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Identification of Expanded Alleles of the *FMR1* Gene in the Childhood Autism Risks from Genes and Environment (CHARGE) Study

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Abstract

Fragile X syndrome (FXS) is a neuro-developmental disorder characterized by intellectual disabilities and autism spectrum disorders (ASD). Expansion of a CGG trinucleotide repeat (>200 repeats) in the 5'UTR of the fragile X mental retardation gene, is the single most prevalent cause of cognitive disabilities. Several screening studies for FXS, among individuals with ID from different ethnic populations, have indicated that the prevalence of the syndrome varies between 0.5 and 16 %. Because the high co-morbidity with autism, we have conducted a screening study of the cohort from CHARGE, a large-scale, population-based, case control study. We have identified six subjects carrying an expanded allele, which emphasize the importance of screening for FXS in a population with intellectual disabilities and ASD.

Keywords

Autism spectrum disorder; Developmental delay; Fragile X; Premutation; Screening; CGG

Introduction

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disabilities (ID), and the most common single gene mutation associated with autism (AUT). It is caused by a trinucleotide repeat expansion (CGG)_n in the 5' untranslated region of the fragile X mental retardation 1 (*FMRI*) gene, located at Xq27.3. The full mutation, present in individuals with more than 200 CGG repeats, typically involves methylation, subsequent transcriptional silencing of the *FMRI* gene, and lack of the *FMRI* protein, FMRP (Fu et al. 1991; Pieretti et al. 1991; Verkerk et al. 1991; Yu et al. 1991).

Although FXS is associated with a characteristic phenotype, there is considerable variation in the severity of clinical presentation and the cadre of cognitive, motor and behavioral impairments, and dysmorphology displayed. Moreover, AUT is frequently co-morbid with FXS, with estimates ranging up to 30 % of FXS patients displaying autism spectrum disorders (ASD), and an additional 30 % of affected patients receiving a diagnosis of pervasive developmental disorder-not otherwise specified, more commonly known by its acronym PDD-NOS (Hagerman 2002; Harris et al. 2008; Rogers et al. 2001).

Individuals with an *FMRI* premutation have an allele with 55–200 CGG repeats that is typically unmethylated, which usually does not result in gene inactivation. They also present with a spectrum of phenotypes including developmental delay, autism spectrum disorder, attention deficit/hyperactivity disorder (ADHD), and behavior problems (Aziz et al. 2003; Bailey et al. 2008; Clifford et al. 2007; Farzin et al. 2006; Loesch et al. 2007). Individuals with FXS + AUT appear more cognitively impaired, especially so in scores on the social and communication domains than in domains of restricted interests and repetitive behaviors. Medical problems that affect the central nervous system, including seizures, malformations,

or other genetic disorders, seem more likely to occur in those individuals presenting with both FXS and ASD compared to those with FXS alone (Garcia-Nonell et al. 2008). Furthermore, the diagnosis of ASD in FXS could be related to other genetic and/or environmental factors leading to brain dysfunction additive to the *FMRI* mutation (Loesch et al. 2007). However, the reason ASD occurs only in a subgroup of individuals with FXS is not known (Loesch et al. 2007). One proximal mechanism contributing to ASD risk in FXS is the loss of the *FMRI* protein (FMRP) (Hagerman et al. 2008). Indeed, recent reports indicate that FMRP may be deficient in other disorders including autism not associated with FXS (Fatemi and Folsom 2011), schizophrenia, bipolar disorder, and even depression (Fatemi et al. 2010). Finally, many studies over the past 25 years have documented the higher incidence of ASD in subjects with FXS (Reddy 2005).

Thus, because of the high co-morbidity with autism, we have conducted a screening for expanded alleles of the *FMRI* gene in a cohort from CHARGE (CHildhood Autism Risks from Genes and Environment), a comprehensive, ongoing, population-based case-control investigation that is sampled from three strata: children with autism, children with developmental delay but not autism, and general population controls (Hertz-Picciotto et al. 2006). We have determined the *FMRI* CGG size distribution in four groups and examined the association with IQ (Vineland and Mullen test scores); in this study, these groups were divided into the neurotypical control group (typical development, TD; n = 346), the developmental delay group without AUT/ASD (DD, n = 152), the autism spectrum disorders group (ASD, n = 144), and the full autism syndrome group (AUT, n = 313).

No expanded alleles were identified in the TD group. However, of the 457 children aged 2–5 years with confirmed diagnosis of AUT or ASD, we identified two subjects (0.4 %) with full mutation allele, in line with previous prevalence reports (Reddy 2005). Two individuals carrying a premutation allele and two others with a full mutation allele were identified in the DD group (1.4 %).

In addition, we have investigated if there was a difference in the location and in the number of AGG interruptions within each *FMRI* allele among the diagnostic groups. Within the CGG repeats, interspersions of one to three AGG “anchors” are commonly found in normal, intermediate, and premutation range alleles and generally occur after 9 or 10 uninterrupted CGG repeats, with a pattern of [(CGG)₉AGG(CGG)₉AGG(CGG)_n]. These AGG interruptions can be stably inherited, such that the number of AGG interruptions and their position is likely to correspond in parent and offspring; however, like the length of CGG-repeat tracts in *FMRI*, there is a level of instability, such as the loss of an interruption during transmission (Dombrowski et al. 2002). Although the presence of AGGs does not influence the transcriptional level of the *FMRI* gene, (Yrigollen et al. 2011) they significantly increase genetic stability by reducing the risk of transmission of a full mutation for all maternal premutation repeat lengths below ~100 CGG repeats (Yrigollen et al. 2012). No differences were observed for both distribution and number of CGG interruptions among the TD, AUT, ASD and DD groups.

Our findings confirm the importance of screening for FXS, individuals with ASD and ID.

