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Chinese women with 29–30 *FMR1* CGG repeats have an earlier menopause

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ABSTRACT

Purpose: A strong, well-established non-linear relationship exists between *fragile X mental retardation (FMR1)* premutation and menopausal age. The aim of this study is to evaluate whether this relationship continues into the normal CGG repeat range.

Methods: *FMR1* CGG repeats of 111 Chinese postmenopausal women from a prospective cohort and the relationship with age at menopause were analyzed. Associations of *FMR1* genotypes with annually measured estradiol and follicle stimulating hormone (FSH) levels were also assessed.

Results: One premutation and two intermediate carriers were identified, with a prevalence of 0.90% and 1.80%, respectively. The age at menopause differed with statistical significance ($p = 0.007$) between women carrying bi-allelic 29–30 repeats (49.66 ± 3.26 years) and those carrying a different number of repeats (51.26 ± 2.74 years). Age at menopause among subgroups (≤ 28 , 29–30, and ≥ 31 repeats) of alleles 1 and 2 were also different ($p = 0.014$, $p = 0.044$). FSH trajectories to final menstrual period differed between women with the bi-allelic 29–30 repeats and others ($p = 0.019$).

Conclusions: Women with 29–30 *FMR1* CGG repeats may experience menopause approximately 2 years earlier than those carrying ≤ 28 or ≥ 31 CGG repeats, and have a longer FSH fluctuant period.

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FMR1; CGG repeats; natural menopause; menopausal age; follicle stimulating hormone

Introduction

The *fragile X mental retardation (FMR1)* gene is located at the X chromosome (Xq27.3). Large numbers of triple CGG repeats of the *FMR1* 5' untranslated region are related to premature ovarian insufficiency (POI) in women¹. It has been estimated that around 11–14% of familial and 2–6% of sporadic POI cases are associated with premutations in *FMR1* (55–199 CGG repeats)². However, the distribution of *FMR1* CGG repeats varies with ethnicity³. The pathogenic role of *FMR1* premutation in Han Chinese women is controversial. Recent studies from China found that premutation carriers represented 0.82% (1/122)⁴, 0.5% (2/379)⁵, and 0.9% (1/117)⁶ of Chinese POI cases, much lower than studies from other countries².

Numerous studies have examined the association between the distribution of *FMR1* CGG repeats in the 'normal' (<45 CGG repeats) or intermediate (45–54 CGG repeats) range and ovarian dysfunction. The results are inconsistent: some studies found that ovarian reserve or ovarian response was not adversely affected by intermediate-range or normal-range CGG repeats^{7–11}. In contrast, others have reported that repeat numbers >36¹², of 41–58¹³, or of 35–54¹⁴ are associated with POI. CGG repeats <26¹⁵, <28¹⁶, of 45–54¹⁷, or >40¹⁸ are also reported to negatively affect ovarian aging. However, none of these studies focused on Chinese women.

The possibility cannot be ruled out that this heterogeneity was partly due to the varied ethnicity of the study participants. Therefore, controlling ethnicity is essential in studies of the association between *FMR1* premutation and ovarian aging. The distributional curve of *FMR1* CGG repeats in Asians was left-shifted compared with that in non-Asians¹⁹. Whether CGG repeats affect the ovarian aging process in Chinese women warrants further investigation. Age at menopause is a reflection of ovarian aging. Information about the association between *FMR1* distribution and age at menopause in Chinese women is scarce.

The Peking Union Medical College Hospital Ageing Longitudinal Cohort of Midlife Women (PALM) is the first longitudinal study of midlife women in China²⁰. We assessed the distribution of *FMR1* CGG repeat numbers in some of the study participants to provide an estimate of the distribution of CGG repeats in a well-characterized population of Chinese women with definite menopause timing.

Materials and methods

Participants

The data for this analysis were part of a prospective, open-cohort, community-based longitudinal study, the PALM study, which aimed to investigate ovarian aging in midlife women in China. The study design and sampling procedures

have been described previously²⁰. The participants were middle-aged female residents of a community in Beijing, China. On enrollment, a questionnaire was completed, which recorded participants' sociodemographic characteristics, menopausal symptoms, and lifestyle behaviors. Women who were still menstruating were given calendars to record their menstrual cycle and related symptoms. Follow-up is conducted regularly every year. The PALM study started in July 2005, and ethical approval (No. JS-2100) was granted by the Institutional Review Board of Peking Union Medical College Hospital. Signed informed consent forms were obtained from all participants.

