Am. J. Hum. Genet. 61:1362–1369, 1997

Fragile X Premutations Are Not a Major Cause of Early Menopause

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Summary

Fragile X syndrome is an X-linked mental retardation condition that usually is due to a trinucleotide-repeat expansion in the FMR1 gene. Whereas full-mutation alleles (>230 repeats) lead to fragile X syndrome, premutation alleles (~60–200 repeats) are apparently nonpenetrant. However, previous studies have suggested that female premutation carriers may have an increased incidence of premature menopause. To test this possible association, we screened for premutation alleles among 216 women with early menopause (at age <47 years), 33 of whom had premature menopause (at age <40 years), as well as among 107 control women, all of whom were ascertained solely on the basis of age at menopause. No full-mutation alleles were found; and only one premutation allele was found, but, it was in a member of the control group. These results are consistent with what would be expected on the basis of chance only. Our sample size was sufficient to rule out a $\geqslant$3-fold increased risk of early menopause and a $\geqslant$9-fold increased risk of premature menopause due to an FMR1 premutation, under a model considering the risk of both sporadic and familial early menopause. Likewise, our results rule out a $\geqslant$4-fold increased risk of familial early menopause and a $\geqslant$26-fold increased risk of familial premature menopause, under a less probable model in which only familial early menopause is considered. These results indicate that the fragile X premutation is not a major risk factor for early menopause and suggest that the risk of premature menopause to fragile X–premutation carriers may not be as great as that reported elsewhere.

Received July 24, 1997; accepted for publication September 15, 1997; electronically published November 14, 1997.

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0002-9297/97/6106-0021$02.00

Introduction

Fragile X syndrome is an X-linked dominant condition with incomplete penetrance. The syndrome is usually the result of an expansion of the CGG-repeat tract in the 5′ UTR of the FMR1 gene (Warren and Nelson 1994; Warren and Ashley 1995). Normal alleles have 6–54 repeats, usually with one to three interrupting AGG triplets (Fu et al. 1991; Snow et al. 1993; Kunst and Warren 1994). Unstable premutation alleles have ~50–200 repeats and are susceptible to expansion when passed from a carrier female to her offspring (Fu et al. 1991; Snow et al. 1993). In the general population, ~1/253 women are carriers of premutation alleles with >54 repeats (Rousseau et al. 1995; Spence et al. 1996). Full mutations have >230 repeats (Rousseau et al. 1991), resulting in hypermethylation of FMR1 and inactivation of transcription (Pieretti et al. 1991; Sutcliffe et al. 1992); the consequent lack of functional FMR protein (FMRP) leads to fragile X syndrome.

For the past decade, investigators have debated the possibility of increased incidence of ovarian dysfunction among carriers of a fragile X mutation. Ovarian response to exogenous stimulation reportedly has been found to be decreased in fragile X carriers (Black et al. 1995). DZ twinning reportedly has been found to be increased in fragile X carriers (Fryns 1986; Tizzano and Baiget 1992), and in one study the increase was observed in premutation carriers but not in full-mutation carriers (Turner et al. 1994). However, Sherman et al. (1988) found no significant difference in the twinning rate between fragile X carriers and carriers of hemophilia A, another X-linked condition, suggesting that the increase in twinning observed in other studies may have been a consequence of the method of ascertainment. In a more recent study, Sherman et al. (1996) followed the pregnancies of fragile X carriers who were known to be carriers before they became pregnant. In this prospective-mother design, no increased twinning rate was observed for either premutation or full-mutation carriers.

There also have been case reports of premature ovarian failure (menopause at age <40 years) cosegregating with a fragile X premutation in families (Conway et al. 1995; Vianna-Morgante et al. 1996). Several reports have suggested that fragile X carriers have a risk of pre-

mature ovarian failure that is increased above the risk in the general population (Cronister et al. 1991; Schwartz et al. 1994; Partington et al. 1996). All three of these studies ascertained fragile X–carrier women and compared their rate of premature ovarian failure with that of controls or with that of the general population. Thus, it is possible that these studies faced the same ascertainment problems seen in the twinning studies discussed above.

