The (TTTA)$_n$ polymorphism of aromatase (CYP19) gene is associated with age at menarche

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BACKGROUND: Twin studies have shown that age at menarche may be subject to hereditary influences but the specific determinants are unknown. Estrogens are known to have an important role in menarche. Since the enzyme aromatase is responsible for the conversion of androgens to estrogens, the aromatase (CYP19) gene could be a candidate gene for the regulation of menarche. The aim of this study was to investigate the possible association of the CYP19(TTTA)$_n$ polymorphism with age at menarche.

METHODS: We studied 130 healthy adolescent females from a closed community in North-Western Greece. Information on menarche was obtained through interviews. The BMI was recorded. The CYP19(TTTA)$_n$ polymorphism was genotyped.

RESULTS: The mean age at menarche was 12.9 ± 1.2 years and the BMI = 19.8 ± 2.3 kg/m$^2$. Genotype analysis revealed 5 CYP19(TTTA)$_n$ alleles containing 7–11 TTTA repeats. Girls homozygous for the allele with 7 TTTA repeats had earlier menarche (12.45 ± 0.9 years) than girls carrying other genotypes (13.0 ± 1.2 years, $P$ = 0.025), whereas the BMI was not different between these two subgroups. Carriers of the allele with 11 TTTA repeats had later menarche compared with non-carriers (14.1 ± 0.75 versus 12.8 ± 1.2 years, $P < 0.001$), whereas no difference was found in BMI values. Comparing girls with early menarche (<12 years, 25th percentile) with girls with late menarche (>13.75 years, 75th percentile), we found that 31% of the girls with early menarche were homozygous for the (TTTA)$_7$ allele compared with 6.9% among girls with late menarche ($P = 0.018$). In addition, none of the girls carrying the (TTTA)$_{11}$ allele was found among the subgroup with early menarche, whereas 24.1% of girls with late menarche had the (TTTA)$_{11}$ allele ($P = 0.001$). No association between other alleles and age at menarche was found.

CONCLUSIONS: There is evidence for a genetic contribution of the CYP19 gene to the age at menarche.

Key words: CYP19 gene / aromatase / menarche / body mass index

Introduction

Differences at the age of menarche may affect endocrine manifestations and influence the reproductive fitness during the female reproductive life. Early menarche is associated with elevated risk of ovarian tumors, endometrial and breast cancer, and also with increased risk of cardiovascular disease events and mortality (Hsieh et al., 1990; Kaaks et al., 2002; Moorman et al., 2008; Lakshman et al., 2009). On the other hand, delayed menarche may affect bone mineral density and increase the risk of osteoporosis (Eastell, 2005; Chevalley et al., 2008). Thus, the identification of factors that contribute to variation of age at menarche could lead to better understanding of many phenotypes.

Twin and family studies have shown that genetic factors play a significant role in influencing the timing of menarche with approximately half of the variation in age at menarche attributable to genetic factors (Towne et al., 2005). However, the genetic basis determining the age at menarche has only partly been explained. Genome-wide linkage analyses designed to identify regions of the genome that harbor gene modulating age at menarche have given several results (Guo et al., 2006; Rothenbuhler et al., 2006; Anderson et al., 2008). Moreover, association studies have mostly focused on genes involved in steroid hormone biosynthesis, action and metabolism, but with inconsistent findings.

We have previously reported an association of polymorphisms of the estrogen receptor-α gene with the age at menarche (Stavrou et al., 2002). The estrogen receptor-β gene has also been associated with the age at menarche by interacting with the estrogen receptor α gene locus (Stavrou et al., 2006). We have also studied the role of
a functional polymorphism of SHBG gene, the SHBG(TAAA)n polymorphism, on the timing of menarche, and we found that carriers of the longer allele genotypes had later menarche than those with shorter allele genotypes (Xita et al., 2005).

Aromatase catalyzes the conversion of androgen to estrogen, and polymorphisms of the aromatase gene (CYP19) that affect gene activity may predispose carriers to conditions influenced by the sex steroid milieu. The most well-studied polymorphism of CYP19 is a tetranucleotide repeat polymorphism in intron 4, the (TTTA)n polymorphism. This polymorphism has been associated with the hyperandrogenic phenotype of polycystic ovary syndrome (PCOS; Xita et al., 2010). It has also been related to increased risk for the development of various estrogen dependent diseases in women such as breast and endometrial cancer and osteoporosis (Berstein et al., 2001; Gennari et al., 2004; Dick et al., 2005; Paynter et al., 2005; Lorentzon et al., 2006; Ma et al., 2010).

The aim of the present study was to investigate the possible role of CYP19(TTTA)n polymorphism in determining the age at menarche.

Materials and Methods

Subjects

The study population consisted of 130 healthy normal-weight adolescent girls from a closed rural community in North-Western Greece. From family history data, no parental or grandparental consanguinity was reported. Information on menarche was obtained through interviews with the adolescents and their mothers, and through diaries. The BMI of each subject, calculated as weight (kg)/height2 (m2), was recorded. Blood samples were obtained from all girls for the genetic analysis. The details of the protocol were explained to the girls and their parents, who gave their consent, and the protocol was approved by the Hospital Ethics Committee.

Genotype analysis

Genomic DNA was isolated from peripheral blood leukocytes of the study population. Amplification of the CYP19(TTTA)n repeat region was accomplished using PCR with a forward primer (5′-CACTCGACCT TCTTTATG-3′) and a reverse primer (5′-GTTTGACTCCGTGAG TTTGA-3′). Amplified products were separated by 12% polyacrylamide gel electrophoresis followed by silver staining and the number of repeats of each allele was determined. The size of PCR products was 356–372 bp in respect of the number of TTTA repeats. The number of TTTA repeats in every particular allele was analyzed by sequencing the appropriate PCR products. A quality control assessment of our PCR method was done by random sampling and sequencing of the PCR products and duplication of PCR assays.