Eligibility criteria were: age 35–64 years; intact uterus and at least one ovary; no history of severe systemic disease, including hematological disorders, autoimmune diseases, and malignancies; no history of chemotherapy or radiotherapy; no gynecological endocrine diseases, such as premature ovarian failure or polycystic ovary syndrome, and so forth; no use of hormonal medications in the previous 3 months; and not pregnant or lactating in the previous 6 months. Eligibility criteria for *FMR1* analysis required women to be in natural menopause before or during follow-up. A total of 111 participants who agreed to participate were enrolled in this study. Estradiol (E_2) and follicle stimulating hormone (FSH) levels of 111 participants at each assessment with 709 observations (a mean of 6.4 observations per participant) were included. The menopausal status was determined according to the 2011 Stages of Reproductive Aging Workshop +10 (STRAW + 10) criteria²¹ by a clinician specialized in gynecologic endocrinology using menstrual calendars. An additional blood sample was obtained from each participant for *FMR1* analysis during follow-up. Performing DNA assays and the analysis of these data were approved by the Institutional Review Board of Peking Union Medical College Hospital.

***FMR1* assay measures**

Genomic DNA was isolated from peripheral blood leukocytes using standard procedures with a Genome DNA Extraction Kit (Qiagen, Germany). Details of the *FMR1* repeat measures are shown in [Supplemental material](#).

Covariates

The final menstrual period (FMP) was identified after 12 months or more of no menstrual bleeding. The time scale was anchored to the FMP date, which facilitated the evaluation of hormone levels at approximately similar time intervals relative to the FMP. The selection of covariates was based on their significance in previous studies and the goals of this study. Body mass index (BMI), level of education, general health status, smoking status, E_2 , and FSH were included in the analysis. BMI was calculated as weight (kg)/height² (m²). General health status was self-reported and classified as poor, good, or excellent. Annual blood samples from premenopausal women were drawn on days 2–4 after bleeding ceased, and were scheduled on any day for postmenopausal women.

Statistical analysis

Because women have two X chromosomes, their *FMR1* results provide two numbers, corresponding to the CGG repeat length in each allele. Consistent with the methodologies of prior studies^{3,22}, the allele with the smaller number of CGG repeats was termed 'allele 1' and the allele with the larger number of CGG repeats was termed 'allele 2'. Continuous variables with normal distributions were expressed as the mean \pm standard deviation. Categorical variables were presented as numbers (percentages). Comparisons of categorical allele variables were made using the non-parametric Kruskal–Wallis test. Association analysis of continuous variables used *t*-testing (two groups) or analysis of variance (three or more groups). Survival analysis was also used to confirm the association of age at menopause between women with different CGG repeats. In the survival analysis, all participants had a definite age at menopause and no censored values were used. To identify the potential effect of several covariates on menopausal age, Cox proportional hazards modeling²³ was used. We used menopausal age as the time scale²⁴, and no values were censored. Some covariates (BMI, education level, general health status, smoking status) that were previously reported to be associated with menopausal age were tested. Variables were retained in Cox models at $p \leq 0.05$, applying forward elimination.

Another set of analyses focused on the longitudinal changes in FSH and E_2 for different numbers of CGG repetitions, in relation to increasing age or years since the FMP. With ages or years relative to FMP as the horizontal axis, and FSH or E_2 observations during follow-up as the vertical axis, scatter diagrams were produced. To show the hormone trajectories of each subgroup, a non-parametric regression model (locally weighted scatterplot smoothing, LOESS) was used for smoothing. A generalized estimating equation model was utilized to study the effect of *FMR1* genotypes on FSH or E_2 measurements, while accounting for correlations within subjects and the confounding effect of time relative to FMP.

