

Association of polymorphisms of the *estrogen receptor α* gene with the age of menarche

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BACKGROUND: The age of menarche may be subject to hereditary influences, but the specific genetic determinants are largely unknown. We evaluated whether the *XbaI* and *PvuII* polymorphisms of the *estrogen receptor α* gene are associated with the age of menarche. **METHODS:** We performed genotyping for *XbaI* and *PvuII* in a cohort of 145 adolescent females from a closed community in North-Western Greece. **RESULTS:** There was strong linkage disequilibrium between the two polymorphisms. Menarche occurred later in girls with the XX genotype than in girls with the Xx or xx genotype (mean \pm SD: 13.36 \pm 1.24 versus 12.80 \pm 1.14 and 12.75 \pm 1.35 years respectively; $P = 0.017$). Menarche also tended to occur later in PP homozygotes than in Pp and pp subjects, but the difference was not significant (mean \pm SD: 13.09 \pm 1.29 versus 12.80 \pm 1.19 and 12.85 \pm 1.33 years respectively). The strongest effect was seen when the PX haplotype was considered [mean \pm SD: 13.43 \pm 1.18 years for homozygotes versus 12.76 \pm 1.25 years in heterozygotes and in subjects without the PX allele, $P = 0.006$]. **CONCLUSIONS:** We document that the *XbaI* polymorphism, and possibly *PvuII*, may be genetic determinants of the age of menarche.

Key words: estrogen receptor α /genetic polymorphisms/menarche/*PvuII/XbaI*

Introduction

Menarche is regulated by a variety of environmental and genetic factors. Twin analyses have estimated that genetic effects may be more important than environmental parameters (van der Akker *et al.*, 1987; Treloar and Martin, 1990; Kaprio *et al.*, 1995). Such studies have indicated that 53–74% of the variation in age of menarche may be attributed to genetic effects. Estrogen exposure of tissues mediated via the estrogen receptor (ER) may be an important determinant of menarche and may be genetically determined in this regard. Conversely, the age of menarche may then influence the total duration of tissue estrogen exposure.

The ER has two major forms, α and β . These two types have a different distribution in the human reproductive organs and may have different contributions to reproductive function (Pelletier and El-Alfy, 2000). Both *ER α* and β genes have polymorphic sites, an increasing number of which have been

recognized in recent years, along with postulated potential associations with reproductive parameters (Jurada *et al.*, 2001; Sundararajan *et al.*, 2001; Westberg *et al.*, 2001). Two polymorphisms of the *ER α* gene in particular, *XbaI* and *PvuII*, have been described for several years and to date have received most of the attention for genetic association studies pertaining to ERs. *ER α* gene polymorphisms have been associated with several important clinical outcomes that may be related to total tissue estrogen exposure, including breast (Andersen *et al.*, 1994) and endometrial (Weiderpass *et al.*, 2000) cancer, cardiovascular risk (Kikuchi *et al.*, 2000) and osteoporosis (Gonnelli *et al.*, 2000; Albagha *et al.*, 2001). However, there are no data on whether these polymorphisms may also regulate the timing of the start of ovulatory cycles. We therefore investigated whether the *XbaI* and *PvuII* polymorphisms of the *ER α* gene are associated with the age of menarche.

Materials and methods

Subjects

We performed genotyping for *ER α* gene polymorphisms in a cohort of 145 healthy adolescent females from the region of Konitsa in North-Western Greece, a mountainous area with a closed, rural population. There is no town with over 3000 people in this area;

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most villages have a population of <200 inhabitants each and typically <50 are of reproductive age. Inbreeding (mating with partners within the same village) has been common and these have been closed communities for many generations. This is a homogeneous population that was selected with the anticipation that cultural and environmental heterogeneity would be minimized, since regional and environmental factors may create some variability in the age of menarche (Rimpela and Rimpela, 1993; Morabia and Costanza, 1998).

Data on menarche

Information on menarche was obtained through interviews with the adolescents and their mothers and through diaries. Informed consent was obtained for genotyping. Menarche information was collected independently from genotype information. Three girls had not reached menarche at the time of evaluation. Menarche for these three girls (aged 14.0, 14.9 and 15.1 years at the time of their evaluation) was assumed to occur 1 year later for the purpose of the main analysis, but different values (0–3 years) yielded very similar results (not shown). We also report analyses excluding these three subjects.

