

Neurobehavioral Phenotype in Carriers of the Fragile X Premutation

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There have been contradictory findings in the fragile X (fraX) literature about possible neurocognitive and psychological symptoms due to the fraX premutation (pM). The purpose of the present study was to investigate the relationship between CGG repeat length and neurobehavioral functioning in carriers of the fraX pM. Eighty-five female carriers of the pM with allele sizes ranging from 59–166 were administered a comprehensive IQ test (WAIS-III) and completed a questionnaire designed to measure psychopathology (Symptom Checklist (SCL)-90-R). No relationship between allele size and cognition was identified. A significant negative relationship between allele size and age was found, as well as a positive relationship between allele size and depression. Follow-up analyses separating small and large allele sizes (below and above 100 CGG repeats) indicated that individuals with larger allele sizes scored significantly higher on the Interpersonal Sensitivity and Depression subscales of a self-administered checklist assessing psychological symptoms (SCL-90-R). Despite the limitation of few individuals with high CGG repeat lengths, our findings suggest that females with larger premutated alleles (≥ 100 repeats) display some clinical manifestations of fraX syndrome.

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KEY WORDS: fragile X syndrome; premutation; allele size; psychological symptoms; cognition

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INTRODUCTION

Fragile X (fraX) syndrome, the most common heritable cause of mental retardation, results from a CGG trinucleotide repeat expansion on the *FMR1* gene. When approximately 200 repeats or more are present, the expanded repeat sequence and an adjacent CpG island are usually hypermethylated, a phenomenon that causes transcriptional silencing and results in decreased levels or absence of the *FMR1* protein (FMRP). The full mutation (FM) is associated with cognitive and neurobehavioral deficits in arithmetic reasoning, visual spatial abilities, processing sequential information, working memory, attention, difficulties with peer social interactions, gaze avoidance, and stereotypical behavior [Theobald et al., 1987; Cohen et al., 1988; Kemper et al., 1988; Freund and Reiss, 1991; Freund et al., 1992, 1993; Hagerman et al., 1992; Lachiewicz, 1992; Mazzocco et al., 1997].

While there are many studies supporting clinical manifestations associated with the FM, there has been an ongoing debate in the literature about potential neurocognitive and psychological symptoms due to the fraX premutation (pM). Premutated alleles exhibit an expansion in the CGG trinucleotide sequence from 50–200 repeats (normal = 6–40 repeats). Since the increase in repeat size is less than 200, hypermethylation does not typically occur. Unlike the fraX FM, it has been hypothesized that there is no interference with transcription of mRNA at the *FMR1* locus in premutated alleles, therefore resulting in normal levels of FMRP. The results of several studies have suggested clinical manifestations associated with fraX may be related to a threshold phenomenon and that there is a distinct separation between nonpenetrant pM carriers and phenotypically affected FM carriers [Reiss et al., 1993; Rousseau et al., 1994].

Recent advances in molecular biology, however, have allowed researchers and clinicians to more precisely measure *FMR1* mRNA and FMRP levels in carriers of premutated alleles. These studies have revealed possible molecular mechanisms that could be responsible for clinical manifestations associated with the fraX pM status. For example, it was recently reported that some

individuals carrying the pM have lower levels of FMRP than normal [Tassone et al., 2000a]. In another study, it was revealed that males with the pM have higher levels of *FMR1* mRNA, suggesting a compensatory mechanism to balance potential dysfunction of translational efficiency [Tassone et al., 2000b].

Although the majority of studies show no differences in cognition between individuals with the pM and normal controls [Mazzocco et al., 1993; Reiss et al., 1993; Rousseau et al., 1994; Riddle et al., 1998], anecdotal findings suggest that some individuals with the pM have impairments in cognitive functioning [Hagerman et al., 1996; Tassone et al., 2000a]. It also has been reported that some males and females with the pM demonstrate psychiatric problems [Dorn et al., 1994; Thompson et al., 1994; Sobesky et al., 1996; Franke et al., 1998]; however, methodological issues (e.g., sample size, potential ascertainment bias, control groups, and interview procedures) limit the conclusions that can be drawn from some of these studies. Although studies of pM-associated clinical features are inconclusive to date, the recent report of *FMR1* mRNA up-regulation in pM carriers [Tassone et al., 2000b] suggests that a more thorough investigation of the fraX pM phenotype is warranted.

