Prevalence and Instability of Fragile X Alleles

Implications for Offering Fragile X Prenatal Diagnosis

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OBJECTIVE: To document fragile X allele frequencies in a national referral population and evaluate CGG repeat expansion in mother-offspring transmissions.

METHODS: Fragile X DNA analysis by Southern blot and polymerase chain reaction was completed for 14,675 women, aged 18 years or older, and 238 mother-offspring pairs between January 1999 and June 2004. Carrier frequencies were compared between groups referred for different clinical indications. Direct comparison of the *FMR1* gene CGG repeat size in mother-offspring pairs determined intermediate and premutation allele stability.

RESULTS: Intermediate fragile X alleles (45–54 CGG repeats) occurred in 257 (1 in 57). The combined total number of patients with a premutation (55–200 CGG repeats) or full mutation (more than 200 CGG repeats) numbered 208 (1 in 71). One in 3.5 women with a family history of fragile X and 1 in 10 with premature ovarian failure had a *FMR1* mutation. This compared with 1 in 86 for those with a family history of mental retardation and 1 in 257 for women with no known risk factors for fragile X. Among 238 mother-offspring pairings, the smallest allele to expand to a full mutation in one generation contained 60 CGG repeats. Although 6.6% (4 of 60) of intermediate repeat alleles did expand, none jumped to a clinically significant full mutation–sized allele.

CONCLUSION: Based on these data and other published literature, offering invasive prenatal diagnosis for fragile X syndrome is not indicated for women with intermediate alleles. Invasive prenatal diagnosis is warranted for those women with a fragile X allele containing 55 or more CGG repeats.

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Fragile X syndrome is the leading inherited cause of mental retardation, affecting approximately 1 in 4,000 males and 1 in 8,000 females.[1] Based on a review of nine population-based studies among low-risk women (those with no family history suspicious for fragile X), the carrier frequency is estimated to be 1 in 303.[2] The prevalence is similar among most racial and ethnic groups[3].

In 1991, the fragile X mental retardation 1 (*FMR1*) gene and the underlying mutation associated with fragile X syndrome was discovered.[4–6] The mutation was shown to be an unstable CGG trinucleotide sequence located in the 5′ untranslated region of the *FMR1* gene. Most people in the general population have stable alleles that range from 6 to 44 CGG repeats. Alleles in the 45–54 CGG repeat range may show some instability and are described as "intermediate." Intermediate alleles have not been observed to expand to full mutations in one generation. Premutation alleles range in size from 55–200 CGG repeats and are generally unstable, resulting in an expansion of the CGG repeat sequence when passed from mother to child. Offspring of women who are premutation carriers are at risk to have fragile X syndrome. Expansion of the repeat region to more than 200 CGG trinucleotide sequences, called a full mutation, leads to methylation of the expanded allele resulting in nonexpression of the fragile X gene and hence the absence of the *FMR1* protein. Both males and females who fail to produce *FMR1* protein develop the classical fragile X phenotype associated with mental retardation.

Carrier testing recommendations, as outlined by the American College of Obstetricians and Gynecologists (ACOG) Committee on Genetics,[7] include pa-

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tients with a family history of mental retardation or a history of fragile X mental retardation, known carriers of the fragile X premutation or full mutation, and any woman who has ovarian failure or an elevated follicle-stimulating hormone level before age 40 years without a known cause. An increasing number of centers are routinely offering fragile X carrier screening to their entire prenatal population.

Although molecular advances have allowed for reliable diagnostic testing, our understanding of the factors that influence the risk of expansion remains limited. For the purposes of genetic counseling today, risk of expansion estimates are limited to one factor, the number of CGG repeats found in the maternal allele.[8] Genetic counseling for individuals shown to have an intermediate allele has proven especially challenging. To date, there is no report of a CGG repeat less than 59 expanding to a full mutation in one generation. However, instability of intermediate alleles has been reported, including expansion to a full mutation in two generations.[9]

Genzyme's Molecular Diagnostic Laboratory is a referral center for patients seeking DNA analysis for many genetic conditions. In this investigation, we report our national experience with samples sent for fragile X testing and examine expansion of intermediate or premutation alleles in mother-offspring transmissions. Although the intermediate allele data presented is the primary focus, fragile X allele detection rates among a large cohort of women referred for various clinical indications are included. This adds to the body of literature available to clinicians and is useful when counseling patients for whom fragile X carrier testing is being considered.