Conway et al. (1995) published a study assessing the presence of the fragile X premutation among 46 women with premature ovarian failure, 9 of whom reported a family history of premature ovarian failure. No premutation carriers were detected among the 37 women with sporadic premature ovarian failure. However, 2 of the 9 women with familial premature ovarian failure were found to carry the premutation. This study avoided some of the possible biases involved in the ascertainment of fragile X carriers but still lacked a comparable control group. These results failed to be replicated by Martin et al. (1997), who found no premutation carriers among 21 women with premature ovarian failure, although only 5 study members reported a family history of premature ovarian failure. To better investigate the alleged association between the fragile X premutation and age at menopause, we have determined the fragile X status for 216 women with early ovarian failure (menopause at age <47 years) and for 107 controls. Although we cannot rule out all ascertainment biases (e.g., incomplete participation in the study and Boston as the study area), we have avoided some of the ascertainment issues raised by previous studies, by ascertaining subjects solely on the basis of age at menopause and by selecting controls through the same methods as those used to select cases.

Subjects and Methods

Subjects

From a population-based survey of 10,600 women at age 45–54 years, in the greater Boston area, 344 cases with early menopause (average age 42.4 years) and 344 age-matched controls who were still menstruating or who had had menopause at age >46 years were selected for the study of epidemiological risk factors. The protocol, approved by the human-subjects committee of the Brigham and Women's Hospital, included collection of a buffy-coat specimen whenever possible. Findings from the study included reproductive history (Cramer et al. 1995*a*) and family history of early menopause (Cramer et al. 1995*b*) as risk factors for early menopause. This study of fragile X premutations included three groups of women who had a buffy coat available: a so-called familial group of 108 early-menopause cases with a family history of early menopause, a so-called sporadic group of 108 randomly selected early-menopause cases with no family history of early menopause, and 108 randomly selected controls with neither early menopause nor a family history of early menopause.

Within the familial group, 17 women had had menopause at age <40 years, 50 women had completed menopause at age 40–43 years, and 41 women had had menopause at age 44–46 years. Within the sporadic group, 16 women had had menopause at age <40 years, 50 women at age 40–43 years, and 42 women at age 44–46 years. The control women had had menopause at age >46 years or were still menstruating at the time of participation in the study. The study participants were ascertained solely on the basis of age at menopause, as described elsewhere (Cramer et al. 1995*a,* 1995*b*).

In this paper, the terms "premature menopause" and "premature ovarian failure" are used interchangeably to refer to the natural completion of menopause at age <40 years. Likewise, the terms "early menopause" and "early ovarian failure" refer to the natural completion of menopause at age <47 years.

DNA Studies

All DNA analyses were performed without knowledge of the study-group status of the subjects. DNA was extracted from frozen buffy coats by treatment with proteinase K and RNase, followed by phenol/chloroform extraction, ethanol precipitation, and resuspension in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). PCR was performed with the incorporation of $\alpha[^{32}P]$-dCTP and by use of the f and c primers, which amplify the region containing the CGG repeat in the 5′ UTR of FMR1 (Fu et al. 1991). The PCR conditions were modified from the conditions described by Brown et al. (1993) and by Kunst and Warren (1994). Ten microliters of PCR mix (1.1 × PCR Buffer II [Perkin Elmer Cetus], 15% dimethyl sulfoxide [Sigma], 1.875 mM $MgCl_2$ [Perkin Elmer Cetus], 0.3 mM each dATP, dCTP, dTTP, and 7-deaza-dGTP [Pharmacia], 0.75 μM each f and c primers, 1 unit perfect match [Stratagene]/ml, and 0.4 mCi $\alpha[^{32}P]$-dCTP/ml) were added to 1 μl of DNA (~50–200 ng). The tubes were heated in a Perkin Elmer 9600 Thermal Cycler at 95°C for 10 min, then at 80°C for 5 min. While holding at 80°C, 4 μl of enzyme mix (1 × PCR Buffer II [Perkin Elmer Cetus] and 0.375 units *Taq*/ml) were added to each reaction tube. The PCR reaction consisted of 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s; this was followed by a final extension of 72°C for 10 min. The PCR products were run on a 5.1% acrylamide gel with formamide, at 80 W for 4.5 h, along with a sequencing ladder, to determine the number of repeats in each allele. DNA from a known premutation carrier with alleles of 29 and ~85 repeats was included, in each reaction, as a positive control.

