific alternative splicing (Ladd et al., 2007; Zafarullah et al., 2020). One of the alternative splicing isoforms, ASFMRI mRNAs Iso131 bp, positively correlates with the CGG repeat number and could distinguish between carriers of the premutation and controls, and its expression levels in premutation carriers, with and without FXTAS, were significantly different from controls (Vittal et al., 2018; Zafarullah et al., 2020).

In 2008, Khalil and his colleagues reported on the identification of FMR4, a novel 2.4-kb lncRNA, which transcribed in an antisense direction upstream of the FMR1 gene. The authors showed that the FMR4 transcript was highly expressed in the heart and kidney of human fetal tissues and in human adult brains including the hippocampus, frontal cortex, and cerebellum. Thus, similarly to the FMR1 gene, the ASFMRI/FMR4 mRNA expression is silenced in the brain of full mutations but highly expressed in premutation carriers. In addition, ASFMRI/FMR4 demonstrated an antiapoptotic function in vitro suggesting it may promote human cell proliferation (Khalil et al., 2008). Further study revealed that the ASFMRI/FMR4 transcript suppresses MBD4 (methyl-CpG-binding domain protein 4), which is a translational repressor, pivotal for DNA mismatch repair, inhibition of transcription, and regulation of apoptosis, in trans-activity (Yakovlev et al., 2017). Interestingly, ASFMRI/FMR4 expression is significantly reduced, while both FMR1 and MBD4 expressions are increased, in differentiating human neural precursor cells proposing a role for ASFMRI/FMR4 as a gene-regulatory lncRNA in normal development (Peschansky et al., 2015). In addition, overexpression and silencing of ASFMRI/FMR4 can induce genome-wide alteration in histone methylation of several genes implicating developmental or neurophysiological roles (Peschansky et al., 2016). These findings suggest that ASFMRI/FMR4...
miR506, miR508, miR509-1, miR509-3, and miR510, all belonging to the "fragile-X miRNA (FX-MIR)" cluster (Ramaiah et al., 2019). miRNAs are crucial for various critical functions of cellular differentiation and homeostasis, as they regulate the expression of hundreds of mRNAs resulting in diverse pathologies, including cancer, neural disorders, and infertility (Greenberg and Soreq, 2014; Khazaie and Nasr Esfahani, 2014; Bracken et al., 2016). miRNAs are approximately 22-nucleotide-long small RNAs that regulate the gene expression at post-transcriptional levels by binding with target mRNAs like FMRP and Argonaute protein incorporated into the RNA-induced silencing complex (RISC) to silence their target mRNAs (Filipowicz et al., 2008; Edbauer et al., 2010; Santhekadur and Kumar, 2020). The FX-MIR is a member of an X chromosome-linked miRNA cluster initially determined in primate testis and found well conserved among placental mammals (Bentwich et al., 2005; Zhang et al., 2007; Li et al., 2010). The FX-MIR consists of 22 miRNAs, located and transcribed from five large clusters on the X chromosome and encompassing approximately 62-kb region. One of these clusters is adjacent to FMR1, which suggests the possibility that the FX-MIR miRNAs might target the FMR1 (Fromm et al., 2015; Wang et al., 2020). Most of them are primarily expressed in the human testis and brain, in which FMR1, their target, is also highly expressed in these two organs. Accordingly, the FX-MIR cluster may play a role in gene expression in both tissues since it can lead to silencing and the consequent absence of FMRP (Tamanini et al., 1997; Peier et al., 2000). Furthermore, the loss of function of FMRP demonstrated deterioration of synaptic formation and plasticity in the central nervous system and macroorchidism by overproduction of Sertoli cells in the testes (Slegtenhorst-Eegdeman et al., 1998; Terracciano et al., 2005). A subset of the FX-MIR cluster has increased expression levels significantly in FXS NPCs and neurons, in which miR-509-3p and miR-890 were upregulated by approximately 78-fold and 106-fold, respectively, compared to the control. Thus, dysregulation of specific miRNAs may play a vital role in FXS (Ramaiah et al., 2019). Consistently with these findings, our proband exhibited moderate ID and ADHD, and macroorchidism, partly due to deletion of the FX-MIR cluster.

Albeit the prevalent mechanism of FXS is the presence of hypermethylation of the CGG repeat tract and the upstream CpG island in the promoter region leading to transcriptional silencing (Bell et al., 1991; Verkerk et al., 1991), several reports involving point mutations and deletions, either small or large, have been described (Sitzmann et al., 2018; Saldarriaga et al., 2020; Erbs et al., 2021). The deletions affecting the FMR1 gene account for the second most common cause of FXS, although they are rare (Coffee et al., 2009). Small deletions (<10 kb) are consistently comitant with full mutation (>200 CGG repeats) or premutation (55-200 CGG repeats) alleles and happen during the transmission of a premutation allele from mother to offspring (Hammond et al., 1997; Han et al., 2006). Rather, large deletions (>10 kb) result in meiotic or mitotic ectopic recombination in which crossing over occurs at non-homologous loci (Coffee et al., 2008). Individuals with large deletions affecting the FMR1 gene usually display typical features of FXS, including seizure and obesity and if the deletions span over adjacent genes, they may present with additional manifestations (Moore et al., 1999).

