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Full-length article

Interaction effects between estrogen receptor α and vitamin D receptor genes on age at menarche in Chinese women

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Key words

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Abstract

Aim: To evaluate whether estrogen receptor α (ER-α) and vitamin D receptor (VDR) genes are associated with the age at menarche in Chinese women. **Methods**: A total of 390 pre-menopausal Chinese women were genotyped at the ER-α *Pvu*II, X*ba*I, and VDR *Apa*I loci using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). **Results**: Neither the ER-α gene nor the VDR gene individually had significant effects on the age at menarche in our subjects (P>0.10). However, evidence of interaction effects between the two genes were observed: with the aa genotype at the VDR *Apa*I locus, subjects with haplotype PX at the ER-α gene had, on average, 6 months later onset of menarche than the non-carriers (P=0.01). **Conclusion:** We found that neither the ER-α gene or the VDR gene had a significant association with the age at menarche individually. However, potential interaction effects between the two genes were observed in Chinese women.

Introduction

Menarche, the first occurrence of menstruation, is a marked characteristic of a woman's sexual maturation and a biological signal for the onset of reproduction. Age at menarche is an important anthropological variable, which influences the total duration of women's estrogen exposure and thus has major implications for a woman's health later in life. Early menarche is associated with an increased risk of breast and endometrial cancer[1]. Delayed menarche increases the risk of Alzheimer's disease[1] and osteoporosis[2], but decreases the incidence of coronary heart disease[1]. Therefore, from a clinical point of view, it is of interest to identify factors that influence the variation of menarcheal age.

Age at menarche is known to be a complex trait that is determined by multiple genetic and environmental factors, including nutrition, exercise, socioeconomic conditions, childhood experience, and general health[1,3–5]. The importance of genetic factors in determining menarcheal age has recently been recognized. Twin studies have shown that genetic factors can explain more than 50% of the variation of menarcheal age[4]. There are highly significant correlations between age at menarche in mothers and daughters[3], and family history is a strong predictor for early menarche[5]. Recently, some candidate genes, such as the androgen receptor (AR) gene[6], the cytochrome P450 c17α (CYP17) gene[7,8] and the CYP3A4 gene[8], have been tested for association with age at menarche. The results so far have been inconsistent, and the specific gene responsible for age at menarche is still not clear.

The onset of menstruation is determined by the hypothalamic-pituitary-gonadal axis and is initiated by an increased amplitude of estrogen exposure of tissues[9]. Estrogen signal receptors including estrogen receptor α (ER-α) play an important role in mediating the specific effects of the estrogen on development, proliferation and differentiation of reproductive tissues[10]. In addition, mice deficient in ER-α are infertile and exhibit atrophy of the oviduct and uterus[11]. Thus, the ER-α gene may be a candidate gene for the onset of menstruation. An association has been observed between the ER-α gene and age at menarche in Greek adolescent females[12] but not in other populations[7,13,14].

The vitamin D receptor (VDR) gene may be another potential candidate gene for age at menarche. The expression of VDR is detected in reproductive organs[15]. In vitamin D-deficient mice, uterine hypoplasia with impaired folliculogenesis was found in the female reproductive organs[16]. The VDR gene has been associated with several diseases that may be related to total tissue estrogen exposure, including breast cancer[17], cardiovascular disease[18], and osteoporosis[19]. Recently, the VDR gene has been associated with the age of menarche in Japanese girls[20].

In the present study, we intended to investigate whether the ER-α and VDR genes, as well as interactions between the two, had effects on the age at menarche in the Chinese female population.

Materials and methods

Subjects The study subjects were selected from 401 nuclear families used in our previous epidemiological study[21], which was approved by the Research Administration Departments of the Shanghai Sixth People's Hospital and Hunan Normal University. All the subjects were recruited from the Shanghai urban area. They were all of the Han ethnic group, which accounts for more than 93% of the total Chinese population. Informed consent was obtained from each subject. For the study subjects, a detailed medical history, including menstrual history, was recorded by nurse-administered questionnaires. The sampling scheme and exclusion criteria have been detailed elsewhere[21].

