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Combined estrogen receptor α and estrogen receptor β genotypes influence the age of menarche

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BACKGROUND: Age at menarche has a strong genetic influence. We reported recently an association between the *Xba*I (351A→C)and *Pvu*II (397T→C) polymorphisms of the estrogen receptor (ER)α gene with the age of menarche in Greek adolescents. In the present study, we examined whether ERβ genotypes alone, or in combination with ERα genotypes, may also influence onset of menarche. METHODS: We performed genotyping for the single nucleotide polymorphisms 1730A→G and 1082G→A of the ERβ gene and examined their association with the age of menarche in the same cohort of 145 Greek girls. We then looked for a possible effect of combined ERα and β genotypes on the age of menarche. RESULTS: Menarche occurred 7 months later in girls with the AA genotype of the 1730A→G polymorphism than in girls with the AG genotype (mean ± SD: 13.23 ± 1.24 versus 12.66 ± 1.26 years, respectively; *P* = 0.005). The 1082G→ A polymorphism was not detected in any of the girls examined. A significant effect of combined ERα and β genotypes was also apparent. Menarche occurred 11 months later in girls bearing the AA/TT,AA (ERα,ERβ) genotypes compared with girls with the CC/CC,AG genotype (13.30 ± 1.27 νversus 12.41 ± 1.28 years; *P* = 0.042). The difference remained significant after adjusting for body mass index (*P* = 0.034). CONCLUSION: Combined ERα and ERβ polymorphisms may influence the age of menarche.

Key words: estrogen receptor α/estrogen receptor β/genotypes/genetic polymorphisms/menarche

Introduction

Menarche depends on the maturation and co-ordination of the hypothalamic–pituitary–ovarian axis with the female reproductive system and other endocrine organs, including the adipose tissue (Carr, 1998). The timing of menarche is regulated by a variety of environmental and genetic factors. Family and twin studies have indicated that the genetic contribution may be more important than environmental effects, since 53–74% of the variation in age of menarche can be attributed to genetic factors (Sharma, 1983, van den Akker *et al.*, 1987, Treloar and Martin, 1990, Kaprio *et al.*, 1995). However, the specific genes involved in this event are not yet well defined.

More than 30 years ago, Frisch and McArthur (1974) proposed that a given amount of body fat is necessary before the onset of menstrual cycles. This claim was recently substantiated by establishing that leptin constitutes the permitting signal, informing the brain on the amount of energy stored in adipose tissue (Kiess *et al.*, 2000; Mantzoros, 2000) and that a polymorphic variant of the leptin gene may influence the onset of menarche in interaction with maternal age (Comings *et al.*, 2001).

Ovarian estrogens appear to play an important role in the differentiation, maturation and function of the reproductive system—and also, in females, the distribution of adipose tissue—through endocrine and paracrine effects mediated by the activation of estrogen receptors (ER) (Enmark and Gustafsson, 1999). Two such receptors have been identified, ERα and ERβ. Both subtypes have been found in the female reproductive organs with overlapping but not identical tissue distribution, and with different or complementary contribution to reproductive functions (Kuiper *et al.*, 1996, Enmark *et al.*, 1997).

Polymorphic variants of both ERα and ERβ genes have been identified in recent years and studied for possible association with reproductive and other clinical outcomes (Georgiou *et al.*, 1999; Syrrou *et al.*, 1999; Weel *et al.*, 1999). Such allelic variants could also account for the genetic variability in the age of menarche. Indeed, we have recently shown that two polymorphisms of the ERα gene, in particular *Xba*I (351A→C) and *Pvu*II (397T→C) may influence the age of menarche in healthy adolescent Greek girls (Stavrou *et al.*, 2002).

In the present study, we examined the association of 1082G→A and 1730A→G polymorphisms of the ERβ gene with the age of menarche and their potential interaction with ERα genotypes in influencing this event in the same study population.