The purpose of the study presented here is to examine the relationship between CGG repeat size and neurobehavioral characteristics in women with an *FMR1* pM. We hypothesized that a possible explanation for discrepant findings in women with the pM was related to the overinclusiveness of this *FMR1* mutation category. Specifically, we predicted that as CGG repeat length increased within the pM repeat length spectrum, carriers would have an increased likelihood of exhibiting clinical features typical of the neurobehavioral phenotype associated with the FM. Based on the work of [Tassone and colleagues \[2000\]^{Q2}](#), we further predicted that this effect would occur exclusively in pM carriers with allele sizes greater than 100 repeats.

METHODS

Subjects

Eighty-five women with an unmethylated fraX pM had a comprehensive evaluation composed of the following: (1) DNA analysis for the fraX genotype with a measurement of CGG amplification and methylation, (2) a thorough cognitive assessment with an IQ test, (3) questionnaires designed to measure psychiatric symptoms, and (4) demographic information, including family income, educational background, age, race, and social class. All subjects had a child affected with the FM and were evaluated in their homes throughout the United States and Canada as a part of a comprehensive study on fraX syndrome.

DNA Testing

Blood samples were obtained from each subject and sent to Kimball Genetics, Inc. (Denver, CO) for DNA analysis. Genomic DNA was isolated from 5 ml of peripheral blood samples using standard methodology

(Puregene kit, Gentra Systems, Inc., Minneapolis, MN). Both Southern blot and PCR analysis were performed on each sample as described by Taylor et al. [1994]. For Southern blot analysis, 5 mg of DNA was digested with EcoR I and Nru I, followed by electrophoresis in a 1% agarose/Tris acetate gel, and transferred to nylon membrane. The blots were hybridized with the *FMR1*-specific probe, StB12.3 [Oberle et al., 1991]. PCR analysis was performed using primers 1 and 3 as described by Brown et al. [1993], with several modifications. PCR products were separated by 6% denaturing polyacrylamide gel electrophoresis, transferred to a nylon membrane, and hybridized with oligonucleotide probe (CGG)₅. The Southern blot testing was used to rule out the possibility of mosaicism for an FM with the pM and also to determine methylation status. The CGG repeat number was calculated from the PCR autoradiograms. To keep our sample homogeneous, individuals who were mosaic or who had partially methylated alleles were excluded from the study.

Measures

Cognitive functioning. The Wechsler Adult Intelligence Scale, Third Edition [Wechsler, 1997] was used to measure overall intellectual functioning in the females with a pM. The Wechsler includes verbal and performance subtests that yield separate IQ scores. The IQ and index scores are standardized to have a mean of 100 and a standard deviation (SD) of 15. The subtest scores are standardized with a mean of 10 and an SD of 3. All scales have demonstrated excellent reliability and validity.

Psychiatric symptoms. The Symptom Checklist (SCL)-90-R [Derogatis, 1997] is a standardized self-report measure of psychological symptoms. This questionnaire was given to all subjects to assess levels of psychological distress. The 90 questions are clustered into the following symptom dimensions: somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. A Positive Symptom Distress Index, a Positive Symptom Total, and a Global Severity Index are generated as indicators of symptom intensity, symptom depth, and level of psychiatric disturbance. The normative mean is 50 with an SD of 10, and individuals are considered at risk if the Global Index score is ≥ 63 , or if any two primary dimension scores are ≥ 63 .