For the present study, we randomly chose one pre-menopausal daughter from each nuclear family. Four hundred and one unrelated pre-menopausal women, who were all in good general health as defined by our exclusion criteria, were sampled. Among these women, 11 had no information of menarcheal age. Thus the 390 remaining pre-menopausal women were available for analysis. The women were unrelated and had regular menstrual cycles.

Genotyping Genomic DNA was isolated using the phenol-chloroform extraction method from whole blood. All subjects were genotyped by polymerase chain reaction followed by restriction fragment length polymorphism procedures (PCR-RFLP). For the ER-α gene, the forward primer (5'-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC-3') and reverse primer (5'-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3') were used to amplify a 1.3 kb DNA fragment in intron 1. For the *Apa*I inside the VDR gene, a 745 bp DNA fragment was produced using the forward primer in intron 8 (5'-CAG AGC ATG GAC AGG GAG CAA G-3') and the reverse primer in exon 9 (5'-GCA ACT CCT CAT GGC TGA GGT CTC A-3'). The PCR mix contained 50 ng genomic DNA, each of the four deoxyribonucleotides (dNTPs; 0.2 mmol/L), 0.6 U *Taq* polymerase (Sangon, Shanghai, China), $MgCl_2$ (1.5 mmol/L), each primer (0.4 μmol/L), and 1×PCR buffer in a total volume of 25 μL. PCR was performed in 38 cycles as follows: denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s on a PE9700 Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). After amplification, 8 μL of the PCR products was digested with the respectively restriction endonucleases *Pvu*II, *Xba*I, and *Apa*I (Promega, Madison, WI, USA) at 65 °C for 4 h and electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light. The genotypes were designated as PP, Pp, and pp for *Pvu*II, XX, Xx, and xx for *Xba*I, and AA, Aa, and aa for *Apa*I. Uppercase and lowercase letters represent the absence and presence of restriction sites, respectively.

Statistical analysis The χ^2 test was performed to test for the Hardy-Weinberg equilibrium (HWE) at the studied marker loci. The phenotypic values were verified for normal distribution by using the Shapiro-Wilks test. The Program SimWalk2 (available at http://www.genetics.ucla.edu/home/software.htm) was used to reconstruct the ER-α haplotypes according to the genotypes of all the subjects in the nuclear families, because the ER-α *Pvu*II and *Xba*I were in strong linkage disequilibrium (LD)[22]. Analysis of variance (ANOVA) was used to evaluate the effects of the ER-α and VDR gene polymorphisms on age at menarche. The frequency of genotype AA for the VDR gene (6.2%) was low in our subjects. If the subjects were divided into three groups, AA, Aa, and aa, the sample sizes for some groups were small, and it was not appropriate to analyze the interaction effect. Then the subjects were divided into two groups, those with and without the minor allele A. To analyze the effect of a special ER-α haplotype, the subjects with this haplotype were denoted as "1"; those without it were denoted as "0". The effect of a given ER-α gene haplotype was tested according to the special VDR genotypes. When a significant result appeared, it was regarded as indicative of an interaction between the ER-α and VDR genes. These statistical analyses were conducted using SAS version 6.12 (SAS Institute, Cary, NC, USA).

Results

Descriptive characteristics of the study subjects The basic characteristics of the 390 unrelated pre-menopausal women are summarized in Table 1. Allele frequencies at the three polymorphic sites in our subjects are summarized in Table 2. All three loci were in HWE (P>0.10). When the genotypes of the ER-α gene were defined according to the

Table 1. Descriptive characteristics of all the subjects. Values are expressed as mean±SD.