Materials and methods

Subjects

The study population consisted of 145 healthy adolescent girls from a closed rural community in northwest Greece as described previously (Stavrou *et al.*, 2002). This homogeneous population was selected with

the anticipation that environmental and cultural heterogeneity, which could possibly create some variability in the age of menarche, would be minimal. Information on the age of menarche was taken through personal interviews with the adolescents and their mothers. Informed consent of the girls and their parents, and approval of the study by the University Hospital of Ioannina Ethics Committee were obtained.

Genotyping

Details on genomic DNA extraction and genotyping for the *Xba*I (351A→C) and *Pvu*II (397T→C) polymorphisms (Herrington and Howard, 2003) of the ERα gene have been described previously (Stavrou *et al.*, 2002).

Genotyping for the 1082G→A *(*Sundarrajan *et al.*, 2001) and 1730A→G (Kealey *et al.*, 2001) polymorphic variants of the ERβ gene was carried out as follows. Specific DNA amplification was performed by PCR using 1 unit of recombinant *Taq* DNA polymerase (Gibco BRL, Göteborg, Sweden) in DNA thermocycler PTC-100 (Peltier-Effect Cycling, MJ Research, Watertown, Massachusetts, USA). The ligand binding domain of exon 5 and the 3′-untranslated region of exon 8 of the ERβ gene were amplified using the following primer pairs: 5′-TCTTGCTTTCCCCAGGCTTT-3′, 5′-ACCTGTCCAGAA CAAGATCT-3′ and 5′-GACCTGCTGCTGGAGATGCT-3′, 5′-AAT GAGGGACCACACAGCA-3′, respectively.

PCR products were analysed for RFLP using *Rsa*I and *Alu*I restriction enzymes (Gibco BRL). Nucleotide exchange G–A at nucleotide 1082 in exon 5 created a recognition site for *RsaI,* and exchange A→G at nucleotide 1730 in the 3′ untranslated region of exon 8 introduced a recognition site for *Alu*I (Fig. 1). Enzyme digestion products underwent electrophoresis on 2% agarose gel and the separation patterns were photographed under ultraviolet illumination. The resulting genotypes for *Rsa*I (1082G→A) and *Alu*I (1730 A→G) polymorphic sites were characterized as GG, GA, AA and AA, AG, GG, respectively.

To meet quality control standards and avoid genotyping errors, all samples were run in duplicate and read by two investigators independently. In addition, genotyping was repeated in 20% of randomly selected samples. There was full agreement between the two investigators reading the gels.

Statistical analysis

The *t*-test and one-way analysis of variance (ANOVA) were used for comparison conducted in Advanced Statistics Package for Social Sciences (SPSS Inc., Chicago, IL, USA). All *P*-values are two-tailed and $P < 0.05$ was considered significant.

Results

Association of ERβ genotypes with the age of menarche

The overall mean (± SD) age at menarche was 12.92 ± 1.26 years; this is a figure typical for the rural population of this region. Of note, heterozygotes for the ERβ 1730A→G polymorphism were over-represented, whereas homozygotes for the presence of the polymorphic site were not detected, deviating from Hardy–Weinberg equilibrium ($P = 0.005$). Regarding the ERβ 1082G→A polymorphism, only one genotype (GG) was present in our population study. A similar deviation from Hardy–Weinberg equilibrium was observed for the ERα 351A→C and 397T→C polymorphisms as previously reported by Stavrou *et al.* (2002). This is highly suggestive of the presence of genetic drift that can be observed in closed communities, as was the case for our study population.

This was further substantiated by comparing the observed allele frequencies (Table I) with allele frequencies derived from other populations. Thus, frequencies in the order of 37– 64% and 94–99% for the A allele of the ERβ 1730A→G and the G allele of the 1082G→A polymorphisms, respectively, have been described in other populations (Rosenkranz *et al.*, 1998; Lambert *et al.*, 2001; Arko *et al.*, 2002). With regard to ERα 397T→C and 351A→C polymorphisms, the frequencies for the T and A alleles were 53.4% and 43.4%, respectively, whereas in the general Greek population these are 48% and 41.5% (I.Georgiou, N.Xita, L.Lazaros, unpublished observation).