Statistical analyses. Analyses were conducted using a specific subset of the cognitive and SCL-90 dependent variables. The cognitive variables included verbal IQ, performance IQ, and full-scale IQ, as well as specific subtests of the WAIS-III. The WAIS-III subtests chosen for analysis included block design, digit span, and arithmetic. These subtests were chosen because previous reports have indicated that females with the fraX FM display deficits in visuospatial, attentional, and arithmetic tasks [Kemper et al., 1986; Freund and Reiss, 1991; Grigsby et al., 1992; Abrams et al., 1994]. SCL-90 variables consisted of the Global Severity score, as well as specific symptom dimensions that have been reported as characteristi-

cally problematic for females with the fraX FM [Reiss et al., 1988; Borghgraef et al., 1990; Hagerman et al., 1992; Lachiewicz, 1992; Freund et al., 1993]. These SCL-90 dimensions included interpersonal sensitivity, depression, and anxiety.

Since there was a significant inverse correlation between repeat size and age (see Results), a residualized variable was created based on the simple linear regression between age and pM repeat size. This residualized repeat size variable was used in all subsequent regression analyses assessing the association between allele size and cognitive or behavioral measures.

Based on the recent findings of an abnormal molecular mechanism in premutated alleles, additional analyses were conducted. A dichotomous variable was created with division of allele size as proposed by Tassone et al. [2000b]. Individuals with CGG repeat sizes less than 100 were in a group (n=66), while individuals with CGG repeats sizes 100 or greater were in a separate group (n=19). Group comparisons were conducted using analyses of covariance (ANCOVA) with age as a covariant. The same dependent variables used in the regression analyses described above were used in the ANCOVA analyses.

RESULTS

Descriptive statistics, including the means and SD of subject age, repeat size, IQ scores, and SCL-90 scores, are shown in Table I. Subjects performed in the normal

range on IQ measures and, as a group, did not display any strengths and weaknesses. The percentage of subjects scoring in the mildly mentally retarded and borderline ranges on the IQ measures was 2.35%, or 2 out of 85, a percentage that is not different from the prevalence of mental retardation and developmental disabilities in the general population (1.49%; chi square=2.61; $P > 0.1$) [Larson et al., 2001]. Mean scores for the SCL-90 indices and subscales were all within the normal range.

Results of the simple linear regression analyses indicated a statistically significant inverse correlation between CGG repeat size and age ($R = -0.349$, $P = 0.001$). After residualizing allele size for age, only the Depression subscale of the SCL-90 was significantly correlated with allele size ($R = 0.220$, $P = 0.043$).

Comparison of ages between the two derived allele size groups indicated significant age differences (see Table II). Therefore, ANCOVAs statistically controlled for age. These analyses indicated no differences in cognitive ability between women with repeat sizes of < 100 and those with repeat sizes of ≥ 100 . Significant differences between groups were found for the SCL-90 dimensions of interpersonal sensitivity and depression (see Table II). Although there were statistical differences between groups, it should be noted that mean scores for both groups were within the normal range. The percentage of subjects that scored in the clinically significant range within each group is displayed in Table II.

TABLE I. Descriptive Statistics of Allele Size, Age, WAIS-III and SCL-90 Scores

	N	Minimum	Maximum	Mean	SD
Allele size	85	59	166	88.76	18.24
Age	85	30.66	51.62	39.92	4.74
IQ test (WAIS-III)					
FSIQ	85	68	140	105.61	14.53
VIQ	85	65	135	103.99	13.43
PIQ	85	64	148	106.78	15.84
Vocabulary	85	5	17	11.13	2.76
Information	83	5	16	10.31	2.62
Comprehension	84	3	17	11.32	2.61
Arithmetic	85	1	16	10.38	2.80
Digit span	83	5	17	10.33	2.59
Picture arrangement	84	3	17	10.77	3.16
Picture completion	84	4	15	9.95	2.90
Block design	85	4	17	10.75	2.72
Matrix reasoning	83	4	18	12.00	2.93
Coding	83	5	19	11.77	2.76
Symbol search	73	5	19	10.79	2.42
Psychiatric symptoms (SCL90)					
Global severity	85	30	69	52.55	10.03
Positive symptom total	85	30	73	51.68	9.97
Positive symptom distress	85	37	69	53.19	8.06
Somatization	85	35	65	49.91	8.58
Obsessive compulsive	85	37	78	54.49	9.75
Interpersonal sensitivity	85	39	71	54.33	9.62
Depression	85	34	72	53.78	10.30
Anxiety	85	37	72	48.47	9.16
Hostility	85	40	74	52.48	8.87
Phobic anxiety	85	44	68	47.22	6.60
Paranoid Ideation	85	41	72	49.56	9.00
Psychoticism	85	44	71	52.88	8.60