Materials and Methods

Subjects

Participants in this study were part of the CHARGE (CHildhood Autism Risks from Genetics and the Environment) study, which includes children from the following groups: children with autism, children with developmental delay but not autism, and general population controls (Hertz-Picciotto et al. 2006). Children with AUT or with other developmental delays were identified through the California Department of Developmental Services (DDS), which provides services regardless of socioeconomic level, citizenship, or racial/ethnic group. Children from the general population were identified in the state birth files and matched to the autism cases on age, sex, and broad geographic area. Children were between the ages of 24 and 60 months and born in California. Children meeting the recruitment criteria (Hertz-Picciotto et al. 2006) were seen at the MIND Institute in Sacramento, California; medical history was collected, and a battery of tests and a physical examination were performed. There were no exclusions for any known genetic or chromosomal disorders; however these were noted in the medical assessment. The only developmentally-related exclusions were for those with serious disabilities that would impede our ability to obtain valid scores on the developmental tests used, e.g., blindness, deafness, or serious motor disability.

Through clinical assessment (see below), diagnosis of AUT, ASD, DD and TD were confirmed. For molecular analysis 313 children with AUT, 144 children with ASD, 152 children with DD, and 346 TD children (Hertz-Picciotto et al. 2006) were screened by PCR. The male to female ratio was 6:1 in children with AUT/ASD (394 males, 63 females) and 3:1 in TDs (262 males, 84 females).

The distribution of ages was similar among diagnoses, with a mean age of 42 months in TD subjects (range 24–61 months), 46 months in ASD subjects (range 26–72 months), 45 months in AUT subjects (range 15–69 months), and 46 months in DD subjects (range 26–61 months).

Clinical Assessment and Assignment of Diagnostic Groups

For all participants, we collected a detailed medical history and conducted a medical examination. All children were also administered tests of IQ and adaptive skills using the Mullen Scales of Early Learning (MSEL) (Mullen 1995) and the Vineland Adaptive Behavior Scales (VABS) (Sparrow et al. 1984). The TD group consisted of children from the general population who scored 70 or above on both MSEL and VABS, and below 15 on the social communication questionnaire. The diagnosis of autism was confirmed using the standardized autism measures: the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al. 1994) given to the primary caregiver, and through direct assessment of the child using the Autism Diagnostic Observation Schedules (Lord et al. 2000). The onset of ASD was also assessed by ADI-R and regression was considered to have occurred if the primary caregiver responded that the child had lost either language (ADI-R question 11 = 1) and/or social skills (ADI-R question 25 = 1). Children that did not meet these criteria were included in the “non-regressive” group. A full autism diagnosis was defined as meeting criteria on the

communication, social interaction, and repetitive behavior domains of the ADI-R with onset prior to 36 months, and scoring at or above the social + communication cutoff for autism on the ADOS module 1 or 2. Children were defined as having ASD if they did not meet full criteria for autism on either or both the ADI-R and ADOS, but did meet criteria on either the communication or the social interaction domain of the ADI-R prior to 36 months, were within two points of the cut-off on the other domain, and were above the social + communication cutoff for ASD on the ADOS module 1 or 2. Parents of children with other developmental delays and those from the general population completed the Social Communication Questionnaire (Rutter et al. 2003) to screen for ASD. Those who screened positive (at or above a score of 16) were then assessed using both the ADOS and ADI-R; children who met criteria for AUT or ASD were then included in the corresponding diagnostic group, regardless of the group from which they were initially recruited. Eleven TD subjects were re-classified as DD, 13 DD subjects were reclassified as ASD, and 16 as AUT.

Molecular Measures

Genomic DNA Isolation—DNA was extracted from 0.5 to 1.5 ml of the cellular fraction of anticoagulated blood collected from children with AUT (n = 313), ASD (n = 144), DD (n = 152), and TD (n = 346). DNA isolation was carried out according to standard procedure (Qiagen, Valencia, CA, USA).

FMR1 Genotyping—*FMR1* allele size was determined by PCR amplification using a combination of an *FMR1*-specific PCR and a two-tier PCR approach as previously described (Chen et al. 2010; Filipovic-Sadic et al. 2010; Tassone et al. 2008). Briefly, samples from males that did not yield a product and females that yielded only one band (amplicon) after the *FMR1*-specific PCR underwent a second PCR screening using the CGG linker primer. Conditions were as detailed previously in Chen et al. (2010) and Filipovic-Sadic et al. (2010). PCR products were stored at 4 °C prior to electrophoresis analyses. PCR products were sized and analyzed using capillary electrophoresis (Applied Biosystems, Foster City, CA, USA). In this study, according to the guidelines of the American College of Medical Genetics (Maddalena et al. 2001), *FMR1* alleles were classified as normal (TD; <45 CGG repeats), gray zone (GZ; 45–54 CGG repeats), premutation (pre CGG; 55–200 CGG repeats), and full mutation (FXS; 200 CGG repeats).

Number and Location of the AGGs—The presence and location of AGG interruptions was determined by PCR using a CGG chimeric primer (CCG)₅ that, when it anneals to the CGG tract, amplifies intermediate fragments of variable length. Because the chimeric primer is unable to anneal to a CGG tract containing an AGG interruption, mapping of the AGGs by identification of a corresponding absence of PCR product, as visualized by capillary electrophoresis, is possible. The position of the AGGs within each allele was determined from raw Gene-scan files imported into Peakscanner software (v1.0) (Applied Biosystems, Foster City, CA, USA). Details of the methodology are reported in Yrigollen et al. (2011).