Genotyping

DNA was extracted from peripheral blood leukocytes using standard procedures. Specific DNA amplification was performed by PCR using 1 unit of recombinant TaqDNA polymerase (Gibco/BRL Life Technologies Inc., Gaithersburg, MD, USA), in a DNA thermocycler PTC-100 (Peltier-Effect Cycling, MJ Research, Waltham, MA, USA). The primers were: 5'-CTGCCACCCTATCTGTATCTTTTCC-TATTCTCC-3' (forward) and 5'-TCTTTCTCTGCCACCCTGGCGT-CGATTATCTGA-3' (reverse). The product contained a part of intron 1 and exon 2 of the ER α gene where both restriction sites are located. PCR products were digested at 37°C by 2 units of *Xba*I (GIBCO BRL) overnight and by 2 units of *Pvu*II (GIBCO BRL) for 2 h. Enzyme digestion products underwent electrophoresis on 2% agarose gel and separation patterns were photographed under ultraviolet illumination. The resulting genotypes for *Pvu*II and *Xba*I restriction sites were characterized as PP/Pp/pp and XX/Xx/xx respectively, with capital letters indicating the absence of the restriction site. More details on the genotyping can be found elsewhere (Mizunuma *et al.*, 1997).

Statistical analyses

Analyses used the *t*-test and one-way analysis of variance (ANOVA) for comparisons of means and the χ^2 with Yates' correction for contingency tables. Adjusted analyses were also performed, taking into consideration the body mass index. We also performed analyses excluding pairs of siblings. The study had 80% power to detect at $\alpha = 0.05$ a difference of 0.5 years in the age of menarche between compared groups where the ratio of subjects would be 2:1 and where the standard deviation of the age of menarche would be 1.2 years. Analyses were conducted in Statistics Package for Social Sciences (SPSS Inc., Chicago, IL, USA). All *P*-values are two-tailed and $P < 0.05$ was considered significant.

Results

Key characteristics of the study population and the frequencies of the different genotypes are shown in Table I. The mean age at menarche was 12.92 years (SD 1.26), a figure that is typical for the rural population of this region. *Xba*I and *Pvu*II polymorphisms were in strong linkage disequilibrium, meaning that P occurred more often than expected with X, and p with x (χ^2 , $P < 0.001$). Therefore, we also considered the PX

Table I. General characteristics of the study population

Characteristic	
Age at menarche, median (IQR), years	13.00 (12.00–13.75)
Age at evaluation, median (IQR), years	16.87 (15.21–18.47)
Height, mean (SD), cm	161.74 (5.80)
Weight, mean (SD), kg	54.17 (10.36)
Body mass index, mean (SD), kg/m ²	20.68 (3.62)
<i>Xba</i> I genotype, <i>n</i> (%)	
XX	35 (24.1)
Xx	56 (38.6)
xx	54 (37.2)
<i>Pvu</i> II genotype, <i>n</i> (%)	
PP	51 (35.2)
Pp	53 (36.6)
pp	41 (28.3)
Haplotypes, <i>n</i> (%)	
PPXX	34 (23.4)
PPXx	12 (8.3)
PPxx	5 (3.4)
PpXX	1 (0.7)
PpXx	43 (29.7)
Ppxx	9 (6.2)
ppXx	1 (0.7)
ppxx	40 (27.6)

IQR = interquartile range.

$P < 0.001$ for linkage disequilibrium between the *Xba*I and *Pvu*II polymorphisms.

haplotype in further analyses. Of note, neither the *Pvu*II nor the *Xba*I genotypes were in Hardy–Weinberg equilibrium (with $P = 0.001$ and $P = 0.01$ respectively). For each polymorphism, both homozygotes were over-represented and the heterozygotes were under-represented. This is usually suggestive of the presence of genetic drift that can be observed in closed rural communities.

In general, menarche occurred ~0.5 years later in girls with the XX genotype than in girls with the Xx or xx genotype (Table II). The difference was significant for the comparison of XX versus Xx and xx ($P = 0.017$, $P = 0.057$ when all three genotypes were compared by ANOVA). There appeared to be a similar trend for later onset of menarche among girls grouped by *Pvu*II genotype with the PP genotype, when compared with Pp or pp, but the difference did not reach formal significance (Table II).