The sample size was determined using PASS 15 Power Analysis and Sample Size Software, version 15.0.5 (NCSS, LLC, Kaysville, UT, USA). Group sample sizes of 37 and 70 were needed to have 80.9% power with a type I error of 5% to detect a statistical difference of menopausal age between the two groups. The present study included 38 and 73 participants in the two groups and the power was 84%. Analyses were performed using SPSS software (version 20.0 for the OS X system; IBM). All tests were two-sided with a 0.05 significance level.

Results

Distribution of *FMR1* alleles

A total of 111 participants were enrolled in the study. Details of the general characteristics of the enrolled women and *FMR1* CGG repeat lengths are reported in [Supplemental Table 1](#). No full mutation subtype was found. Only one pre-mutation carrier with repeat lengths of 40/59 was identified,

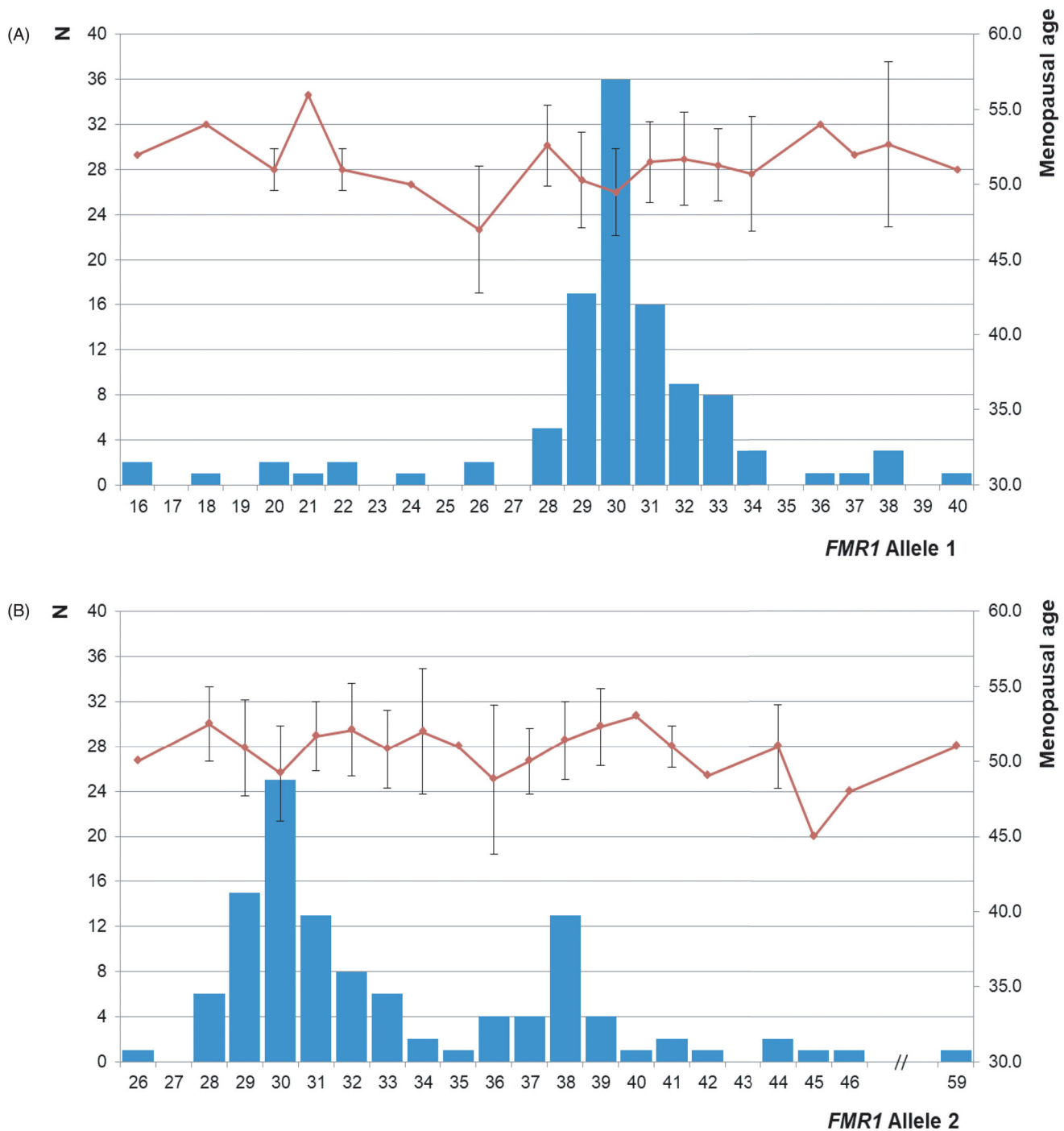


Figure 1. Distribution of *fragile X mental retardation* (*FMR1*) CGG repeat numbers and menopausal age in the cohort. (A) Results of *FMR1* allele 1; (B) results of *FMR1* allele 2. The left vertical axis represents numbers of women with specific CGG number. The right vertical axis and line chart represent mean values of menopausal age of specific CGG number. Error bars indicate the standard deviation.

with a prevalence of 0.90%. The proportion of women with an intermediate repeat was 1.80% (two carriers: one with repeat lengths of 34/46, and the other with repeat lengths of 29/45).

The overall distribution of various repeat numbers in the women is presented in Figure 1. The CGG repeats data for both alleles have a major peak at 30 repeats, followed by 29. The distribution for allele 1 was mainly clustered between 25 and 34 CGG repeats (86.5%). This is in line with the results of the Study of Women's Health Across the Nation (SWAN, a large longitudinal multi-ethnic community-based study of

menopausal transition), in which the proportion of 25–34 repeats was 92.7%. The repeat lengths for allele 2 had a secondary peak at 38 repeats. The CGG repeats were also divided into groups of five consecutive values, shown in Supplemental Table 2, and the results for Chinese women in the SWAN³ are also presented for comparison. No statistically significant differences were found on allele 2 ($p=0.184$), whereas the distribution for allele 1 differed ($p=0.003$). Homozygous CGG repeat lengths were common (66.7%), and 30/30 and 29/29 repeats were the most and second most prevalent, respectively.