Statistical Analysis

A total of 955 samples were screened for the presence of an *FMRI* expanded allele. Six subjects identified with an expanded allele (four with FXS, two AUT and two DD; and two premutations, both DD) were excluded from analyses of CGG-repeat size distribution and number of AGG interruptions. Four male subjects (two AUT, two DD) showed two bands (corresponding to two alleles) using two different sets of primers and were not analyzed further. Although we cannot completely exclude that some of these samples may have been mislabeled with respect to the gender, it is also possible that some could have been samples from Klinefelter subjects (XXY). After these exclusions, data from 945 participants remained for the statistical analysis: 346 TD (262 males, 84 females), 309 AUT (270 males, 39 females), 144 ASD (121 males, 23 females), and 146 DD (95 males, 51 females). The mean total CGG length was compared across all four diagnosis categories, and between the AUT and ASD groups pooled versus subjects with TD, using one-way analysis of variance (ANOVA) models; CGG length was used as the response, and diagnosis as the independent variable. Chi-square tests were used to look for an association between diagnosis and number of AGG interruptions. Fisher's exact test was used to compare the proportion of GZ alleles between AUT/ASD and TD groups.

Results

According to our workflow, only DNA samples from male subjects that do not yield a band (no amplified allele obtained) after the first round of PCR with primers c and f (*FMRI*-specific PCR), and DNA samples from female subjects that yield only one band (one allele) are usually subjected to a second PCR round using the CGG primer-based PCR methodology (Chen et al. 2010; Filipovic-Sadic et al. 2010; Tassone et al. 2008). However, as the use of the CGG primer allows for the detection and characterization of the AGG interruptions (location and number) within each *FMRI* allele on capillary electrophoresis, all samples underwent the second PCR round using the CGG primer as previously described (Yrigollen et al. 2011). Six subjects carrying an *FMRI* expanded allele were identified (see Fig. 1): one male and one female with an expanded allele in the full mutation range (FXS; >200 CGG repeats) in the AUT group, and two full mutation males in the DD group. One pre CGG male and one pre CGG female (55–200 CGG repeats) were also identified in the DD group. Allele size was determined for all subjects, and the summary statistics for total CGG length (mean, median, standard deviation, and range) was calculated for subjects with alleles in the normal or intermediate range in each diagnosis group (Table 1).

Alleles in the GZ range were identified in five females (two ASD/AUT; two TD; one DD) and six male carriers (five ASD/AUT; one TD). The observed frequency of GZ alleles in subjects in this sample with AUT/ASD was 3.2 % in females (95 % CI 0.9 %, 11.0 %) and 1.3 % in males (95 % CI 0.5 %, 2.9 %). The observed frequency of GZ alleles in TD subjects in this sample was 2.4 % in females (95 % CI 0.7 %, 8.3 %) and 0.4 % in males (95 % CI 0.0 %, 2.1 %). No statistical significant difference was observed between the frequency of GZ alleles in the AUT/ASD group compared to TD subjects in females (Fisher's exact test $p > 0.99$) or in males (Fisher's exact test $p = 0.41$).

For purposes of comparison, we used the GZ allele frequencies observed in a newborn screening pilot study ongoing in California (Long et al. 2011). Specifically, a GZ allele frequency of 1.4 % was observed in a sample of 6,013 newborn females from the general population and a GZ allele of 0.7 % was observed in a sample of 6,441 newborn males from the general population. While the observed frequency of GZ alleles in AUT/ASD subjects in this study sample is numerically higher than the frequencies observed for males and females in the newborn screening sample, GZ allele frequencies in AUT/ASD males and females in this study do not significantly differ from the observed newborn screening frequencies. The frequency of GZ alleles observed in the DD subjects in this study was 2.0 % in females (95 % CI 0.1 %, 10.3 %) and 0 % in males (95 % CI 0 %, 3.8 %), which did not differ significantly compared to the TD subjects in either females or males (Fisher's exact test $p > 0.99$ in both cases). Lack of significance was also obtained when the frequency of the GZ alleles in the DD group was compared to those observed in the newborn screening subjects. The CGG-repeat length distribution was also measured and means were found not to differ significantly among the four groups ($F(3, 941) = 0.32, p = 0.81$). Full mutation ($n = 4$) and premutation ($n = 2$) alleles were excluded from the analysis. Allele size distribution is shown in Fig. 2 by diagnosis. The data show similar distributions within each diagnosis group. In addition, no significant difference between groups in total CGG-repeat length was observed when the AUT and ASD groups were pooled together ($F(1, 797) = 0.003, p = 0.96$).

We also determined the distribution of the number of AGG interruptions in the four diagnostic groups. No significant difference in the distribution and numbers of AGG interruptions between diagnoses was observed (Table 2) either when all four diagnoses were included separately, $\chi^2(9, N = 945) = 5.35, p = 0.80$, or when the AUT/ASD groups were pooled and the DD group was excluded, $\chi^2(3, N = 799) = 1.63, p = 0.65$. No differences in either the mean CGG size or in the number and location of the AGG interruptions were detected between the regressive and non-regressive AUT/ASD groups.

The association of IQ with CGG-repeat number was investigated in the three groups (AUT, ASD and DD). Both Vineland and Mullen test scores were regressed on total CGG length using data from patients with the ASD, AUT, and DD diagnoses, and TD. The estimated change in Vineland test score for each additional repeat was $b = 0.01$, 95 % confidence interval $(-0.31, 0.34)$, $t(914) = 0.08, p = 0.94$ (Supplemental Fig. 1). The estimated change in Mullen test score for each additional repeat was $b = 0.11$, 95 % confidence interval $(-0.27, 0.50)$, $t(914) = 0.58, p = 0.56$. (Supplemental Fig. 2). Models were also fitted separately for each diagnosis group; conclusions were the same as when the AUT and ASD diagnosis categories were combined.

Discussion

FXS is an X-linked disorder characterized by intellectual disability, and behavioral and physical features (Hagerman 2002); and is caused by a CGG trinucleotide expansion in the 5'UTR of the *FMR1* gene. Behavioral features present in both males and females with FXS can also include impairments in social interaction and communication, social anxiety, gaze avoidance, hand and finger mannerisms, and repetitive and stereotypic behavior (Hagerman

2002; Lachiewicz and Dawson 1994; Miller et al. 1999; Sudhalter et al. 1990), typical of autism.