Menarche occurred 7 months later in girls with the AA genotype (mean ± SD: 13.23 ± 1.24 years) than in girls with the AG genotype (12.66 ± 1.26 years) of the ERβ 1730A→G polymorphism ($P = 0.005$).

Effect of combined ERα and ERβ genotypes on the age of menarche

Since ERα and ERβ genes may interact in their biological effects on reproductive functions, we investigated whether combined genotypes of both ERα 351A→C and 397T→C and ERβ 1730A→G polymorphisms may also influence the age of menarche. As shown in Table II, girls bearing the AA/TT haplotype of ERα polymorphisms in combination with the AA genotype of ERβ (Group 1) had 11 months delay in menarche compared with girls bearing the genotype combination CC/CC,AG (Group 2). In addition, menarche occurred 4 months later when girls in Group 1 were compared with girls carrying all other genotype combinations (Group 3). The differences were significant when the groups were compared with each other ($P = 0.04$). There appeared to be a trend for earlier onset of menarche for girls carrying one or more of the

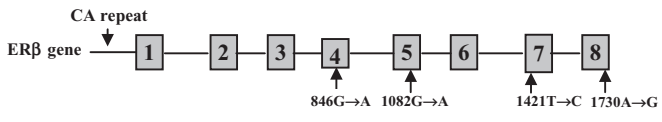

Figure 1. The gene encoding estrogen receptor β (ERβ) with the positions of known polymorphisms

Table I. Demographic characteristics of the study population and frequency of the different estrogen receptor β (ERβ) genotypes

Characteristics				
Age at menarche, median (IQR), years		13.0 (12.0–15.75)		
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 (10.35)		
Body mass index, mean (SD), kg/m^2		20.68 (5.62)		
Polymorphism	*Genotype frequency, n (%)*		*Allele frequency (%)*	
ERβ-1082G→A	GG	145 (100%	G	100
	GA	–	A	–
	AA	–	–	–
ERβ-1730A→G	AA	65 (44.8%)	A	72
	AG	80 (55 .2%)	G	28
	GG	–	–	–

IQR: interquartile range.

Table II. Comparison for age at menarche between different estrogen receptor α (ERα)and estrogen receptor β (ERβ) genotype combinations

Genotype groups	Number of subjects	Mean age (SD) at menarche (years)	*P* value
Group 1	22	13.30 (1.27)	0.042
Group 2	29	12.41 (1.28)	
Group 3	94	12.96 (1.23)	

Group 1: AA/TT,AA (ERα, ERβ). Group 2: CC/CC,AG (ERα, ERβ). Group 3: all other genotype combinations.

polymorphic C/C,G alleles of ERα and ERβ, respectively—the greater the number of these alleles, the earlier the age of menarche.

After adjusting for BMI (recorded at the time of evaluation), the difference in menarcheal age between AA homozygotes and the AG heterozygotes for the 1730A→G polymorphism was still significant ($P = 0.006$). Similarly, the difference in age of menarche between the girls with the genotype combination AA, AA/TT in Group 1 compared with the groups 2 and 3 remained the same ($P = 0.034$).

Discussion

Family and twin studies have shown that genetic factors are related to age of menarche. Thus, significant correlations between age at menarche in mothers and daughters along with significant differences in menarcheal age between monozygotic and dizygotic twin pairs have been described (Treloar and Martin, 1990). We reported recently that age at menarche was associated with the *Xba*I (351A→C) and possibly the *Pvu*II (397T→C) polymorphism of the ERα gene in healthy adolescent Greek girls (Stavrou *et al.*, 2002). In particular, menarche occurred 6 months later in girls with the AA genotype of the 351 A→C polymorphism than in girls with AC or CC genotypes and tended to occur later in TT homozygotes of the 397T→C polymorphism than in TC and CC genotype carriers. In the current study, we examined whether there was also an association between ERβ genotypes and age at menarche in the same study population, since ERβ receptors are also distributed in reproductive tissues and may influence reproductive function (Kuiper *et al.*, 1997; Hiroi *et al.*, 1999; Sar and Welsch, 1999; Pelletier and El-Alfy, 2000).