The association between *FMR1* CGG repeats and age at menopause

The average age at menopause was 50.71 ± 3.01 years in this study. An exploration was performed into whether possible associations between the number of CGG repetitions and the timing of menopause are driven by alleles on the *FMR1* gene. Scatter diagrams with LOESS regression of menopausal age for the two *FMR1* alleles are shown in [Supplementary Figure 1](#). Mean menopausal age for each particular quantity of CGG repetitions is shown in [Figure 1](#). Menopausal ages seemed slightly earlier for samples featuring 29 and 30 CGG repeats in alleles 1 and 2. For further analysis, subgroups of alleles 1 and 2 were defined as ≤ 28 , 29–30, and ≥ 31 repeats. When comparing the mean menopausal ages for the three subgroups for alleles 1 and 2, we found statistically significant differences among three of the subgroups ($p = 0.006$ and $p = 0.044$, [Table 1](#)). Then, a subgroup was formed including women with bi-allelic 29–30 genotypes, comparing them with women with one or more alleles outside the range 29–30. The menopausal age varied with statistical significance ($p = 0.007$, [Table 1](#)). In the survival analysis, there was a left shift in menopausal age in the bi-allelic 29–30 group ([Supplemental Figure 2](#), $p = 0.02$). In the Cox proportional hazards model used to evaluate risk factors for menopausal age, BMI ($P = 0.31$), smoking ($p = 0.24$), health status ($p = 0.40$), and education level ($p = 0.44$) had a p -value higher than 0.05, and were not included in the final model. The *FMR1* bi-allelic 29–30 CGG repeats subgroup was retained and had an effect on menopausal age ($p = 0.04$).

Hormonal trajectories around menopause in different *FMR1* allele groups

The serum FSH and E_2 levels for different CGG repeats groups in relation to increasing age or years relative to time since FMP are shown in [Figure 2](#). The FSH level began to ascend around 5 years before the FMP in all groups. FSH levels in the ≥ 31 repeats group had a steeper increase than the other groups and stabilizes approximately 2 years after the FMP; FSH in the 29–30 repeats group ascended slower and gradually stabilizes approximately 4 years after the FMP.