Studies assessing the prevalence of autism among subjects with FXS range from 5 to 60 % depending on the diagnostic criteria used (Hagerman 2002; Harris et al. 2008; Rogers et al. 2001), but it is lower in females with FXS (Bailey et al. 1993; Hagerman 2002). Several studies have also been carried out in different populations of subjects including ID, AUT, or those with special educational needs, to both assess the prevalence of FXS among these populations and to identify subjects with FXS, which is very important for families. In the above studies, using both cytogenetic and molecular approaches, the full mutation rate varies between 0.5 and 16 % and the premutation rate varies between 0 and 0.8 % (Biancalana et al. 2004; Blomquist et al. 1985; Brown et al. 1986; de Vries et al. 1998; de Vries et al. 1997; Hecimovic et al. 2002; Major et al. 2003; Mazzocco et al. 1997; Mila et al. 1997; Pandey et al. 2002; Patsalis et al. 1999; Pouya et al. 2009; Reddy 2005; Schaefer and Lutz 2006; Sharma et al. 2001; Syrrou et al. 1998; Watson et al. 1984; Yuhas et al. 2009). A multicenter study conducted in Sweden (Blomquist et al. 1985) found FXS in 16 % of boys with infantile autism, and a multicenter survey among individuals with autism found 13 % were positive for FXS (Brown et al. 1986). Bailey et al. (1993) determined the prevalence of FXS among males and females with autism in three groups, 45 pairs of twins, 2 sets of triplets, and clinical control samples, and found FXS mutations in 1.6 % of the individuals with autism in the twin and clinical sample sets, with a similar prevalence in both sexes. The variation of the incidence among these studies and many others (also reviewed in (Reddy 2005)) are likely due to different clinical criteria utilized for the definition of positive cases, and to different sample sizes. Thus, although the prevalence of FXS among individuals with ASD differs between these studies and samples, strong evidence points toward an association between ASD and FXS. However, the underlying cause of this co-morbidity is unknown.

We screened the CHARGE study for FXS and identified six subjects with an expanded *FMRI* allele, suggesting that the presence of an expansion of the trinucleotide repeats within the *FMRI* gene could account for the etiology of the developmental delay in these children. Two individuals with a premutation allele were also identified.

The premutation alleles are common in the general population with a prevalence of approximately 1 in 113–259 females (0.3–0.8 %) and 1 in 251–813 males (0.1–0.3 %) (Dombrowski et al. 2002; Fernandez-Carvajal et al. 2009; Rousseau et al. 1995; Toledano-Alhadeff et al. 2001). Individuals with the premutation can present with a range of clinical involvement including fragile X-associated primary ovarian insufficiency (FXPOI) (Cronister et al. 1991; Sullivan et al. 2005; Wittenberger et al. 2007) and fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman et al. 2001; Jacquemont et al. 2003), in addition to emotional problems—particularly depression and anxiety (Bourgeois et al. 2009; Hessler et al. 2005; Roberts et al. 2009). Furthermore, ASD and ADHD have been found to be significantly more prevalent in boys with the premutation who presented clinically as probands, compared with their non-probandbrothers and with their brothers without the premutation (Aziz et al. 2003; Bailey et al. 2008; Chonchaiya et al. 2012; Farzin et al. 2006). Causes of these neurodevelopmental and behavioral problems need to be further

investigated to better understand the nature of such problems, and to possibly develop targeted treatments for those with the premutation.

Interestingly, we have identified five female (2 ASD/AUT, 2 TD, 1 DD) and six male carriers (5 ASD/AU, 1TD) of a GZ allele with a twofold higher frequency in AUT/ASD subjects than in the general population. Understanding the phenotypic effects of small CGG-repeat alleles of the *FMRI* gene is of great importance considering the bridging position between the pathogenic premutation and the normal allele categories. Indeed, GZ alleles are considered quite unstable and both abnormal molecular and clinical phenotypes have been recently reported, including a higher frequency of GZ among females with premature ovarian failure (Bodega et al. 2006; Bretherick et al. 2005; Haddad et al. 1999; Hall et al. 2011; Loesch et al. 2009; Murray et al. 1996; Sherman 2000; Sullivan et al. 2002), and among females with parkinsonism. In addition, a recent study reported on four subjects, carriers of a gray-zone allele, with symptoms of the late-onset neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome (Hall et al. 2011).

Clinical involvement is observed in premutation carriers and caused by an RNA toxic gain-of-function due to increased *FMRI* mRNA levels observed in subjects with an allele in this repeat range (Tassone et al. 2000). Increased *FMRI* mRNA levels have also been detected in individuals with GZ alleles; therefore, it is possible that RNA toxicity occurs in smaller expanded alleles, though to a lesser degree (Loesch et al. 2007). Since the frequency of gray-zone alleles in the general population is quite frequent (~1 %) (Long et al. 2011), even modest deleterious contributions, if substantiated, are likely to be borne by a large number of people.

In conclusion, this study screened a population of young children recruited for participation in the CHARGE study, including subjects with confirmed diagnoses of ASD, AUT, and DD, and determined the prevalence of *FMRI* expanded alleles; the results correlate with similar findings reported from previous studies. The identification of six individuals with an expanded *FMRI* allele in this study population confirms and suggests the role played by the *FMRI* gene in the etiology of developmental delay in children. Detailed characterization of the molecular variation of the *FMRI* gene within the CHARGE cohort demonstrates that both CGG-repeat size distribution and AGG interruptions are equally dispersed in the ASD and DD groups, and therefore do not appear to play a distinctively different role in the physiopathology of ASD versus that of ID. The findings further demonstrate the importance of the FXS DNA screening test in individuals with ID and ASD, which should be strongly encouraged to rule out FXS as well as to reveal potential causes of intellectual disabilities. As several studies have demonstrated that children with FXS and autism have poorer outcomes than children with only FXS or only autism (Bailey et al. 2000, 2001; Hatton et al. 2000, 2006; Kau et al. 2004; Lewis et al. 2006; Rogers et al. 2001), and because early intervention can improve outcomes for young children with autism (National Research Council 2001), it is very important that young children with FXS and autism be identified as early as possible.