The difference was even larger when we considered the PX haplotype. On average, PX homozygotes ($n = 34$) had ~8 months delay in menarche compared with PX heterozygotes and subjects without the PX allele combined (Table II). Separation of PX heterozygotes from subjects without the PX allele is not as straightforward, because there is uncertainty about whether the 43 PpXx individuals are all PX heterozygotes (carrying the PX and px alleles), or if some have no PX allele at all (i.e. they carry two Px alleles). Nevertheless, given the strong linkage disequilibrium between P and X, the occurrence of two Px alleles in the same individual is expected to be very rare. Assuming the 43 PpXx individuals to be PX heterozygotes, there was no difference in the age of menarche between the group of PX heterozygotes ($n = 56$; mean 12.76 years, SD 1.17), and subjects without any PX allele ($n = 55$; mean 12.76 years, SD 1.34). The mean age of menarche was

Table II. Comparisons for age of menarche between different genotypes and haplotypes

	Mean age (SD)	P-value for comparison	
		Homozygotes without restriction site	All three groups
<i>Xba</i> I polymorphism		0.017	0.057
XX	13.36 (1.24)		
Xx	12.80 (1.14)		
xx	12.75 (1.35)		
<i>Pvu</i> II polymorphism		0.21	0.45
PP	13.09 (1.29)		
Pp	12.80 (1.19)		
pp	12.85 (1.33)		
PX haplotype		0.006	NP
PX homozygotes	13.43 (1.18)		
PX heterozygotes or no PX allele	12.76 (1.25)		

NP = not pertinent (analysis cannot be performed).

also similar between the 43 PpXx individuals (mean 12.82 years, SD 1.16) and the remaining 13 certain PX heterozygotes (mean 12.54 years, SD 1.21). Thus the genetic effect was limited to PX homozygosity and there was no apparent evidence of a dose-response effect.

After adjusting for body mass index, the difference between XX homozygotes and other subjects was 0.57 years ($P = 0.021$). The difference between PP homozygotes and other subjects was still not significant (0.26 years, $P = 0.26$). Finally, the difference between PX homozygotes and other subjects was still 0.67 years ($P = 0.008$). Excluding the three girls who had not reached menarche at the time of the evaluation, the results were very similar: PX homozygotes had a mean age of menarche of 13.38 years versus 12.70 for PX heterozygotes and for subjects lacking the PX allele ($P = 0.004$).

There were 11 pairs of siblings included in the study, six of which had similar haplotypes for *Xba*I and *Pvu*II. Excluding these 11 pairs from the analysis yielded similar results for the association of the X allele with the age of menarche [mean age (years) 13.41 for XX ($n = 31$), 12.73 for Xx ($n = 45$) and 12.70 for xx ($n = 47$)].

Discussion

*Xba*I XX homozygotes or, in more general terms, subjects homozygous for the PX haplotype seem to have a modest delay in the age of menarche. The average time difference compared with other subjects is slightly >0.5 years and it is ~ 8 months when haplotypes are considered. The effects of *Pvu*II are not as clear on their own as the effects of *Xba*I. The trends we have observed may simply reflect the strong linkage disequilibrium with *Xba*I, and the PX haplotype shows the strongest association with the age of menarche. We did not observe any trend of a dose-response effect, but we should acknowledge that our study was underpowered to detect dose-responses. Alternatively, perhaps the menarche-modulating effect applies only to PX homozygotes.

XX homozygotes are protected from breast cancer and endometrial cancer (Andersen *et al.*, 1994; Weiderpass *et al.*,

2000). A non-significant trend for protection against endometrial cancer has also been seen for PP homozygotes (Weiderpass *et al.*, 2000). It is unknown to what extent these findings reflect differential cumulative exposure to estrogens due to different estrogen levels, different expression of ERs, different time duration of the estrogen exposure, or a combination of these factors. Other polymorphisms of steroid metabolizing enzymes have also been found to be associated with the risk of breast cancer. One of them, *CYP17*, is also associated with the age of menarche (Haiman *et al.*, 1999). Part of the protective effect may be mediated by a delayed menarche and this may also be the case for *Xba*I XX homozygotes. A delayed menarche is a strong protective factor against breast and endometrial cancer and is related to reduced lifetime estrogen exposure of the target tissues, as has been shown in several studies, including studies before the widespread advent of oral contraceptives (Hsieh *et al.*, 1990), which may also contribute to the cumulative estrogen exposure.