The hormonal trajectories with increasing age for different *FMR1* genotypes was calculated, and we found the FSH trajectories in relation to FMP differed between the bi-allelic 29–30 group and others ($p = 0.019$), as well as among the allele 2 subgroups ≤ 28 , 29–30, and ≥ 31 ($p = 0.046$). In the different allele 1 subgroups, no statistically significant difference was observed ($p = 0.122$).

Discussion

The *FMR1* CGG repeat sizes might play a role in the mechanisms of the ovarian aging process and thereby affect the timing of menopause. In this study, we conducted a detailed analysis of the distribution of *FMR1* CGG repeat numbers in 111 naturally menopausal Chinese women in the PALM study. We found that the number of CGG repeats was associated with age at natural menopause in Chinese women. Having alleles with CGG repeats in the range of 29–30 might lower the menopausal age.

The *FMR1* distribution varies according to ethnicity. Therefore, we must attach importance to control for ethnicity when designing and analyzing studies in the field of ovarian aging. Genereux and Laird showed that Asian and non-Asian populations followed similar distributional curves, but the Asian curve was left-shifted¹⁹. Among the SWAN Chinese women, 55.2% were homozygous with 29/29 and 30/30 repeats equally observed; among the SWAN Japanese women, 63.7% were homozygous³. The distribution features of our study population are consistent with those mentioned in previous reports on Asian women. In our cohort, 66.7% were homozygous, and 30/30 and 29/29 repeats were the most and second most common. Reports showed that CGG repeats in Asians also have a secondary peak of 33, 34, or 36 repeats, which have not been identified in western populations¹². The highest peak for CGG repeats in this study was 30 and the secondary peak was 38. Another feature of Asian women was that allele lengths of < 25 CGG repeats on one or both alleles occurs much less frequently than in Caucasians and African Americans³. In this study, no women had allele 2 lengths < 25 and only 8.1% of women had allele 1 lengths < 25 .

Table 1. Association between CGG categories and age at menopause.

Allele	CGG repeats	N (%)	Menopausal age, mean \pm standard deviation	p-Value
1	≤ 28	16	51.56 \pm 2.92	0.006
	29–30	53	49.77 \pm 2.95	
	≥ 31	42	51.57 \pm 2.82	
2	≤ 28	7	52.14 \pm 2.41	0.044
	29–30	40	49.83 \pm 3.27	
	≥ 31	64	51.11 \pm 2.79	
1/2	Bi-allelic 29–30	38	49.66 \pm 3.26	0.007
	Others	73	51.26 \pm 2.74	
	Both alleles ≤ 28	7	52.146 \pm 2.41 ^a	
	Both alleles ≥ 31	40	51.686 \pm 2.83 ^a	
	Allele 1 ≤ 28 and allele 2 ≥ 31	7	50.57 \pm 3.60	
	Allele 1 ≤ 28 and allele 2 29–30	2	53.00 \pm 1.41	
	Allele 1 29–30 and allele 2 ≥ 31	17	50.00 \pm 2.00	
Allele 1 29–30 and allele 2 ≤ 28	0	NA		

^a $p < 0.05$ when compared with the bi-allelic 29–30 subgroup by t -testing. NA, not available.