MATERIALS AND METHODS

This study included patients referred for fragile X DNA analysis between January 1999 and June 2004. Institutional review board approval was obtained from Lankenau Institute for Medical Research. Samples originated from a wide geographic distribution throughout the United States. DNA testing was completed for 14,675 women referred for fragile X carrier testing. Results, including the number of CGG repeats for each sample tested, were documented. All patients included in the carrier frequency comparison analysis were age 18 years or older and provided informed consent for testing. Obligate carriers and women already known to have an intermediate or premutation allele at the time of the referral were excluded for purposes of frequency determination but were included for mother-offspring transmission analysis. The study population was divided into four groups based on the primary referral indication provided by the referring physician. Comparison groups were as follows: 1) family history of fragile X; 2) family history of mental retardation of unknown cause, developmental delay, or autism; 3) personal history of premature ovarian failure; and 4) no family history suggestive of fragile X syndrome. Women for whom we did not have personal or family history information were excluded. Although this study population was not intentionally preselected, we acknowledge that it may not represent the general population of the United States; some samples sent for a positive family history may have been selected using screening criteria established by the referring physician.

To examine expansion of the fragile X CGG repeat, DNA from 238 mothers and their offspring was analyzed and the *FMR1* gene characterized. All women included in this aspect of the study had CGG repeats in the intermediate or premutation range and were requesting fragile X prenatal or postnatal DNA evaluation of their fetus or child. Transmission of the intermediate and premutation allele was documented as stable (no change in the number of CGG repeat sequences), expanded (an increase in the number of CGG repeat sequences), or contracted (a reduction in the number of CGG repeat sequences). The change in the number of CGG repeats was documented. The referral indication for maternal fragile X carrier testing was also recorded.

Extracted DNA was tested by Southern blot and polymerase chain reaction analysis to determine *FMR1* gene allele size(s) and methylation status. Polymerase chain reaction analysis was performed using the method of Brown et al,[10] with modification. The region surrounding the *FMR1* CGG repeat was amplified using primers from Fu et al.[4] The resulting amplification products were separated by electrophoresis through a 6% acylamide gel, immobilized on nylon membrane, hybridized with a ^{32}P-labeled 15-mer CGG probe, washed, and exposed to film. The amplification products were sized by comparison with a ^{32}P-labeled size standard that was included on the gel. Southern blot analysis was performed by the method of Rousseau et al,[11] with modification. The extracted DNA was digested with *Eco*RI and *Eag*I for 4 hours at 37°. This was followed by separation of the DNA fragments through a 0.8% agarose gel by electrophoresis for 16 hours at 100 volts. The DNA was transferred to nylon membrane, immobilized by exposure to ultraviolet light, and hybridized with a ^{32}P-labeled StB 12.3 probe. After washing, the nylon membrane was exposed to film to visualize the bands. *FMR1* allele size was interpreted as normal (fewer

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Table 1. Carrier Frequencies Observed in Genzyme Genetics Samples Tested Between January 1999 and June 2004

	No Family History	Suspicious Family History	Fragile X Family History	History of POF	Combined
Normal	9,538 (97.7)	4,357 (97.5)	262 (67.3)	53 (88.3)	14,210 (96.8)
Intermediate	183 (1.9)	58 (1.3)	15 (3.9)	1 (1.7)	257 (1.8)
Premutation	38 (0.4)	48 (1.1)	97 (24.9)	6 (10)	189 (1.3)
Full	0	4 (0.1)	15 (3.9)	0	19 (0.1)
Premutation and full combined	38 (0.4)	52 (1.2)	112 (28.8)	6 (10)	208 (1.4)
Total	9,759 (66.5)	4,467 (30.4)	389 (2.7)	60 (0.4)	14,675

POF, premature ovarian failure.
Data are expressed as n (%).

than 45 repeats), intermediate (45–54 repeats), premutation (55–200 repeats), or full mutation (more than 200 repeats) in accordance with the American College of Medical Genetics' Technical Standards and Guidelines for fragile X.[12] Precise comparison of maternal and fetal alleles for each mother-offspring pair was performed using side-by-side analysis of their samples on the same polymerase chain reaction gels.