The *Xba*I and *Pvu*II polymorphisms of the *ER* α gene have also been implicated in other major diseases where lifetime estrogen exposure is considered to be a potentially important risk modifier. For example, in a study of 102 Japanese school children, XX homozygosity was associated with higher levels of low-density lipoprotein (LDL) cholesterol (Kikuchi *et al.*, 2000). High LDL cholesterol is a strong risk factor for cardiovascular disease and estrogen exposure may have a protective effect by reducing LDL cholesterol. The same polymorphisms have also been implicated as potential regulators of bone mineral density and the risk of osteoporosis, which is also affected by estrogen exposure. However, studies have not been consistent on whether Px haplotype effects (Albagha *et al.*, 2001) or some other polymorphism in linkage disequilibrium with *Xba*I and *Pvu*II, such as the TA dinucleotide repeat polymorphism 5' upstream of exon 1 of the *ER* α gene, may be implicated (Georgiou *et al.*, 1999; Gonnelli *et al.*, 2000). In a population of postmenopausal women from the wider geographic area of Epirus (mostly an urban population), we did not observe any significant association between these two polymorphisms and bone mineral density (Efsthadiadou *et al.*, 2001); however, modest differences for XX homozygotes

could have been missed in that study, because XX was somewhat more rare than in the current study population.

The biological pathway for *Xba*I and *Pvu*II that may affect the age of menarche is unknown. Restriction sites of both polymorphisms are located in the intron 1 of the *ER* α gene. Some introns contain regulatory sequences such as enhancers (Bornstein *et al.*, 1987; Gasch *et al.*, 1989), i.e. binding sites for elements that regulate the level of gene expression and thus also affect protein synthesis (Laurie and Stam, 1994). Alternatively, the observed association may reflect linkage disequilibrium with some other functional polymorphism in the *Xba*I vicinity. Regardless of the exact mechanism, if *ER* α gene polymorphisms can alter the estrogenic biological activity at the cellular level, this may influence the maturation of the hypothalamic–pituitary–gonadal axis, which determines the onset of menarche. Additional studies should also investigate the effects on the age of menarche of other *ER* α polymorphisms (TA repeat, BstUI and codon 325) (Jurada *et al.*, 2001; Westberg *et al.*, 2001) and *ER* β polymorphisms (Syrrou *et al.*, 1999; Sundarajan *et al.*, 2001; Westberg *et al.*, 2001).

This is the first study to report a significant difference in the age of menarche for subjects with different *Xba*I genotypes and/or PX haplotypes. One other group of investigators has suggested that *ER* α gene polymorphisms, in particular *Pvu*II, may affect the age of menopause (Weel *et al.*, 1999). In that study, the P allele showed a dose–effect relationship with a 0.5 year earlier onset of natural menopause per each copy of the P allele and the risk of surgical menopause was higher, by 2.4-fold, in women carrying the PP genotype as compared with pp homozygotes (Weel *et al.*, 1999). The same investigators had observed a non-significant trend for later menarche in PP homozygotes (0.2 years as compared with pp homozygotes), a finding consistent with our results. Thus, the PX haplotype may be important in regulating not only the onset, but also the end of high tissue estrogen exposure during the lifetime of an individual.

Our study was performed in a homogeneous population in a closed rural community. In the study population, menarche occurred slightly later on average than has been reported for other populations in Greece (Prapas *et al.*, 1989; Georgiadis *et al.*, 1997; Papadimitriou *et al.*, 1999), but the difference was small. Rural versus urban differences have diminished in more recent years in European countries (Marrodan *et al.*, 2000). We aimed for a population that would have relatively later menarche on average and more homogeneity for cultural parameters and environmental exposures. The lack of Hardy–Weinberg equilibrium also proves that this is a closed community with fairly substantial genetic drift due to inbreeding in small communities (Rousset and Raymond, 1995; Vogel and Motulsky, 1997). Genetic drift in these circumstances would tend to diminish the relative representation of heterozygotes and increase the percentage of homozygotes. The later age of menarche and the larger representation of homozygotes were helpful in giving the study adequate power to detect clinically meaningful differences. Although the population studied is a closed community with probably considerable genetic drift, the studied adolescents did not in the large

majority belong to the same families. The study included only 11 pairs of siblings and, excluding these pairs, yielded similar results for the importance of the X allele. Therefore, it is unlikely that what we have observed can be explained simply by familial clustering that may reflect some other, totally unrelated, genetic factors.

We should acknowledge that the actual age of menarche may differ across diverse populations and one should be cautious in extrapolating to other populations, especially more heterogeneous ones. The measurement of the age of menarche is also subject to error due to subjective interpretation, wrong reporting or other misconceptions across different subjects. Such error would introduce some noise into the derivation of any differences between groups of subjects with different genotypes. However, this noise would tend to diminish the power of a study to detect a significant difference among groups, if one truly exists (Kelsey *et al.*, 1996). Therefore, if a difference is detected, it is likely to be real. Nevertheless, it would be worthwhile to conduct studies in other heterogeneous populations in order to assess the replication validity (Ioannidis *et al.*, 2001) of our findings.

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