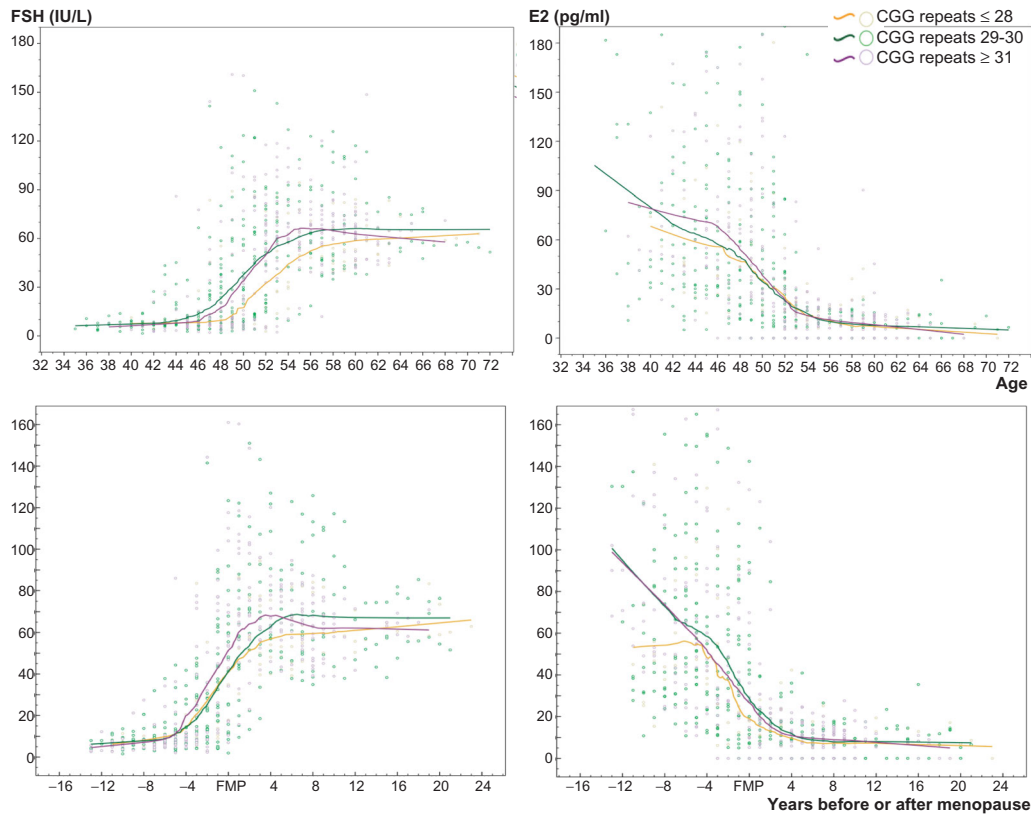
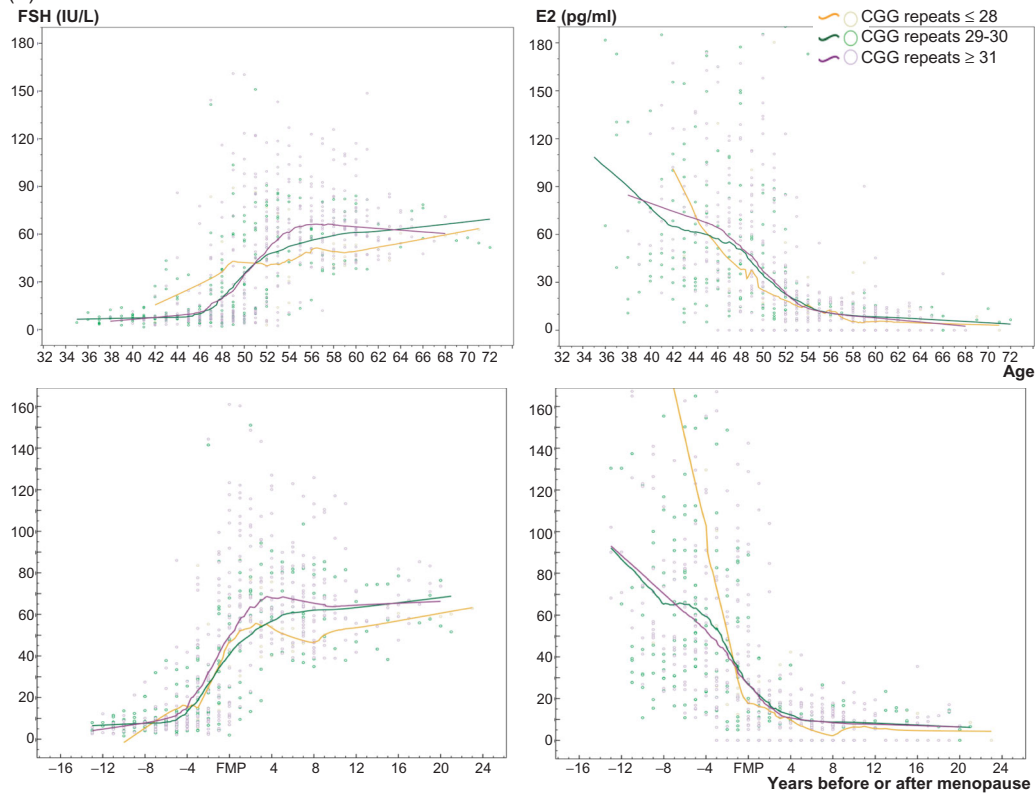
(A) Allele1**(B) Allele2**