RESULTS

Among the 14,675 women who underwent fragile X carrier testing, 2.6% had a family history of fragile X syndrome, fewer than 1% had a personal history of premature ovarian failure, approximately 30% had a family history of mental retardation, developmental delay, or autism (increased-suspicion group), and approximately 65% had no family history suggestive of fragile X syndrome (low-risk group). Data are provided in Table 1. Poisson regression technique,[13] with χ^2 testing to compare the groups, was applied. Overall, 1 in 71 women were found to have a premutation (55–200 CGG repeats) or full mutation (more than 200 CGG repeats). As expected, carrier identification was highest among women with a family history of fragile X and women with a personal history of premature ovarian failure. The difference in combined carrier frequency between these two high-risk groups and the remaining two groups (those with a family history of mental retardation, developmental delay, or autism, as well as those women at low risk with no known risk factors), was statistically significant ($P<.001$). Among women with a known fragile X family history, 97 (1 in 4) had a premutation, and 15 (1 in 26) had a full mutation, for a combined carrier frequency of 1 in 3.5. Six (1 in 10) women referred because of premature ovarian failure were identified as premutation carriers. Five had CGG repeat sizes under 100 (56, 64, 67, 80, 80), and one had a CGG trinucleotide repeat number of 120. There was also a significant difference between carrier frequencies in those with a family history of mental retardation, developmental delay, or autism and the low-risk (no family history) group ($P<.001$). Among those with a suspicious family history, 48 (1 in 93) had premutations, and 4 (1 in 1,117) had full mutations (combined carrier frequency of 1 in 86), whereas 38 (1 in 257) women with no family history had a CGG repeat in the premutation range.

The observed overall intermediate allele frequency in our study population was 1 in 57 (1.8%). Intermediate allele frequencies between the referral groups were compared using a Poisson regression technique with a χ^2 test. Detection rates were highest among women with a family history of fragile X syndrome (1 in 26) and significantly different ($P<.001$) from those with a suspicious family history (1 in 77). Women with a family history of fragile X had a 2.28-fold higher risk to be an intermediate carrier (95% confidence interval for relative risk: 1.36–3.80) than those with either a family history of mental retardation, developmental delay, or autism or those with no known family history.

Among the 238 mother-offspring pairs tested, 111 involved transmission of the normal allele from the unaffected X chromosome. Of the remaining 127 pairs, 60 mothers had intermediate alleles (45–54 CGG repeats), and 67 were premutation carriers (55–200 CGG repeats). Further details of the transmission data are shown in Tables 2 and 3.

The vast majority of the intermediate alleles (56 of 60 or 93.4%) remained stable through transmission from mother to fetus or child (no change in CGG repeat size). Of the 6.6% (4 of 60) of intermediate repeat alleles that expanded, none jumped to a clinically significant full mutation–sized allele. Thirty-nine of 40 (97.5%) alleles with 45–49 CGG repeats remained stable during transmission; one expanded from 46 to 47 CGG repeat sequences. Of the 20 transmissions involving maternal CGG repeats in the 50–54 CGG repeat range, three were unstable. Two expanded into the premutation range; one mother

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Table 2. Referral Indication and *FMR1* Allele Instability Data for Mother-Offspring Transmissions