The gene encoding ERβ is located in chromosome 14q 22–24 (Enmark *et al.*, 1997). Five novel polymorphisms have been identified so far within the ERβ gene (Rosenkranz *et al.*, 1998). Among these, two silent mutations, 1082 G→A within exon 5 (*Rsa*I polymorphism) and 1730 A→G in the 3′-untranslated region of exon 8 (*Alu*I polymorphism) were selected in the present study, since these polymorphisms have been implicated in other conditions where estrogen exposure is considered to be an important risk modifier (Rosenkranz *et al.*, 1998; Eastwood *et al.*, 2002).

In our study, the 1082G→A polymorphic variant was not found in any of the girls examined, indicating that this polymorphism may be rare in Greek or South European populations. With regard to 1730A→G polymorphism, however, AA homozygotes appeared to delay menarche by 7 months compared with heterozygotes for the polymorphism indicating that, in addition to ERα genotypes, ERβ genotypes may also contribute to genetic variability of the age of menarche.

In our study, we aimed for a population that would have homogeneity for cultural parameters and environmental influences. The lack of Hardy–Weinberg equilibrium for both ERα and ERβ polymorphisms also suggests that this is a closed community with probably considerable genetic drift due to inbreeding in small communities (Rousset and Raymond, 1995; Vogel and Motulsky, 1997).

Previous studies have shown significant associations of the 1082G→A and 1730A→G polymorphisms with reproductive dysfunctions (Sundarrajan *et al.*, 2001). In addition, the 1082G→ A polymorphism has been associated with anorexia nervosa (Rosenkraz *et al.*, 1998; Eastwood *et al.*, 2002).

Although the above polymorphisms do not lead to amino acid changes in the ERβ protein, it is possible that this polymorphism is in linkage disequilibrium with other regulatory sequence variations that may affect gene expression or function (Yaich *et al.*, 1992). Alternatively, it is believed that single nucleotide polymorphisms—even when situated in the untranslated region—may cause different structural folds of mRNA, and thus influence the expression of the gene (Shen *et al.*, 1999).

The second hypothesis we tested in this study was whether combined genotypes of both ERα and ERβ polymorphisms may also influence the age of menarche. This hypothesis was based on the knowledge that, in addition to homodimers, functional ERα/ERβ heterodimers may interact in their biological effects (Cowley *et al.*, 1997). Indeed, we observed a positive additive effect of combined 351A→C, (397T→C) (ERα) and 1730A→G (ERβ) genotypes on the age of menarche. In particular, the AA/TT haplotype of ERα combined with the AA genotype of ERβ is associated with later onset of menarche by 11 months compared with the genotype combination CC/CC,AG. Furthermore, there appeared to be a trend for earlier menarche for the girls with one or more of the polymorphic C/C,G alleles of the respective genes in that, the higher the number of these alleles, the earlier the onset of menarche—suggesting a dose-response effect. This implies that ERα and ERβ genes may interact in their effects on the age of menarche and this effect may be modified by the presence of certain genotype combinations of both ER subtypes.

Significant interactions between the ERβ 1730A→G polymorphism and the ERα 351A→C and 397T→C polymorphisms were also reported in a study on Alzheimer's disease in the U.K (Lambert *et al.*, 2001). This study suggested that the risk for Alzheimer's disease may be modulated by certain ERα and ERβ variants, which influence their expression and/or biological activities.

In conclusion, the findings of the present study suggest that the 1730A→G polymorphism of the ERβ gene is associated with the age of menarche among healthy adolescent girls. In addition, combined genotypes of both ERα and ERβ polymorphisms may further influence menarcheal age, suggesting interaction between these two ER subtypes in affecting this event. Thus, allelic variants of both ERα and β

genes may contribute to the genetic variability in the age of menarche.

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