Figure 2. Scatter plots of serum follicle stimulating hormone (FSH) and estradiol (E_2) levels with different CGG repeats groups in relation to increasing age (upper) or years relative to final menstrual period (lower). (A) Allele 1; (B) allele 2; (C) bi-alleles. Corresponding locally weighted scatterplot smoothing (LOESS) curves are shown in each figure. The number of women with ≤ 28 CGG repeats is relatively small, so the corresponding LOESS curves are not smooth, with poor representation.

(C) Both Allele1 and 2

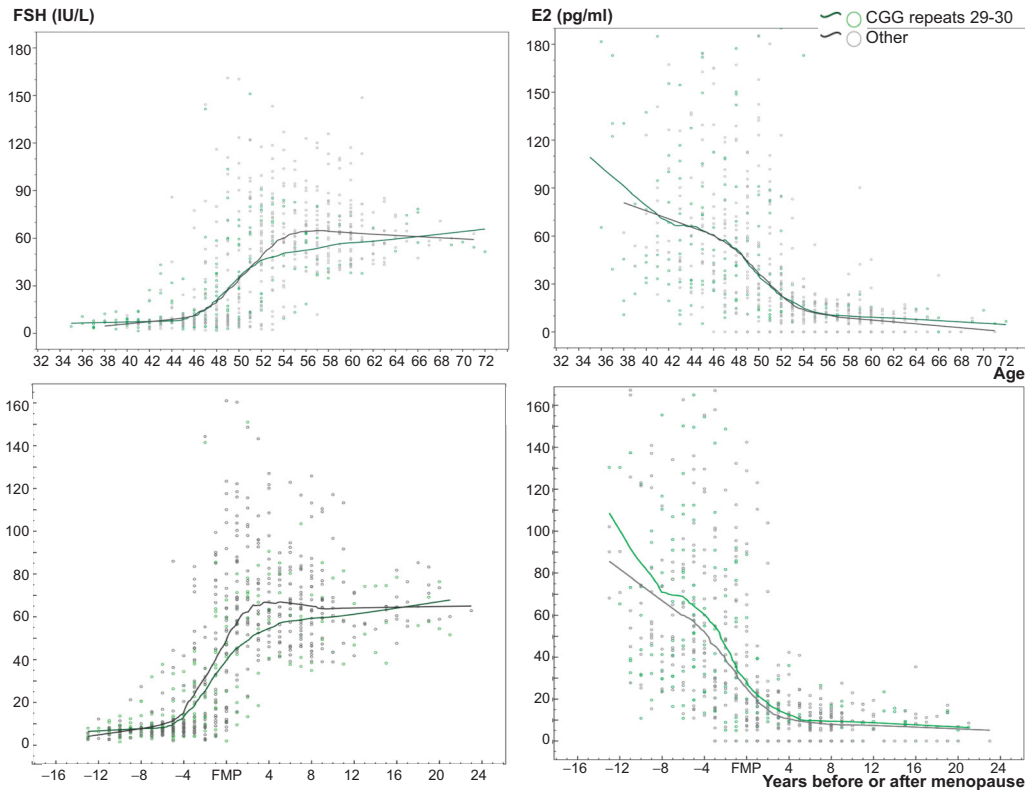


Figure 2. Continued.

FMR1 premutation and intermediate repeats have been major focuses in several studies. Asian populations have markedly lower frequencies of premutation alleles¹⁹. One premutation carrier and two intermediate cases were identified (0.90% and 1.80%) in our study, which are higher than those reported amongst Chinese women in the SWAN (both 0%)³. In the studies from China⁴⁻⁶ that have compared Chinese POI patients with controls, all control groups comprised young women who were still menstruating with apparently normal ovarian function. Nevertheless, the controls have the potential to develop POI or other diseases in the future. The enrolled women in our data were from a longitudinal cohort and had a definite natural menopausal age. We postulated that these data offered a better comparative reference for ovarian aging research.