TABLE II. Group Comparisons of Allele Size, Age, IQ Scores, and SCL-90 Dimension Scores for Premutation Carriers With CGG Alleles Sizes Below and Above 100 Repeats

	pM ^a allele size < 100 (n = 66)		pM allele size ≥ 100 (n = 19)		ANCOVA	
	Mean±SD	% in clinical range	Mean±SD	% in clinical range	F	P
Allele size	80.97 ± 8.15	NA	115.84 ± 17.79	NA	14.16	< .0001
Age	40.63 ± 4.52	NA	37.41 ± 4.81	NA	7.296	.008
SCL-90 scores						
Global severity	51.65 ± 10.28	18.2	55.68 ± 8.61	26.3	2.579	NS
Interpersonal sensitivity	53.2 ± 9.7	22.7	58.3 ± 8.6	36.8	4.3	.041
Depression	52.6 ± 9.9	19.7	57.8 ± 10.8	36.8	5.6	.020
Anxiety	47.9 ± 9.3	4.5	50.6 ± 8.4	5.3	1.0	NS
WAIS IQ scores						
FSIQ	106.64 ± 13.77	NA	102.05 ± 16.81	NA	.495	NS
VIQ	104.71 ± 13.24	NA	101.47 ± 14.16	NA	.136	NS
PIQ	108.03 ± 14.52	NA	102.42 ± 19.59	NA	.982	NS
Arithmetic	10.47 ± 2.77	NA	10.05 ± 2.93	NA	.089	NS
Digit span ^b	10.49 ± 2.43	NA	9.72 ± 3.08	NA	2.061	NS
Block design	10.91 ± 2.69	NA	10.21 ± 2.84	NA	.826	NS

Means and standard deviations (SD) of allele size, age, IQ and SCL-90 scores for individuals separated into groups based on premutation (pM) allele size < 100 and allele size ≥ 100 (df = 1,83). Percentage of individuals from each group in the clinical range on SCL-90-R subscales. ANCOVA's with age as a covariate.

^apM, premutation.

^bn, 66,18.

DISCUSSION

This is the first study of large sample size to find a significant association between CGG repeat length and neurobehavioral dysfunction in female carriers of the fraX pM. The hypothesis that more clinical features characteristic of fraX would be apparent as CGG length increases within the entire range of premutated alleles (i.e., 50–200) was not strongly supported by our linear regression analyses. The finding that carriers of the pM are not cognitively impaired is consistent with previous studies [Mazzocco et al., 1993; Reiss et al., 1993; Rousseau et al., 1994; Riddle et al., 1998].

Whereas the results of our regression analyses only supported the correlation of CGG repeat size and depression, the ANCOVA analyses demonstrated that larger allele sizes, as defined categorically, could be related to symptoms falling under both the interpersonal sensitivity and depression dimensions of the SCL-90. The behaviors encompassing these dimensions include deficits with interpersonal skills, withdrawn behavior, and depressed mood. These behaviors are often reported as problematic in adult females with the fraX FM [Hagerman and Sobesky, 1989; Borghgraef et al., 1990; Hagerman et al., 1992; Lachiewicz, 1992; Freund et al., 1993], and our results suggest that females in the upper pM range may have greater susceptibility to the psychological problems often associated with the FM phenotype. Similar to our findings, it has been reported by other research groups that females with a pM have schizotypal traits, emotional difficulties, including social anxiety, and an increased prevalence of mood disorders [Thompson et al., 1994; Sobesky et al., 1996; Franke et al., 1998]. Contrary to our hypothesis, individuals with larger allele sizes did not score significantly higher on the

anxiety dimension of the SCL-90. One possible explanation for this negative finding is that the construct of anxiety in the SCL-90 scale does not capture social anxiety, but rather symptoms of panic and generalized anxiety. Given that previous studies support mainly social anxiety in fraX females [Reiss et al., 1988; Freund et al., 1993; Steyaert et al., 1994; Franke et al., 1998], a different scale measuring this specific type of anxiety is recommended for future studies.