Referral Indication	Number of Stable Transmissions	Number of Unstable Transmissions	Total Transmissions
Maternal *FMR1* CGG repeat size 45–49			
Family history of FXS	1	0	1
Family history suspicious of FXS	10	0	10
No family history	21	0	21
Mother known carrier	6	0	6
Indication unknown	1	1	2
A. Subtotal for 45–49 range	**39**	**1**	**40**
Maternal *FMR1* CGG repeat size 50–54			
Family history of FXS	0	0	0
Family history suspicious of FXS	1	0	1
No family history	8	2	10
Mother known carrier	7	1	8
Indication unknown	1	0	1
B. Subtotal for 50–54 range	**17**	**3**	**20**
Intermediate allele total (A+B)	**56**	**4**	**60**
Maternal *FMR1* CGG repeat size 55–59			
Family history of FXS	1	0	1
Family history suspicious of FXS	1	0	1
No family history	3	0	3
Mother known carrier	8	1	9
Indication unknown	1	0	1
C. Subtotal for 55–59 range	**14**	**1**	**15**
Maternal *FMR1* CGG repeat size greater than 60			
Family history of FXS	0	21	21
Family history suspicious of FXS	1	5	6
No family history	0	1	1
Mother known carrier	1	18	19
Indication unknown	2	3	5
D. Subtotal for greater than 60 range	**4**	**48**	**52**
Premutation and full mutation allele total (C+D)	**18**	**49**	**67**
Total all alleles	**74**	**53**	**127**

FXS, fragile X syndrome.
Bold lines indicate subtotals for the different allele size ranges.

with 52 CGG repeats expanded to 55 during transmission to her fetus, and another mother with 54 CGG repeats expanded to 59 fetal CGG trinucleotide sequences. One contracted from 52 repeats to 51 repeats.

Premutation allele instability in mother-offspring transmissions (maternal CGG repeats ranged in size from 55 to 120) was 73.1% (49 of 67). Of those that demonstrated instability, 10 premutations (all less than 80 CGG repeats) remained in the premutation

Table 3. CGG Repeat Size Change in Unstable Transmissions for Carriers of Alleles With 45–60 CGG Repeats

Referral Indication	Maternal *FMR1* CGG Repeat Size	Offspring *FMR1* CGG Repeat Size	*FMR1* CGG Repeat Size Change
Indication unknown	46	47	1
No family history	52	51	−1
No family history	52	55	3
Mother known carrier	54	59	5
Mother known carrier	56	60	4
Family history of fragile X	60	62	2
Family history of fragile X	60	Greater than 200	Greater than 140
Family history of fragile X	60	68	8
Family history suspicious of fragile X	60	63	3

Note that 44 alleles with more than 60 CGG repeats also showed unstable transmission but are not documented in this table.
Each table row represents a single mother-offspring transmission.

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range when transmitted from mother to offspring while 39 expanded to a full mutation. None of the premutations contracted. The smallest premutation to expand to a full mutation in a single transmission step was 60 repeats. All mutations of more than 85 CGG repeats expanded to a full mutation.

DISCUSSION

As expected, premutation and full mutation carrier frequency was highest in women with a family history of fragile X syndrome. Our study also demonstrated a relatively high frequency of premutation carriers (1 in 10) in women with a personal history of premature ovarian failure. This is consistent with previous reports that estimate the premutation carrier frequency as 2% among women with sporadic premature ovarian failure and 14% for women with familial premature ovarian failure.[14] Our data supports the current screening recommendations for this subset of women. Our investigation is consistent with previous reports that document the risk for premature ovarian failure to be directly proportional to the number of CGG repeats. The risk of premature ovarian failure increases as CGG repeat size expands from 59 to 99 CGG repeats, but then decreases with alleles greater than 100 repeats.[15]

Numerous studies have proven the efficacy of fragile X testing for women with a family history of mental retardation, but only recently has the feasibility of general population screening been evaluated.[16–19] Toledano-Alhadef et al[19] reported a premutation carrier frequency (more than 55 CGG repeats) of 1 in 113 among 14,334 Israeli women with no family history of mental retardation. Most other studies report a carrier frequency in the 1/250–1/300 range.[2] In our study population of 9,759 low-risk women with no suspicious family history of fragile X syndrome, mental retardation, developmental delay, autism, or premature ovarian failure, 38 (1 in 257) had a premutation. It is apparent that, for a prenatal fragile X screening program to be effective, screening of all expectant mothers (regardless of background risk) is necessary.