	Subjects
Sample size	390
Age/a	31.0±5.5
Height/cm	159.8±5.3
Weight/kg	55.0±8.1
Age at menarche/a	13.6±1.4

Table 2. Allele frequencies at the three polymorphic sites.

	Allele frequencies/%
ER-α *Pvu*II	P=37.1
	p=62.9
ER-α *Xba*I	X=22.4
	x=77.6
ER-α haplotype	PX=16.0
	px=56.6
	Px=21.0
	pX=6.4
VDR *Apa*I	A=27.4
	a=72.6

The *Pvu*II and *Xba*I polymorphisms of the ER-α and the *Apa*I polymorphism of the VDR were in Hardy-Weinberg equilibrium (P>0.10).

haplotypes, ten genotypes were found, with pxpx as the most common (frequency=0.331) and pXpX as the least common (frequency=0.002).

Association of the ER-α and VDR genes with the age at menarche We did not find a significant association between age at menarche and either the ER-α individual polymorphisms or the ER-α haplotypes, or the VDR *Apa*I locus (P> 0.10) (Table 3). Evidence of an interaction between the ER-α and VDR genes was observed. With the aa genotype at the VDR gene, subjects with haplotype PX at the ER-α gene had, on average, 6 months later onset of menarche than the non-carriers (P=0.01) (Table 4).

Discussion

Age at menarche is an important complex trait, which is controlled by both genetic and environmental factors. Some genetic studies have been performed in white and black women, and significant differences between different ethnic populations have been observed, that is, the average onset of menstruation in African-American girls is 9 months earlier than that in Caucasians[23]. However, few genetic studies

Table 3. Association analyses for the ER-α and VDR genes with age at menarche.

Polymorphisms		*n*	Age at menarche/ years
ER-α *Pvu*II	PP	50	13.4±1.5
	Pp	189	13.7±1.4
	pp	151	13.5±1.4
	P value		0.36
ER-α *Xba*I	XX	14	13.4±1.3
	Xx	147	13.7±1.4
	xx	229	13.5±1.4
	P value		0.57
ER-α haplotype	PX/px	73	13.7±1.2
	PX/Px	29	13.8±1.7
	px/px	129	13.5±1.4
	px/Px	89	13.7±1.5
	px/pX	21	13.6±1.2
	Px/pX	24	13.6±1.4
	P value		0.74
VDR *Apa*I	AA	24	13.4±1.3
	Aa	166	13.5±1.3
	aa	200	13.7±1.4
	P value		0.52

All data are presented as mean±SD of the age of menarche. Only 6 combinations of *Pvu*II and *Xba*I genotypes were included in the analysis, because the number of other possible genotypes is small. *P* values are the results of ANOVA.

Table 4. Interaction effects between the ER-α and VDR genes on the age at menarche.

		VDR gene	
		A	B
ER-α PX	0	13.6±1.4 (127)	13.5±1.4 (148)
	1	13.3±1.2 (63)	14.0±1.4 (52)
	P value	0.19	0.01
ER-α px	0	13.1±1.3 (33)	13.8±1.6 (45)
	1	13.6±1.4 (157)	13.6±1.4 (155)
	P value	0.10	0.45
ER-α Px	0	13.5±1.3 (118)	13.6±1.4 (119)
	1	13.5±1.4 (72)	13.8±1.6 (81)
	P value	1.00	0.23
ER-α pX	0	13.5±1.4 (167)	13.6±1.5 (174)
	1	13.7±1.3 (23)	13.6±1.4 (26)
	P value	0.56	1.00

All data are presented as mean±SD of the age of menarche. The sample size is listed in parentheses. "1" denotes carriers and "0" denotes non-carriers of the corresponding ER-α haplotype. "A" denotes the subjects with allele A (the AA and Aa genotypes) at the VDR *Apa*I locus and "B" denotes the subjects without allele A (the aa genotype) at the VDR *Apa*I locus.

have been conducted in Chinese. In this study, the potential interaction effects between the ER-α and VDR genes were observed, although neither of them was significantly associated with the age at menarche individually.