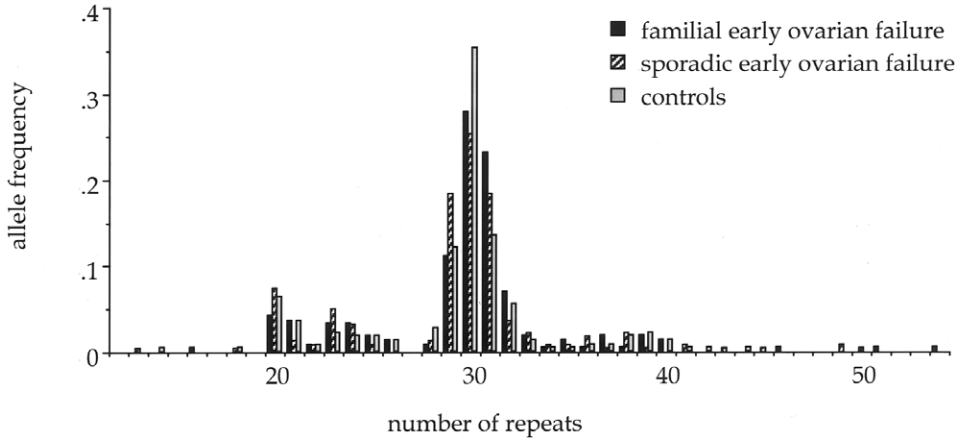

Figure 1 Distribution of FMR1 trinucleotide-repeat lengths, for all three study groups

To ensure that no underamplified alleles >85 repeats were missed, apparently homozygous samples that produced only one band were reanalyzed by a more sensitive PCR method modified from the study by Brown et al. (1993). The PCR was performed as described above, without $\alpha[^{32}P]$-dCTP and with the following modifications: the enzyme mix consisted of 0.375 units of 15:1 *Taq:Pfu* (Stratagene) blend and 1 × PCR Buffer II, and the PCR cycle consisted of 94°C for 30 s, 65°C for 60 s, and 72°C for 120 s. The PCR product was run on a 6% acrylamide gel, at 80 W for 3.25 h, was blotted overnight onto a Hybond-N^+ nylon transfer membrane (Amersham), and was probed with a ^{32}P-end-labeled $(CGG)_{10}$ probe for 2.5 h at 63°C, in Rapid-hyb buffer (Amersham). DNA from a known premutation carrier with a normal allele of 30 repeats and a premutation allele of ~100 repeats was included as a positive control.

On the basis of the observed allele distribution in the study group, we calculated the probability of finding at least 1 individual of the 324 who was homozygous, for each of the possible number of repeats within the normal range. By use of a probability of $\leqslant 10\%$, individuals who appeared to be homozygous for the less common alleles were analyzed further to help determine whether these individuals were indeed homozygous or whether they had one allele deleted on one of their X chromosomes. These samples were typed for two polymorphisms flanking the FMR1 repeat region, FRAX-AC1 and FMRa (Kunst and Warren 1994). One sample that was apparently homozygous for these markers also was examined for DXS548 (Riggins et al. 1992) and FRAX-AC2 (Zhong et al. 1993).

Data Analysis

The frequency of early ovarian failure in the general population is 10% (Coulam et al. 1986), and 37.5% of these women report a family history of early ovarian failure (Cramer et al. 1995*b*). Using these frequencies, we calculated the expected frequency of premutation carriers in each of the three study groups. The calculations were performed with the assumption that there was no association between premutation and early ovarian failure or that having a premutation doubles (or triples, etc.) the risk of early ovarian failure above that of the general population. Two different models were used in these calculations. In model 1, the premutation allele increases equally the risk of familial early ovarian failure and of sporadic early ovarian failure. In model 2, the premutation allele increases only the risk of familial early ovarian failure. A similar set of calculations was completed for premature ovarian failure, based on a frequency of premature ovarian failure of 1% and a frequency of family history of same of 37.5% among women with premature ovarian failure (Cramer et al. 1995*b*).

Statistical methods were performed as indicated in the Results section, using an alpha level of .05 to determine significance. The Bonferroni method was used to correct for multiple comparisons.

Results

Allele Distributions

In this study, the overall distribution of normal FMR1 alleles was similar to previously reported distributions (Brown et al. 1996; Meadows et al. 1996). The three study groups did not differ in allele distribution, by χ^2 analysis (fig. 1). The rate of homozygosity of FMR1 repeat lengths was 58% in the familial early–ovarian failure group, 53% in the sporadic early–ovarian failure group, and 46% in the control group.