In future studies using the CHARGE cohort, it is important to consider the *FMRI* status reported in the present study. As mentioned earlier, children with FXS and autism are at a

greater disadvantage than children with only FXS or only autism, thus young children with FXS and autism need to be identified as soon as possible so early intervention can improve outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Aziz M, Stathopulu E, Callias M, Taylor C, Turk J, Oostra B, et al. Clinical features of boys with fragile X premutations and intermediate alleles. *American Journal of Medical Genetics*. 2003; 121B(1):119–127. [PubMed: 12898586]
- Bailey A, Bolton P, Butler L, Le Couteur A, Murphy M, Scott S, et al. Prevalence of the fragile X anomaly amongst autistic twins and singletons. *J of Child Psychology and Psychiatry*. 1993; 34(5): 673–688.
- Bailey DB Jr, Hatton DD, Mesibov GB, Ament N, Skinner M. Early development, temperament and functional impairment in autism and fragile X syndrome. *Journal of Autism and Developmental Disorders*. 2000; 30(1):49–59. [PubMed: 10819120]
- Bailey DB Jr, Hatton DD, Skinner M, Mesibov GB. Autistic behavior, FMR1 protein, and developmental trajectories in young males with fragile X syndrome. *Journal of Autism and Developmental Disorder*. 2001; 31(2):165–174.
- Bailey DB Jr, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: Findings from a national parent survey. *American Journal of Medical Genetics, Part A*. 2008; 146A(16):2060–2069. [PubMed: 18570292]
- Biancalana V, Beldjord C, Taillandier A, Szpiro-Tapia S, Cusin V, Gerson F, et al. Five years of molecular diagnosis of Fragile X syndrome (1997–2001): A collaborative study reporting 95 % of the activity in France. *American Journal of Medical Genetics*. 2004; 129A(3):218–224. [PubMed: 15326620]
- Blomquist HK, Bohman M, Edvinsson SO, Gillberg C, Gustavson KH, Holmgren G, et al. Frequency of the fragile X syndrome in infantile autism: A Swedish multicenter study. *Clinical Genetics*. 1985; 27(2):113–117. [PubMed: 3978844]
- Bodega B, Bione S, Dalpra L, Toniolo D, Ornaghi F, Vegetti W, et al. Influence of intermediate and uninterrupted FMR1 CGG expansions in premature ovarian failure manifestation. *Human Reproduction*. 2006; 21(4):952–957. [PubMed: 16361284]
- Bourgeois JA, Coffey SM, Rivera SM, Hessl D, Gane LW, Tassone F, et al. A review of fragile X premutation disorders: Expanding the psychiatric perspective. *Journal of Clinical Psychiatry*. 2009; 70(6):852–862. [PubMed: 19422761]
- Bretherick KL, Fluker MR, Robinson WP. FMR1 repeat sizes in the gray zone and high end of the normal range are associated with premature ovarian failure. *Human Genetics*. 2005; 117(4):376–382. [PubMed: 16078053]
- Brown WT, Jenkins EC, Cohen IL, Fisch GS, Wolf-Schein EG, Gross A, et al. Fragile X and autism: A multicenter survey. *American Journal of Medical Genetics*. 1986; 23(1–2):341–352. [PubMed: 3513570]