A review of the current literature found six male individuals with de novo large deletion (>10 kb) as their mothers did not carry either a premutation or a full mutation allele, or a deleted allele and index cases did not have an expansion of CGG repeat but only large deletions harboring the FMR1 gene and contiguous DNA sequences (Wöhrl et al., 1992; Tarleton et al., 1993; Gu et al., 1994; Hirst et al., 1995; Nagamani et al., 2012; Jirananont et al., 2016). The reported deletions encompassing the FMR1 gene range from 35 kb to 3 Mb; they all have typical features of FXS involving ID from moderate to severe and developmental delay (DD) including motor and language development at various severities. Regarding physical anomalies, four out of seven cases, including our case, had dysmorphic features including macroorchidism and shawl scrotum, and two cases presented with macrocephaly. Interestingly, three of them had vision-related problems including epicanthus inversus, esotropia, and our case presents with mild deficits in visual-related cognitive abilities. Three cases had neurodevelopmental disorders, including autistic behaviors and attention and concentration problems. This evidence strongly supports that the development of typical functions of cognition and behavior are regulated by FMR1.

Our patient manifested typical FXS characteristics with distinct facial features, macroorchidism, and moderate ID with ADHD. However, he is a well-developed physical boy with good social interaction and delay in fine motor and subtle visual deficits. To date, there are only two studies mentioning de novo FXS cases with gain and loss CNV of the ASFMR1/FMR4 gene (Nagamani et al., 2012; Vengeoochea et al., 2012). Our colleagues reported a 4-year-old boy with 86-kb microduplication of the FMR1 and of the ASFMR1/ FMR4 genes, together with a 363-kb duplication on 1q44 and a 168-kb deletion on 4p15.31 inherited from a healthy father. The boy presented with myoclonic seizures and later developed absence seizures, persistent speech, and fine motor delay. He also had signs and symptoms of hyperactivity (Vengeoochea et al., 2012). (Nagamani et al., 2012) reported a 5-year-old boy with a deletion
genomic region containing the FMR1 and the ASFMRI/FMR4 genes. This boy had relatively macrocephaly and large ears, epicanthus inversus, and bilateral trigones of the lateral ventricles. He could not speak at 4-years of age indicating significant language delay. Our case phenotypes are more like a boy with microduplication of the FMR1 and of the ASFMRI/FMR4 genes with a relatively milder phenotype, fine motor delay and ADHD although this boy did not have distinctive facial features and macroorchidism, as in our case. The author suggested that either a loss or a gain copy number of the FMR1 gene, which is tightly regulated, is essential for the normal development of neurocognitive structures and functions, and can lead to neurodevelopmental disorders (Nagamani et al., 2012). Finally, we report that the deletion observed in the proband may be due to a de novo deletion, as the two probands’ sisters, the mother and maternal uncle do not have the deletion (Supplementary Figure 1). However, although unlikely, given the two probands’ normal sisters, we cannot completely exclude the possibility of germinal mosaicism in his mother.

Importantly, this study, suggested a complex transcription within the FMR1 locus, and further advanced investigation would be needed to determine the exact nature and function of all the transcripts, and their relevance to the FMR1-associated disorders. An Mb deletion encompassing the FMR1, ASFMRI/FMR4 genes, and FX-MIR cluster detected in the proband may provoke the observed clinical phenotype including intellectual disability, attention-deficit hyperactivity disorder, distinct facial features, and macroorchidism, which are typical characteristics exhibited in individuals with FXS. Importantly, we verified that the ASFMRI/FMR4 is the same genes.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Request to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UC Davis Institutional Review Board. Written informed consent was obtained from the minor(s)’ legal guardian/next of kin. Written informed consent was obtained from the participant’s legal guardian/next of kin. Written informed consent was obtained from the minor(s)’ legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

FT, PJ, NT, and EM conceptualized the manuscript. FT and NT discussed and designed the study. PJ, EM, MZ, and FT conducted the methods and data analysis. PJ drafted the manuscript, and all authors edited and revised the manuscript.

FUNDING

This study was funded by the National Institute of Health HD024356.

ACKNOWLEDGMENTS

We are grateful to this family for their willingness and enthusiasm to participate in our investigation. This article is dedicated to the memory of Matteo. We thank Hiu-Tung Tang for assistance with figures, generated using Adobe Photoshop CS2. Figure 2 was generated by Biorender, and Figure 3 was generated by MZ using Integrative Genomics Viewer (IGV) software.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.884424/full#supplementary-material

Supplementary Figure S1 | Southern blot analysis of genomic DNA isolated from a negative control (lane 1, normal female) and from a positive control (lane 5, full mutation male). Lane 2 shows lack of hybridization to the FMR1-specific probe Stb12.3 in the proband’s DNA indicating the presence of a deletion. Lanes 2 and 3 show a normal band pattern in the proband’s mother and in the maternal uncle, respectively. One-kilobase ladder is shown in lane M. The normal unmethylated band (2.8 kb) and normal methylated band (5.2 kb) in a female individual are shown on the left side. PCR analysis using FMR1 specific primers did not yield any amplicons (data not shown).

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