Although these rates of repeat homozygosity are not

Table 1

Proportion of Premutation Carriers Predicted among Women with Familial or Sporadic *Early* Menopause or among Controls, at Various Levels of Relative Risk

	Proportion of Premutation Carriers (%)		
Relative Risk	Familial Early Menopause	Sporadic Early Menopause	Controls
Model 1:			
1	.4	.4	.4
2	.8	.8	.4
3	1.2	1.2	.3
4	1.6	1.6	.3
5	2.0	2.0	.2
6	2.4	2.4	.2
7	2.8	2.8	.1
8	3.2	3.2	.1
9	3.6	3.6	.0
Model 2:			
1	.4	.4	.4
2	.8	.4	.4
3	1.2	.4	.4
4	1.6	.4	.3
5	2.0	.4	.3
6	2.4	.4	.3
7	2.8	.4	.3
8	3.2	.4	.3
9	3.6	.4	.3

significantly different, by χ^2 analysis, we characterized nine women in the study group who appeared to be homozygous for uncommon FMR1 repeat lengths (i.e., alleles for which there was a <10% chance of finding at least one homozygote in the study group of 324 women, on the basis of the observed allele frequencies in the study group). Of these nine women, three were from the familial early-menopause group, two were from the sporadic early-menopause group, and four were from the control group. To examine the possibility that any of these individuals carried a deletion encompassing the FMR1 trinucleotide-repeat region, we analyzed these samples for two polymorphic loci in the area. FRAX-AC1 is a dinucleotide polymorphism that is ~7 kb upstream of the FMR1 repeat tract (Richards et al. 1991), and FMRa is a biallelic marker exhibiting a 1-bp polymorphism in intron 1 of FMR1, 6 kb downstream of the repeat (Kunst and Warren 1994). Three individuals were heterozygous for FRAX-AC1, and five individuals were heterozygous for both markers. Hence, it is unlikely that any of these eight individuals carry a large deletion of this area. Although this analysis would not have detected a small deletion involving the repeat region, such deletions cause fragile X syndrome (de Graaff et al. 1995), and none of these individuals reported a family history of mental retardation. One individual from the control group was homozygous for both FRAX-AC1 and FMRa, as well as for DXS548 and FRAX-AC2 (150 kb upstream and 11 kb downstream of the repeat, respectively) (Richards et al. 1991; Riggins et al. 1992). Because we could not rule out the possibility of a deletion in FMR1 in this individual, she was eliminated from the control group for the remainder of the study, although her inclusion would not have modified our conclusions.

Early Menopause and Fragile X Premutations

Among all three groups, only one premutation carrier was identified. She had alleles with 29 and 61 repeats and was a member of the control group. Because the frequency of premutation carriers in the general population is ~1/253 and because there were a total of 323 women in this study, the finding of one premutation carrier is entirely consistent with the expected results based on chance only (i.e., no association between FMR1 premutation carriers and early ovarian failure).

By use of Fisher's exact test, the observed results were compared with the expected results from the models described in the Subjects and Methods section. Under model 1, the observed results did not differ significantly from those expected, under the assumption that there was no association between premutation and early ovarian failure or that carrying a premutation allele doubles the chance of early ovarian failure (table 1). However, the results were significantly different from those predicted by a $\geqslant$3-fold increased risk of early ovarian fail-

Table 2

Proportion of Premutation Carriers Predicted among Women with Familial or Sporadic *Premature* Menopause or among Controls, at Various Levels of Relative Risk

	Proportion of Premutation Carriers (%)		
Relative Risk	Familial Premature Menopause	Sporadic Premature Menopause	Controls
Model 1:			
1	.4	.4	.4
2	.8	.8	.4
3	1.2	1.2	.4
4	1.6	1.6	.4
5	2.0	2.0	.4
6	2.4	2.4	.4
7	2.8	2.8	.4
8	3.2	3.2	.4
9	3.6	3.6	.4
Model 2:			
1	.4	.4	.4
2	.8	.4	.4
3	1.2	.4	.4
4	1.6	.4	.4
5	2.0	.4	.4
6	2.4	.4	.4
7	2.8	.4	.4
8	3.2	.4	.4
9	3.6	.4	.4
10	4.0	.4	.4
20	8.0	.4	.4
26	10.4	.4	.4

ure for premutation carriers. In this study, the power for detection of a 3-fold increase in risk was 93%.

Under model 2, the observed results were significantly different from those expected for a $\geqslant$4-fold increased risk of familial early ovarian failure due to a premutation (table 1). Under this model, the power for detection of a 4-fold increase in risk was 86%.