- Chen L, Hadd A, Sah S, Filipovic-Sadic S, Krosting J, Sekinger E, et al. An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis. *Journal of Molecular Diagnostics*. 2010; 12(5):589–600. [PubMed: 20616364]
- Chonchaiya W, Au J, Schneider A, Hessler D, Harris SW, Laird M, Mu Y, Tassone F, Nguyen DV, Hagerman RJ. Increased prevalence of seizures in boys who were probands with the FMR1 premutation and co-morbid autism spectrum disorder. *Human Genetics*. 2012; 131(4):581–589. [PubMed: 22001913]
- Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, Loesch DZ. Autism spectrum phenotype in males and females with fragile X full mutation and premutation. *Journal of Autism and Developmental Disorders*. 2007; 37(4):738–747. [PubMed: 17031449]
- Cronister A, Schreiner R, Wittenberger M, Amiri K, Harris K, Hagerman RJ. Heterozygous fragile X female: Historical, physical, cognitive, and cytogenetic features. *American Journal of Medical Genetics*. 1991; 38(2–3):269–274. [PubMed: 2018071]
- de Vries BB, Mohkamsing S, van den Ouweland AM, Halley DJ, Niermeijer MF, Oostra BA, et al. Screening with the FMR1 protein test among mentally retarded males. *Human Genetics*. 1998; 103(4):520–522. [PubMed: 9856500]
- de Vries BB, van den Ouweland AM, Mohkamsing S, Duivenvoorden HJ, Mol E, Gelsema K, et al. Screening and diagnosis for the fragile X syndrome among the mentally retarded: An epidemiological and psychological survey. Collaborative Fragile X Study Group. *American Journal of Human Genetics*. 1997; 61(3):660–667. [PubMed: 9326332]
- Dombrowski C, Levesque ML, Morel ML, Rouillard P, Morgan K, Rousseau F. Premutation and intermediate-size FMR1 alleles in 10 572 males from the general population: Loss of an AGG interruption is a late event in the generation of fragile X syndrome alleles. *Human Molecular Genetics*. 2002; 11(4):371–378. [PubMed: 11854169]
- Farzin F, Perry H, Hessler D, Loesch D, Cohen J, Bacalman S, et al. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. *Journal of Developmental and Behavioral Pediatrics*. 2006; 27(2 Suppl):S137–S144. [PubMed: 16685180]
- Fatemi SH, Folsom TD. The role of fragile X mental retardation protein in major mental disorders. *Neuropharmacology*. 2011; 60(7–8):1221–1226. [PubMed: 21108954]
- Fatemi SH, Kneeland RE, Liesch SB, Folsom TD. Fragile X mental retardation protein levels are decreased in major psychiatric disorders. *Schizophrenia Research*. 2010; 124(1–3):246–247. [PubMed: 20727716]
- Fernandez-Carvajal I, Walichiewicz P, Xiaosen X, Pan R, Hagerman PJ, Tassone F. Screening for expanded alleles of the FMR1 gene in blood spots from newborn males in a Spanish population. *Journal of Molecular Diagnostics*. 2009; 11(4):324–329. [PubMed: 19460941]
- Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, et al. A novel FMR1 PCR method for the routine detection of low-abundance expanded alleles and full mutations in fragile X syndrome. *Clinical Chemistry*. 2010; 56(3):399–408. [PubMed: 20056738]
- Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. *Cell*. 1991; 67(6):1047–1058. [PubMed: 1760838]
- Garcia-Nonell C, Ratera ER, Harris S, Hessler D, Ono MY, Tartaglia N, et al. Secondary medical diagnosis in fragile X syndrome with and without autism spectrum disorder. *American Journal of Medical Genetics, Part A*. 2008; 146A(15):1911–1916. [PubMed: 18627038]
- Haddad LA, Aguiar MJ, Costa SS, Mingroni-Netto RC, Vianna-Morgante AM, Pena SD. Fully mutated and gray-zone FRAXA alleles in Brazilian mentally retarded boys. *American Journal of Medical Genetics*. 1999; 84(3):198–201. [PubMed: 10331590]
- Hagerman, PJ. Gene expression and molecular approaches to therapy. In: Hagerman, RJ.; Hagerman, PJ., editors. *Fragile X syndrome: Diagnosis, treatment and research*. 3rd ed.. Baltimore, MD: The Johns Hopkins University Press; 2002.
- Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R, Hills J, et al. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology*. 2001; 57:127–130. [PubMed: 11445641]

- Hagerman RJ, Rivera SM, Hagerman PJ. The fragile X family of disorders: A model for autism and targeted treatments. *Current Pediatric Reviews*. 2008; 4:40–52.
- Hall DA, Berry-Kravis E, Zhang W, Tassone F, Spector E, Zerbe G, et al. FMR1 gray-zone alleles: association with Parkinson's disease in women? *Movement Disorders: Official Journal of the Movement Disorder Society*. 2011; 26(10):1900–1906. [PubMed: 21567456]
- Harris SW, Hessel D, Goodlin-Jones B, Ferranti J, Bacalman S, Barbato I, et al. Autism profiles of males with fragile X syndrome. *American Journal of Mental Retardation*. 2008; 113(6):427–438. [PubMed: 19127654]
- Hatton D, Bailey DB, Roberts J, Skinner M, Mayher L, Duffee Clark R, et al. Early intervention services for young boys with fragile X syndrome. *Journal of Early Intervention*. 2000; 23(4):235–251.
- Hatton DD, Sideris J, Skinner M, Mankowski J, Bailey DB Jr, Roberts JE, et al. Autistic behavior in children with fragile X syndrome: Prevalence, stability, and the impact of FMRP. *American Journal of Medical Genetics, Part A*. 2006; 140(17):1804–1813. [PubMed: 16700053]
- Hecimovic S, Tarnik IP, Baric I, Cakarun Z, Pavelic K. Screening for fragile X syndrome: Results from a school for mentally retarded children. *Acta Paediatrica*. 2002; 91(5):535–539. [PubMed: 12113322]
- Hertz-Picciotto I, Croen LA, Hansen R, Jones CR, van de Water J, Pessah IN. The CHARGE study: An epidemiologic investigation of genetic and environmental factors contributing to autism. *Environmental Health Perspectives*. 2006; 114(7):1119–1125. [PubMed: 16835068]
- Hessel D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA, Gane LW, et al. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. *American Journal of Medical Genetics Part B Neuropsychiatric Genetics*. 2005; 139(1):115–121.
- Long, K.; Tong, T.; Gane, L.; Sorensen, P.; Berry-Kravis, E.; Nguyen, D.; Mu, Y.; Skinner, D.; Bailey, D.; Hagerman, R.; Tassone, F. Newborn screening in fragile X syndrome: prevalence and allele distribution of the FMR1 gene; Paper presented at the American College Medical Genetics; Vancouver. 2011.
- Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L, Brunberg JA, et al. Fragile X premutation tremor/ataxia syndrome: Molecular, clinical, and neuroimaging correlates. *American Journal of Human Genetics*. 2003; 72(4):869–878. [PubMed: 12638084]
- Kau ASM, Tierney E, Bukelis I, Stump MH, Kates WR, Trescher WH, et al. Social behavior profile in young males with fragile X syndrome: Characteristics and specificity. *American Journal of Medical Genetics*. 2004; 126A:9–17. [PubMed: 15039968]
- Lachiewicz AM, Dawson DV. Behavior problems of young girls with fragile X syndrome: Factor scores on the Conners' Parent's Questionnaire. *American Journal of Medical Genetics*. 1994; 51(4):364–369. [PubMed: 7943000]
- Lewis P, Abbeduto L, Murphy M, Richmond E, Giles N, Bruno L, et al. Cognitive, language and social-cognitive skills of individuals with fragile X syndrome with and without autism. *Journal of Intellectual Disability Research*. 2006; 50(Pt 7):532–545. [PubMed: 16774638]
- Loesch DZ, Bui QM, Dissanayake C, Clifford S, Gould E, Bulhak-Paterson D, et al. Molecular and cognitive predictors of the continuum of autistic behaviours in fragile X. *Neuroscience and Biobehavioral Reviews*. 2007; 31(3):315–326. [PubMed: 17097142]
- Loesch DZ, Godler DE, Khaniani M, Gould E, Gehling F, Dissanayake C, et al. Linking the FMR1 alleles with small CGG expansions with neurodevelopmental disorders: Preliminary data suggest an involvement of epigenetic mechanisms. *American Journal of Medical Genetics, Part A*. 2009; 149A(10):2306–2310. [PubMed: 19760650]
- Lord C, Risi S, Lambrecht L, Cook EH Jr, Leventhal BL, DiLavore PC, et al. The Autism Diagnostic Observation Schedule-Generic: A standard measure of social and communication deficits associated with the spectrum of autism. *Journal of Autism and Developmental Disorder*. 2000; 30(3):205–223.
- Lord C, Rutter M, Le Couteur A. Autism Diagnostic Interview-Revised: A revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *Journal of Autism and Developmental Disorders*. 1994; 24(5):659–685. [PubMed: 7814313]