A preliminary study in Greek adolescent females has shown an association between ER-α *Xba*I and *Pvu*II and age at menarche[12]. Subjects with the genotype XX had later onset of menarche than those with genotypes Xx or xx. The study also found that haplotype PX homozygotes were correlated with later onset of age at menarche. However, such an association was not observed in Japanese women[7] or Dutch women[13,14]. Similar to these findings, we did not find any significant association between the ER-α *Xba*I or *Pvu*II polymorphisms and age at menarche in Chinese women.

For the VDR *Apa*I locus, no association was obtained in the present study. However, in a Japanese population, a significant association was found between the VDR gene and age at menarche[20].

Discrepancies between our study and the other studies may be due to differences in ethnic background, sample size, ascertainment schemes, and statistical methods. For example, if the sample sizes are limited, the power to detect the association will be very low. However, for a single candidate gene that can explain approximately 10% of the variation of menarcheal age, our study sample has about 70% power to detect its association with age at menarche. In addition, gene-by-gene or gene-by-environment interactions may also influence the results of association. For example, interaction between the ER-α and VDR genes was observed in our study. To our best knowledge, this is the first study to find an interaction effect between these two genes on age at menarche. This finding suggests that the magnitude and the direction of the effects of genotypes at one locus may be affected by the specific genotype at the other loci. It also implies that the combination of genotypes at several loci may be more important than a single one. The gene-by-gene interactions may be different in different populations, so the interaction effects observed here have yet to be confirmed by separate analyses in various populations or ethnic groups.

The mechanism by which these two genes interact with each other to affect the onset of menstruation is not clear; however, from a physiological point of view, interaction is possible. An estrogen-responsive promoter region has been characterized in the VDR gene. Transcription of the VDR promoter is dependent on the estrogen receptor[24]. On the other hand, vitamin D may influence the balance between androgens and estrogens, which in turn modulates the availability of steroid hormones for their receptors[25]. In addition, vitamin D may act at several points along the estrogen response pathway, affecting the levels of estrogen receptor as well as their ability to function as enhancers of transactivation[26].

Noticeably, there was a multiple-testing problem, because we tested multiple alleles in our analyses. If we use the *Bonferroni* correction to adjust for the multiple testing ($P \leq 0.006$ as the significant level), the interaction between the ER-α and VDR genes ($P=0.01$) will be close to, but not reach, statistical significance. In this situation, the *Bonferroni* correction is likely to be too conservative because it assumes that all variants are independent[27]. However, the statistical tests in the present study are expected to be highly correlated. For example, the *Xba*I and *Pvu*II polymorphisms are only 45 bp apart and in strong LD. Thus multiple testing corrections may lead to power loss and an increased rate of false-negative associations. To report some potentially important associations that are likely to be worthwhile pursuing further, we report the original statistical results.

For genetic investigations of complex traits, phenotype definition is a critical issue. In the present study, age at menarche was determined retrospectively based on self-report, and recall error may be inevitable. However, menarche is one of the most important milestones in a woman's life and retrospective recall is known to be reasonably accurate: recent studies have shown a high correlation between the recalled and actual ages at menarche[28]. Furthermore, it seems unlikely that this recall bias differs in different genotypes. Thus, our results based on the recalled age at menarche may be valid. Menarcheal age is affected by nutritional level and other living environmental factors. Such information was not recorded and was not used as covariates to adjust the raw data in the present study. In this study, we tried to limit the unrelated subjects to a similar age, so they would have a similar living environment. Such a method of subject ascertainment will have improved the accuracy of our association results.

In summary, the ER-α and VDR genes individually were not associated with the age of menarche in Chinese women. However, potential interaction effects between them were observed. Further studies in other populations with larger sample sizes and denser markers are required to confirm the findings reported here.

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