Premature Menopause and Fragile X Premutations

No FMR1 premutation carriers were found among the subset of 33 samples from women with premature ovarian failure. As in the case of early ovarian failure, described above, these results were not significantly different from the results expected if there were no association between FMR1 premutation alleles and premature ovarian failure. Of the 33 women with premature ovarian failure, 17 were familial cases. On the basis of results from the study by Conway et al. (1995), we would have expected to find four premutation carriers in our familial premature–ovarian failure group. By use of Fisher's exact test, these expected results were found to be significantly different from the observed results of no premutation carriers in this group.

Under model 1, the observed results were significantly different from those expected if the premutation increases the risk of both familial and sporadic premature ovarian failure by $\geqslant$9-fold above the 1% risk to the general population (table 2). The power for detection of a 9-fold increase in the risk of premature ovarian failure was 93%.

Under model 2, in which only the risk of familial premature ovarian failure is increased in premutation carriers, there is an increase in risk that is <26-fold above the general-population risk of 1% (table 2). Under this model, the power for detection of a 26-fold increase was 88%.

Discussion

The results of this study do not support the hypothesis that fragile X–premutation carriers are at a high risk of early menopause (at age <47 years). The observed results do not differ significantly from those expected, by chance, with no association between the premutation and early menopause. From these results, we can conclude that if having a premutation does increase the risk of early menopause, it increases the risk by <3-fold above the risk to the general population. These results indicate a much lower risk of early menopause associated with a premutation than that found by some previous reports.

For example, when the frequency of early ovarian failure in the general English population is also assumed to be 10%, the data reported by Partington et al. (1996) suggest that premutation carriers have at least a 7-fold increase in the risk of early ovarian failure.

Conway et al. (1995) suggested that premutation carriers have an increased risk of familial premature ovarian failure but not of sporadic premature ovarian failure. Under this model, our results indicate that an increased risk of familial early ovarian failure due to a premutation is <4-fold above the risk of familial early menopause in the general population. However, it seems unlikely that a premutation would increase just the familial early-menopause cases. Under the assumption that a premutation increases the risk for early menopause, not all premutation carriers would have early menopause, and not all family structures would make familial early ovarian failure obvious; such cases would be ascertained as sporadic. Therefore, a woman with the premutation and early menopause may or may not have similarly affected female relatives.

The actual prevalence of premutation carriers among the general population is an important factor in the statistical analysis of the data presented here. The figure 1/253 was derived from two different general-population screens (Rousseau et al. 1995; Spence et al. 1996). The data in the study by Spence et al. (1996) were collected from a Virginia population and therefore probably represent a reasonable estimate for our study population. However, the population screened in the study by Rousseau et al. (1995) was predominantly French Canadian, and haplotype analysis of the fragile X families in this population suggested a possible founder effect. Consequently, the premutation-carrier prevalence of 1/259, reported by Rousseau et al. (1995), may not reflect a general carrier frequency. Sherman (1995) estimated the frequency of premutation carriers in a general population of primarily northern European descent to be ~1/342–875. Repetition of this analysis by use of a premutation-carrier frequency of 1/342 does not change the results for the risk of early menopause, under either model. A carrier frequency of 1/875 only changes the results for model 2 (familial), in which a $\geqslant$5-fold increased risk of early menopause due to a fragile X premutation is ruled out.

However, if only the women with menopause at age <40 years (premature ovarian failure) are included in our analysis, there is not sufficient power to rule out a substantial increase in the risk of premature menopause due to a fragile X premutation. Unlike the results for the early-menopause analysis discussed above, these results are significantly affected by the prevalence of fragile X–premutation carriers. Under model 1 (sporadic and familial), we can rule out a 9-fold, 15-fold, or 19-fold increase in premature-menopause risk based on premutation-carrier frequencies of 1/253, 1/342, and 1/875, respectively. Under model 2 (familial), a 26–35-fold increase in risk is ruled out, for the three different carrier frequencies.