Since the lack of FMRP is ultimately what causes the phenotype in the FM, it has been suggested that the mild phenotype in premutated individuals is caused by lower than normal levels of protein. A recent study has demonstrated that six individuals with the pM had a significant deficit of FMRP, as well as behaviors associated with the fraX FM phenotype [Tassone et al., 2000a]. It is widely recognized that a repeat above 200 usually results in hypermethylation and silencing of the *FMR1* gene, thereby causing neurobehavioral manifestations of the fraX FM [Pieretti et al., 1991; Hansen et al., 1992; Pai et al., 1994; Reiss et al., 1995]. Recent findings suggest that there is a different molecular mechanism responsible for lower levels of FMRP in premutated alleles beyond a certain repeat threshold [Tassone et al., 2000b]. Specifically, *FMR1* mRNA levels are elevated up to fivefold in individuals with a pM allele size of ≥ 100 repeats. A current hypothesis to explain this phenomenon is that the high levels of mRNA are produced to compensate for a translational deficiency of the premutated *FMR1* mRNA, although translational deficiency ultimately results in lower levels of FMRP, even with up-regulation of mRNA production [Tassone et al., 2000b].

The results indicating that age is inversely related to repeat size is a robust statistical finding, which

suggests a biological disadvantage of lymphoblasts with larger premutated alleles. A similar age-dependent decrease in CGG repeat length has been observed in females with an FM [Rousseau et al., 1991]. This age-dependent finding for individuals with a pM implies that there is also a Darwinian-type selection in premutated blood stem cells for smaller allele sizes. It should be noted, however, that the inverse relationship of age and allele size was based on cross-sectional data and requires longitudinal follow-up of subjects with the pM, preferably both male and female, to verify the validity of the results.

One limitation of the current study is that our hypothesis was based mainly on the work of [Tassone and colleagues \[2000b\]^{Q3}](#), whose sample size was relatively restricted. Another limitation includes the small number of subjects with allele sizes above 100 repeats ($n=19$), compared to the number of subjects with fewer than 100 repeats ($n=66$). Future studies should seek to include a comparable number of subjects with a low and high range of repeats. Finally, this study would have been strengthened with the availability of comparison groups. For example, it would be of interest to compare the psychiatric profiles of individuals with a pM in the high repeat range to those of normal controls and individuals with an FM.

In summary, the cellular selection of smaller allele sizes and the recent findings of lower than normal levels of FMRP in premutated alleles suggest a potential continuum of clinical involvement for individuals in the 100–200 repeat range. Our findings support the hypothesis that while demonstrating cognitive abilities within the normal range, females with premutated alleles of high repeat length may be at greater risk for psychological symptoms characteristic of the fraX FM. Accordingly, a possible explanation for the varying results in the fraX pM literature is that individuals with low and high repeat sizes, as well as partially methylated and unmethylated pMs, have been included in the same group sample. In future studies, individuals with partially methylated and unmethylated pMs should be analyzed separately. In addition, a nonlinear regression model should be used to explore the relationships between repeat size, FMRP, and neurobehavioral variables since these relationships are most likely nonlinear throughout the pM range (50–200). The continued study of individuals with the pM is an important line of research that will aid in determining the spectrum of clinical manifestations associated with *FMR1* mutations. This research will have potential implications for prenatal counseling and identifying individuals who may be in need of therapeutic intervention.