Current clinical practice is to estimate the risk of expansion based on the number of maternal CGG repeats and interpret this in the context of the family history. In a large multicenter study, Nolin et al[8] evaluated mother-offspring transmissions in women with 49–65 CGG repeats who reported no family history of fragile X. Among the 84 women with 49–54 CGG repeats, 19% of the transmissions demonstrated instability, with the largest expansion being from 50 to 66 CGG repeats. Comparing data from women with 50–54 repeats, the instability rates are remarkably similar: 17.6% (3 of 17) in our data set compared with 17.3% (14 of 81) in the Nolin et al[8] data. In addition, this study concurs with the findings of Nolin et al[8] and Strom et al[20], in that no intermediate allele expanded to a full mutation, and the smallest-sized CGG repeat to expand to a full mutation was 60 (compared with 59 in the Nolin study and 64 in the Strom study).

The intermediate frequency noted in our study was 1 in 57. This compares with the 1 in 72 to 1 in 145 reported by Murray et al.[21] As this study shows, identification of fragile X intermediate repeat alleles is an issue for all referral populations. The fact that intermediate alleles were significantly more frequent in women with a family history of fragile X is of interest. Intermediates identified within fragile X families are presumed to not be segregating with the fragile X allele, but this needs to be further evaluated.

A frequent concern associated with fragile X prenatal population screening is the follow-up and counseling for women who are identified as carriers of intermediate alleles (45–54 CGG repeats). Expectant parents may experience significant anxiety and fear associated with this finding and are often given the option of invasive prenatal diagnosis to determine fetal CGG repeat allele size(s). Our data and that of others demonstrate that some intermediates are unstable, but the magnitude of the instability is minimal: only a few CGG repeats. The research question under investigation by many is why some of the intermediates are stable, generation after generation, and why others of the exact same size expand. If the intermediates that expand can be further characterized, the hope is that clinicians could have a clinically applicable test that distinguishes intermediate alleles that are precursors in future generations to a fragile X premutation from those that are not. In the absence of this knowledge, clinicians can be reassuring and stress that an intermediate allele, regardless of family history, is of no immediate clinical significance. Patients with intermediates are not at increased risk for medical problems, and their offspring are not at immediate risk for classic symptoms of fragile X syndrome. Genetic counseling should stress that the presence of an intermediate mutation of fewer than 55 repeats is not associated with a significant risk of expansion to more than 200 CGG repeats and, therefore, there is little risk of the fragile X syndrome phenotype in offspring. Regardless, many women may proceed to a chorionic villus sampling (CVS) or amniocentesis once informed that they are an intermediate fragile X carrier. Based on our investigation and the previously cited Nolin study[8] of more than 1,500 women with intermediate alleles and Strom et al study[20] of 307

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prenatal cases, the risk of expansion to a clinically significant full mutation is likely to be extremely rare and has not yet been documented for an allele with fewer than 59 CGG repeats.

A basic principle of any effective prenatal screening program is that it leads to the identification of a fetus with a clinically significant disorder.[22] Performing a CVS or amniocentesis on a woman with an intermediate fragile X expansion does not meet these criteria because there is an extremely low risk of fragile X syndrome in the fetus. Our analysis demonstrates that there is poor clinical utility in offering invasive prenatal diagnosis to women with intermediate alleles because expansion to a full mutation has not been demonstrated. Invasive prenatal diagnosis should be offered only if the mother possesses a premutation or a full mutation. By avoiding invasive prenatal diagnosis in intermediate allele carriers, the cost of a fragile X screening program and the iatrogenic pregnancy loss from CVS and amniocentesis can be significantly reduced.

In conclusion, these data, as well as information from other published reports, reveal that identification of all fetuses at risk for fragile X–related mental retardation requires general population prenatal screening because many affected fetuses are borne by women with no known risk factors for this disorder. However, invasive prenatal diagnosis for fragile X syndrome is not indicated unless the CGG repeat size is in the premutation or full mutation range (more than 55 CGG repeats). Performing invasive prenatal diagnosis in women with intermediate range alleles exposes women to the risk of miscarriage from the procedure that is not justified, given the extremely low probability that a CGG repeat of less than 55 will expand to a clinically significant full mutation.

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