- Maddalena A, Richards CS, McGinniss MJ, Brothman A, Desnick RJ, Grier RE, et al. Technical standards and guidelines for fragile X: The first of a series of disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. Quality Assurance Subcommittee of the Laboratory Practice Committee. *Genetic Medicine*. 2001; 3(3):200–205.
- Major T, Culjkovic B, Stojkovic O, Gucsekic M, Lakic A, Romac S. Prevalence of the fragile X syndrome in Yugoslav patients with non-specific mental retardation. *Journal of Neurogenetics*. 2003; 17(2–3):223–230. [PubMed: 14668200]
- Mazzocco MM, Kates WR, Baumgardner TL, Freund LS, Reiss AL. Autistic behaviors among girls with fragile X syndrome. *Journal of Autism and Developmental Disorder*. 1997; 27(4):415–435.
- Mila M, Sanchez A, Badenas C, Brun C, Jimenez D, Villa MP, et al. Screening for FMR1 and FMR2 mutations in 222 individuals from Spanish special schools: Identification of a case of FRAXE-associated mental retardation. *Human Genetics*. 1997; 100:503–507. [PubMed: 9341861]
- Miller LJ, McIntosh DN, McGrath J, Shyu V, Lampe M, Taylor AK, et al. Electrodermal responses to sensory stimuli in individuals with fragile X syndrome: A preliminary report. *American Journal of Medical Genetics*. 1999; 83(4):268–279. [PubMed: 10208160]
- Mullen, EM. Mullen Scales of Early Learning. Circle Pines: American Guidance Service; 1995.
- Murray A, Youings S, Dennis N, Latsky L, Linehan P, McKechnie N, et al. Population screening at the FRAXA and FRAXE loci: Molecular analyses of boys with learning difficulties and their mothers. *Human Molecular Genetics*. 1996; 5(6):727–735. [PubMed: 8776586]
- Pandey UB, Phadke S, Mittal B. Molecular screening of FRAXA and FRAXE in Indian patients with unexplained mental retardation. *Genetic Testing*. 2002; 6(4):335–339. [PubMed: 12537661]
- Patsalis PC, Sismani C, Hettinger JA, Boumba I, Georgiou I, Stylianidou G, et al. Molecular screening of fragile X (FRAXA) and FRAXE mental retardation syndromes in the Hellenic population of Greece and Cyprus: incidence, genetic variation, and stability. *American Journal of Medical Genetics*. 1999; 84(3):184–190. [PubMed: 10331587]
- Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, et al. Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell*. 1991; 66(4):817–822. [PubMed: 1878973]
- Pouya AR, Abedini SS, Mansoorian N, Behjati F, Nikzat N, Mohseni M, et al. Fragile X syndrome screening of families with consanguineous and non-consanguineous parents in the Iranian population. *European Journal of Medical Genetics*. 2009; 52(4):170–173. [PubMed: 19361583]
- Reddy KS. Cytogenetic abnormalities and fragile-X syndrome in Autism Spectrum Disorder. *BMC Medical Genetics*. 2005; 6(1):3. [PubMed: 15655077]
- Roberts JE, Bailey DB Jr, Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *American Journal of Medical Genetics, Part B Neuropsychiatric Genetics*. 2009; 150B(1):130–139.
- Rogers SJ, Wehner EA, Hagerman RJ. The behavioral phenotype in fragile X: Symptoms of autism in very young children with fragile X syndrome, idiopathic autism, and other developmental disorders. *Journal of Developmental and Behavioral Pediatrics*. 2001; 22(6):409–417. [PubMed: 11773805]
- Rousseau F, Rouillard P, Morel ML, Khandjian EW, Morgan K. Prevalence of carriers of premutation-size alleles of the FMRI gene—and implications for the population genetics of the fragile X syndrome. *American Journal of Human Genetics*. 1995; 57(5):1006–1018. [PubMed: 7485149]
- Rutter, M.; Bailey, A.; Berument, SK.; Lord, C.; Pickles, A. Social Communication Questionnaire (SCQ). Los Angeles: Western Psychological Services; 2003.
- Schaefer GB, Lutz RE. Diagnostic yield in the clinical genetic evaluation of autism spectrum disorders. *Genetic Medicine*. 2006; 8(9):549–556.
- Sharma D, Gupta M, Thelma BK. Expansion mutation frequency and CGG/GCC repeat polymorphism in *FMR1* and *FMR2* genes in an Indian population. *Genetic Epidemiology*. 2001; 20(1):129–144. [PubMed: 11119302]
- Sherman SL. Premature ovarian failure in the fragile X syndrome. *American Journal of Medical Genetics (Seminars in Medical Genetics)*. 2000; 97(3):189–194. [PubMed: 11449487]
- Sparrow, SS.; Balla, DA.; Cicchetti, DV. Vineland Adaptive Behavior Scales Survey Form Manual. Circle Pines: American Guidance Service; 1984.