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REFERENCES

- Abrams MT, Reiss AL, Freund LS, Baumgardner TL, Chase GA, Denckla MB. 1994. Molecular-neurobehavioral associations in females with the fragile X full mutation. *Am J Med Genet* 51:317–327.
- Borghgraef M, Fryns JP, van den Berghe H. 1990. The female and the fragile X syndrome: data on clinical and psychological findings in 7 fra(X) carriers. *Clin Genet* 37:341–346.
- Brown WT, Houck GE Jr, Jeziorowska A, Levinson FN, Ding X, Dobkin C, Zhong N, Henderson J, Brooks SS, Jenkins EC. 1993. Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test [published erratum appears in *JAMA* 1994;271:28]. *JAMA* 270:1569–1575.
- Cohen IL, Fisch GS, Sudhalter V, Wolf-Schein EG, Hanson D, Hagerman R, Jenkins EC, Brown WT. 1988. Social gaze, social avoidance, and repetitive behavior in fragile X males: a controlled study. *Am J Ment Retard* 92:436–446.
- Derogatis LR. 1997. Symptom checklist-90-R. Administration, scoring, and procedures manual, 3rd ed. Minneapolis: National Computer Systems, Inc. 123 p.
- Dorn MB, Mazzocco MM, Hagerman RJ. 1994. Behavioral and psychiatric disorders in adult male carriers of fragile X. *J Am Acad Child Adolesc Psychiatry* 33:256–264.
- Franke P, Leboyer M, Gansicke M, Weiffenbach O, Biancalana V, Cornillet-Lefebvre P, Croquette MF, Froster U, Schwab SG, Poustka F, Hautzinger M, Maier W. 1998. Genotype-phenotype relationship in female carriers of the premutation and full mutation of *FMR1*. *Psychiatry Res* 80:113–127.
- Freund LS, Reiss AL. 1991. Cognitive profiles associated with the fra(X) syndrome in males and females. *Am J Med Genet* 38:542–547.
- Freund LS, Reiss AL, Hagerman R, Vinogradov S. 1992. Chromosome fragility and psychopathology in obligate female carriers of the fragile X chromosome. *Arch Gen Psychiatry* 49:54–60.
- Freund LS, Reiss AL, Abrams MT. 1993. Psychiatric disorders associated with fragile X in the young female. *Pediatrics* 91:321–329.
- Grigsby J, Kemper MB, Hagerman RJ. 1992. Verbal learning and memory among heterozygous fragile X females. *Am J Med Genet* 43:111–115.
- Hagerman RJ, Sobesky WE. 1989. Psychopathology in fragile X syndrome. *Am J Orthopsychiatry* 59:142–152.
- Hagerman RJ, Jackson C, Amiri K, Silverman AC, O'Connor R, Sobesky W. 1992. Girls with fragile X syndrome: physical and neurocognitive status and outcome. *Pediatrics* 89:395–400.
- Hagerman RJ, Staley LW, O'Connor R, Lugenbeel K, Nelson D, McLean SD, Taylor A. 1996. Learning-disabled males with a fragile X CGG expansion in the upper premutation size range. *Pediatrics* 97:122–126.
- Hansen RS, Gartler SM, Scott CR, Chen SH, Laird CD. 1992. Methylation analysis of CGG sites in the CpG island of the human *FMR1* gene. *Hum Mol Genet* 1:571–578.
- Kemper MB, Hagerman RJ, Ahmad RS, Mariner R. 1986. Cognitive profiles and the spectrum of clinical manifestations in heterozygous fra (X) females. *Am J Med Genet* 23:139–156.
- Kemper MB, Hagerman RJ, Altshul-Stark D. 1988. Cognitive profiles of boys with the fragile X syndrome. *Am J Med Genet* 30:191–200.
- Lachiewicz AM. 1992. Abnormal behaviors of young girls with fragile X syndrome. *Am J Med Genet* 43:72–77.
- Larson SA, Lakin KC, Anderson L, Kwak Lee N, Anderson D. 2001. Prevalence of mental retardation and developmental disabilities: estimates from the 1994/1995 national health interview survey disability supplements. *Am J Ment Retard* 106:231–252.
- Mazzocco MM, Pennington BF, Hagerman RJ. 1993. The neurocognitive phenotype of female carriers of fragile X: additional evidence for specificity. *J Dev Behav Pediatr* 14:328–335.
- Mazzocco MM, Kates WR, Baumgardner TL, Freund LS, Reiss AL. 1997. Autistic behaviors among girls with fragile X syndrome. *J Autism Dev Disord* 27:415–435.
- Oberle I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas MF, Mandel JL. 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science* 252:1097–1102.
- Pai JT, Tsai SF, Horng CJ, Chiu PC, Cheng MY, Hsiao KJ, Wu KD. 1994. Absence of *FMR1* gene expression can be detected with RNA extracted from dried blood specimens. *Hum Genet* 93:488–493.