Menopause is the endpoint of the follicle wastage process and an inevitable part of ovarian aging. The high heritability of age at menopause implies a strong genetic component and some variants are associated with age at natural menopause^{25,26}. *FMR1* variations are known to affect follicle wastage²⁷, thus possibly affecting age at menopause. The association between *FMR1* premutation carriers and menopausal age has been investigated; a strong, well-established non-linear relationship exists^{28,29}. It was not known whether this relationship continued into the normal range. In a large cross-sectional study of Caucasian women, there appeared to be no relationship between CGG repeats in the normal and intermediate range and age at natural menopause²². A study from the UK demonstrated that *FMR1* alleles in the normal range do not influence risk of early menopause in Caucasian

women³⁰. Because both *FMR1* CGG repeats and menopause vary according to ethnicity^{7,25}, research focusing on populations of targeted ethnicity is necessary.

We investigated the relationship between *FMR1* CGG repeat sizes and age at natural menopause in the Chinese population. From previous studies, the median menopausal age ascertained by follow-up interviews was about 52 years^{20,31}. We found a slightly decreased menopausal age in women with 29–30 CGG repeats for either allele 1 or 2 (about 50 years old), and ≤ 28 or ≥ 31 repeats in both alleles had menopausal age of approximately 52 years, which indicates that 29–30 repeats may be deleterious to the ovarian aging process. One strength of the present study is that we considered alternative potential reasons for earlier menopause in the analysis. Previous studies have demonstrated that smoking, teetotalism, lower weight, lower educational level, and poor health significantly associate with younger age at menopause^{31,32}. In the present study, even after considering the effect of confounding factors, including BMI, smoking status, health status, and education level, which were previously reported to be related to menopausal age, *FMR1* 29–30 CGG repeats were still found to be associated with menopausal age.

Hormonal trajectories around menopause have long been a concern of researchers; these were first described for different *FMR1* genotypes. Hormonal fluctuations around menopause may be a reflection of undulated ovarian function. Women in the 29–30 CGG repeats groups showed longer (~2 years) hormonal fluctuant periods. FSH levels in women with 29–30 repeats increase more slowly than in other

women, which means the perimenopausal period may be longer in this group.

The discrepancy between the findings and previous reports can be explained by several factors. First, ethnic differences could be a cause, since the distribution of CGG repeats differs significantly between ethnic groups and was seen to be left-shifted in Asians¹⁹. It could be possible that the association between CGG repeats and the ovarian aging process is fundamentally different between ethnicities. Therefore, the observed discrepancy with other studies could be explained by the fact that the current study was performed in a Chinese population. Another explanation may stem from the fact that risk factors of menopausal age are heterogeneous. Menopausal age is influenced by its interaction with genes and/or other factors^{31,33}. The complex genetic background and surrounding environment affects menopausal age and causes differences in associations. A study suggesting that the association between *BRCA1* carriers and diminished ovarian function is actually *FMR1*-mediated³⁴ strengthens this hypothesis. In addition, socioeconomic and lifestyle factors are thought to be associated with menopausal age³², which should be taken into account in analyses. The present study adjusted for confounding factors.

In summary, *FMR1* CGG repeat numbers may be associated with age of natural menopause in Chinese women; women with 29–30 repeats may go through menopause at an earlier age and have a longer and slowly increased FSH fluctuant patterns. This work could serve as a comparative reference for research on early ovarian aging. Although the earlier menopause related to individuals with 29–30 CGG repeats was only approximately 2 years earlier than in other women, this may be a related genetic factor with a deleterious effect. When comprehensively evaluating a woman's age at menopause, in addition to other known associated factors, the *FMR1* genotype should be considered. One important limitation of the present study was the small sample size; it is therefore desirable to confirm our findings in studies with a larger sample size, especially in longitudinal cohorts.

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