- Sudhalter V, Cohen IL, Silverman W, Wolf-Schein EG. Conversational analyses of males with fragile X, Down syndrome, and autism: Comparison of the emergence of deviant language. *American Journal of Mental Retardation*. 1990; 94(4):431–441. [PubMed: 2137003]
- Sullivan AK, Crawford DC, Scott EH, Leslie ML, Sherman SL. Paternally transmitted FMR1 alleles are less stable than maternally transmitted alleles in the common and intermediate size range. *American Journal of Human Genetics*. 2002; 70(6):1532–1544. [PubMed: 11992259]
- Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE, Paquin JJ, et al. Association of FMR1 repeat size with ovarian dysfunction. *Human Reproduction*. 2005; 20(2):402–412. [PubMed: 15608041]
- Syrrou M, Georgiou I, Grigoriadou M, Petersen MB, Kitsiou S, Pagoulatos G, et al. FRAXA and FRAXE prevalence in patients with nonspecific mental retardation in the Hellenic population. *Genetic Epidemiology*. 1998; 15(1):103–109. [PubMed: 9523214]
- Tassone F, Hagerman RJ, Chamberlain WD, Hagerman PJ. Transcription of the FMR1 gene in individuals with fragile X syndrome. *American Journal of Medical Genetics (Seminars in Medical Genetics)*. 2000; 97(3):195–203. [PubMed: 11449488]
- Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ. A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. *Journal of Molecular Diagnostics*. 2008; 10(1):43–49. [PubMed: 18165273]
- Toledano-Alhadeff H, Basel-Vanagaite L, Magal N, Davidov B, Ehrlich S, Drasinover V, et al. Fragile-X carrier screening and the prevalence of premutation and full-mutation carriers in Israel. *American Journal of Human Genetics*. 2001; 69(2):351–360. [PubMed: 11443541]
- Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*. 1991; 65(5):905–914. [PubMed: 1710175]
- Watson MS, Breg WR, Hobbins JC, Mahoney MJ. Cytogenetic diagnosis using midtrimester fetal blood samples: Application to suspected mosaicism and other diagnostic problems. *American Journal of Medical Genetics*. 1984; 19(4):805–813. [PubMed: 6517103]
- Wittenberger MD, Hagerman RJ, Sherman SL, McConkie-Rosell A, Welt CK, Rebar RW, et al. The FMR1 premutation and reproduction. *Fertility and Sterility*. 2007; 87(3):456–465. [PubMed: 17074338]
- Yrigollen CM, Durbin-Johnson B, Gane L, Nelson DL, Hagerman R, Hagerman PJ, Tassone F. AGG interruptions within the maternal FMR1 gene reduce the risk of offspring with fragile X syndrome. *Genetics in Medicine*. 2012
- Yrigollen CM, Tassone F, Durbin-Johnson B. The role of AGG interruptions in the transcription of FMR1 premutation alleles. *PLoS One*. 2011; 6(7):e21728. [PubMed: 21818263]
- Yu S, Pritchard M, Kremer E, Lynch M, Nancarrow J, Baker E, et al. Fragile X genotype characterized by an unstable region of DNA. *Science*. 1991; 252(5010):1179–1181. [PubMed: 2031189]
- Yuhas J, Walichiewicz P, Pan R, Zhang W, Casillas EM, Hagerman RJ, et al. High-risk fragile x screening in Guatemala: Use of a new blood spot polymerase chain reaction technique. *Genetic Testing and Molecular Biomarkers*. 2009; 13(6):855–859. [PubMed: 19810826]

Fig. 1.

Capillary electrophoresis (CE) analysis showing the presence of an expanded *FMRI* allele in a DD male (**a** and **b**), in an autism female (**c**), and in an autism male (**d**). Two premutation alleles were also identified in the DD group (1 female, **e**; 1 male, **f**). The *x-axis* indicates the number of base pairs and the *y-axis* indicates relative fluorescence intensity

Fig. 2.

Boxplots of CGG-repeat number by diagnosis. The *solid lines* inside each *box* represent the medians in each diagnosis group, the *lower* and *upper edges* of each *box* represent the 25th and 75th percentiles in each group, respectively; the lower and upper 'whiskers' represent the smallest and largest observations, respectively, that lie within 1.5 times the interquartile range away from the edges of each box. Outlying observations are indicated with a *circle*

Table 1CGG repeat number by diagnosis^a

Diagnosis	Mean (SD)	Median (Range)
Typical development (n = 346)	30.0 (4.3)	30 (17–50)
Autism (n = 309)	30.1 (4.9)	30 (17–50)
ASD (n = 144)	29.6 (4.8)	30 (13–48)
Developmental delayed (n = 146)	29.8 (4.6)	30 (15–46)
Autism/ASD pooled (n = 453)	29.9 (4.8)	30 (13–50)

^aExpanded alleles or males with 2 alleles not included in summaries

Table 2

Number of AGG interruptions by diagnosis

	0 interruptions	1 interruption	2 interruptions	3 or 4 interruptions
Typical development (n = 346)	11 (3 %)	72 (21 %)	256 (74 %)	7 (2 %)
Autism (n = 309)	6 (2 %)	68 (22 %)	225 (73 %)	10 (3 %)
ASD (n = 144)	5 (3 %)	34 (24 %)	101 (70 %)	4 (3 %)
Developmental delayed (n = 146)	7 (5 %)	31 (21 %)	102 (70 %)	6 (4 %)