- Pieretti M, Zhang FP, Fu YH, Warren ST, Oostra BA, Caskey CT, Nelson DL. 1991. Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell* 66:817–822.
- Reiss AL, Hagerman RJ, Vinogradov S, Abrams M, King RJ. 1988. Psychiatric disability in female carriers of the fragile X chromosome. *Arch Gen Psychiatry* 45:25–30.
- Reiss AL, Freund L, Abrams MT, Boehm C, Kazazian H. 1993. Neurobehavioral effects of the fragile X premutation in adult women: a controlled study. *Am J Hum Genet* 52:884–894.
- Reiss AL, Freund LS, Baumgardner TL, Abrams MT, Denckla MB. 1995. Contribution of the FMR1 gene mutation to human intellectual dysfunction. *Nat Genet* 11:331–334.
- Riddle JE, Cheema A, Sobesky WE, Gardner SC, Taylor AK, Pennington BF, Hagerman RJ. 1998. Phenotypic involvement in females with the FMR1 gene mutation. *Am J Ment Retard* 102:590–601.
- Rousseau F, Heitz D, Oberle I, Mandel JL. 1991. Selection in blood cells from female carriers of the fragile X syndrome: inverse correlation between age and proportion of active X chromosomes carrying the full mutation. *J Med Genet* 28:830–836.
- Rousseau F, Heitz D, Tarleton J, MacPherson J, Malmgren H, Dahl N, Barnicoat A, Mathew C, Mornet E, Tejada I, [et al](#)^{Q4}. 1994. A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases. *Am J Hum Genet* 55:225–237.
- Sobesky WE, Taylor AK, Pennington BF, Bennetto L, Porter D, Riddle J, Hagerman RJ. 1996. Molecular/clinical correlations in females with fragile X. *Am J Med Genet* 64:340–345.
- Steyaert J, Decruyenaere M, Borghgraef M, Fryns JP. 1994. Personality profile in adult female fragile X carriers: assessed with the Minnesota Multiphasic Personality Profile (MMPI). *Am J Med Genet* 51:370–373.
- Tassone F, Hagerman RJ, Taylor AK, Mills JB, Harris SW, Gane LW, Hagerman PJ. 2000a. Clinical involvement and protein expression in individuals with the FMR1 premutation. *Am J Med Genet* 91:144–152.
- Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE, Hagerman PJ. 2000b. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am J Hum Genet* 66:6–15.
- Taylor AK, Safanda JF, Fall MZ, Quince C, Lang KA, Hull CE, Carpenter I, Staley LW, Hagerman RJ. 1994. Molecular predictors of cognitive involvement in female carriers of fragile X syndrome [comments]. *JAMA* 271:507–514.
- Theobald TM, Hay DA, Judge C. 1987. Individual variation and specific cognitive deficits in the fra(X) syndrome. *Am J Med Genet* 28:1–11.
- Thompson NM, Gulley ML, Rogeness GA, Clayton RJ, Johnson C, Hazelton B, Cho CG, Zellmer VT. 1994. Neurobehavioral characteristics of CGG amplification status in fragile X females. *Am J Med Genet* 54:378–383.
- Wechsler D. 1997. Wechsler adult intelligence scale, In: Administration and scoring manual. 3rd ed. San Antonio: The Psychological Corporation. 194 p.

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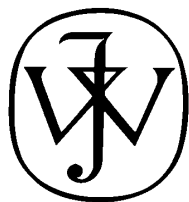
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