Because the fragile X premutation-carrier frequency of 1/253, used in this analysis, was derived in part from the study by Spence et al. (1996), it is likely that the lower frequency of 1/875 represents a conservative estimate for our analysis. In their Virginia population, 4/745 women carried FMR1 alleles with >54 repeats, giving an overall premutation frequency of 1/186 females. Thus, the results from our analysis using a frequency of 1/253 may be reasonable estimates of the true population characteristics. The results based on this premutation-carrier frequency are much lower than those suggested in previous studies. For example, among premutation carriers, Partington et al. (1996) found a frequency of premature ovarian failure of 28%. When a general-population frequency of premature ovarian failure in the English population is assumed to be 1%, these results suggest that premutation carriers have an increased risk of premature ovarian failure that is 28-fold above that of the general population. The data in the study by Conway et al. (1995) suggest that premutation carriers have a >50-fold increased risk for familial premature ovarian failure. These data predicted that we would find four premutation carriers among our 17 samples from women with familial premature ovarian failure, a predication that is significantly different from our observed results of no premutation carriers in this group.

It is also possible that the results from the early-menopause analysis are applicable to premature menopause. There is no clinical reason to distinguish between the two categories, and the use of the age of 40 years as a cutoff for premature ovarian failure is a fairly arbitrary choice (Alper et al. 1986). Furthermore, among cases with a family history of early or premature menopause, Cramer et al. (1995*b*) found no difference in the proportions of women with menopause at age <40 years, at age 40–43 years, and at age 44–46 years, suggesting that a genetic component contributes equally to menopause in all three age groups. Therefore, our data suggest that the association between fragile X premutations and premature menopause may not be quite as strong as that suggested in previous reports.

Conway et al. (1995) proposed that a premutation allele is underexpressed in fetal ovaries, leading to a reduction in the number of oocytes at birth. This hypothesis was used to explain the reported association between premutation carriers and premature ovarian failure. However, premutation alleles do not appear to reduce the expression of the FMR1 gene product, in other tissues (Devys et al. 1993; Feng et al. 1995*a,* 1995*b*). Moreover, analysis of the ovaries from a 16-wk-gestation fetus

carrying a full-mutation allele that did not express FMRP suggested that the number of oocytes was not markedly reduced (Malter et al. 1997).

A more congruent explanation is that the FMR1 repeat is in linkage disequilibrium with a nearby mutation that leads to ovarian dysfunction, including decreased ovarian reserve, increased twinning rates, and premature menopause. Such a mutation could be at a nearby locus that is involved in ovarian function, but this hypothesis makes it difficult to explain the apparently normal ovarian function in full-mutation carriers (Schwartz et al. 1994; Turner et al. 1994) whose full mutation still should be associated with such a nearby mutant allele. Alternatively, the mutation could be an unrecognized mutation within the FMR1 gene itself that either interacts with premutation-sized repeats to cause ovarian dysfunction or is in linkage disequilibrium with large repeat tracts. FMR1 is expressed in proliferating germ cells (Bachner et al. 1993) and, in adult ovaries, in the granulosa cells of developing follicles (Hinds et al. 1993; Hergersberg et al. 1995). Therefore, mutations that lead to an altered pattern of ovarian FMRP expression, such as promoter mutations, could lead to ovarian dysfunction in premutation carriers while leaving full-mutation carriers unaffected, since FMRP is not expressed from full-mutation alleles (Pieretti et al. 1991; Sutcliffe et al. 1992). This hypothesis is supported by the observations that the FMR1 repeat is in linkage disequilibrium with nearby loci (Kunst and Warren 1994; Eichler et al. 1996; Kunst et al. 1996) and that FMR1 premutations cosegregate with premature menopause, in some fragile X families (Conway et al. 1995; Vianna-Morgante et al. 1996).

Our hypothesis also could explain the different reported levels of association between fragile X premutations and premature menopause, since the populations chosen may have different proportions of fragile X premutations that are linked to such a putative genetic factor leading to ovarian dysfunction. Thus, further studies of this kind are needed to elucidate the relationship between fragile X and ovarian function, with special attention given to both population choice and ascertainment. In addition, since most of the premature–ovarian failure cases in our study had completed menopause in their 30s, it may be necessary to ascertain women who have had an even earlier menopause, in order to find premutation carriers.

Acknowledgments

We thank Eleanor Feingold, Stephanie L. Sherman, Cam Fraer, Huijuan Xu, Jane Iber, Fuping Zhang, and Chris Gunter for their assistance and discussion. The collection of samples was supported by grant R01 HD23661 (to D.W.C.). This project was supported, in part, by grants R37 HD20521 and P01 HD35576 (to S.T.W.). S.T.W. is an investigator with the Howard Hughes Medical Institute.

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