Statistical analysis

Statistical analysis of differences in allele and genotype frequencies between girls with earlier menarche and those with later menarche was performed using the χ2 test with the Yates correction. Differences in menarche were assessed with the non-parametric Mann–Whitney U-test. A P-value of <0.05 was set as statistically significant. All results are reported as mean ± SD. All analyses used the SPSS statistical package (version 15.0, SPSS Inc., Chicago, IL, USA).

Results

The general characteristics of the study population are presented in Table I. The mean age at menarche was 12.9 ± 1.2 years and the mean BMI was 19.8 ± 2.3 kg/m2. Although there is no consensus definition of a normal range for age at menarche, none of the girls reported menarche sooner than 8 years or later than 15 years. Genotype analysis revealed 5 CYP19(TTTA)n alleles containing 7–11 TTTA repeats. The genotype frequency is presented in Fig. 1. The effect of each allele on age at menarche was examined by comparing carriers with no carriers of each allele. Girls homozygous for the allele with 7 TTTA repeats had earlier menarche (12.45 ± 0.9 years) than girls carrying other genotypes (13.0 ± 1.2 years, P = 0.025), whereas no difference was found in BMI values between these two subgroups (19.7 ± 2.7 versus 19.8 ± 2.2 kg/m2, Fig. 2). We also compared carriers of each allele with non-carriers. It was found that carriers of the allele with 11 TTTA repeats (none of the girls in our population were homozygous for the 11 TTTA allele) had later menarche compared with non-carriers (14.1 ± 0.75 versus 12.8 ± 1.2 years, P < 0.001), whereas the BMI values were no different (19.4 ± 2.9 versus 19.8 ± 2.2 kg/m2, Fig. 3). No difference in the age at menarche was found when we compared carriers of other alleles.

This association between genotype and age at menarche was also found when girls were subdivided into two groups based on the median age at menarche, and genotype frequencies were compared.

| Table I General characteristics of the study population |
|---------------------------------------------|-----------|
| Characteristics                             |           |
| Age at menarche, median (IQR) (years)        | 13.0 (12.0–13.7) |
| Age at evaluation median (IQR) (years)       | 16.9 (15.2–18.5) |
| Height, mean (SD) (cm)                       | 161.6 (5.0)  |
| Weight, mean (SD) (kg)                       | 51.8 (6.8)  |
| BMI, mean (SD) (kg/m2)                       | 19.8 (2.3)  |
| (TTTA)n alleles                              | n (%)     |
| (TTTA)7                                     | 115 (44.2) |
| (TTTA)8                                     | 38 (14.6)  |
| (TTTA)9                                     | 27 (10.4)  |
| (TTTA)10                                    | 69 (26.5)  |
| (TTTA)11                                    | 11 (4.2)   |

IQR, inter-quartile range.

![Figure 1](image-url) Distribution of CYP19 genotypes in the study population.
24.1% of girls with late menarche had this allele (girls with early menarche was carrying the (TTTA)11 allele, whereas among girls with late menarche (were homozygous for the (TTTA)7 allele compared with 6.9% percentile). It was found that 31% of the girls with early menarche were compared with girls with late menarche (evident when girls with early menarche (compared with 14.9 % in Group 2 having this allele (CYP19(TTTA)n repeat polymorphism, the CYP19 gene and age at menarche

The present study supports an association between a tetranucleotide repeat polymorphism, the CYP19(TTTA)n, with age at menarche. More precisely, girls who were homozygous for the (TTTA)7 allele have earlier menarche than girls with other genotypes and, in addition, girls carrying the (TTTA)11 allele have later menarche compared with non-carriers of this allele.

These finding are in accordance with a previous study that investigated the role of genes involved in estrogen synthesis metabolism and estrogen action, in regulating menarche and menopause and reported that homozygotes for the (TTTA)7 allele had earlier menarche than heterozygotes for this allele (Mitchell et al., 2008). The advantage over the previous report is that our population consisted of adolescent girls who participated in the study 1 or 2 years after menarche and they or their mothers were able to remember the exact year and month of menarche and so recall bias was minimized. Moreover, they were girls of normal weight and therefore bias due to effects of body weight on menarche was avoided. The study population was homogeneous from a closed rural community in North-Western Greece. Although a genetic drift due to inbreeding cannot be excluded, the study population was selected with the anticipation that cultural and environmental heterogeneity would be minimized. Regional and environmental factors may create some variability in the age of menarche and, as is the case in family-based studies, the homogeneity of the population could represent an advantage of the study.

In vitro studies in tumor tissues of endometrial cancer and skin fibroblasts have shown an association between the presence of long CYP19 alleles and enhanced aromatase activity (Berstein et al., 2004; Gennari et al., 2004). Clinical studies also investigated the association between this CYP19 polymorphism and hormonal status with inconsistent results. Thus, Haiman et al. (2000) found lower levels of estrogen sulfate (but not estradiol) among post-menopausal women with at least one (TTTA)7 allele. On the other hand, Dick et al. (2005) found an association between the presence of (TTTA)7 allele with higher free estradiol concentration. Tworoger et al. (2004) reported increased estrogen levels among post-menopausal women having the (TTTA)n allele, whereas other studies showed no association between the CYP19(TTTA)n polymorphism and hormonal status (Probst-Hensch et al., 1999; García-Closas et al., 2002; Salmen et al., 2003; Van Pottelbergh et al., 2003; Travis et al., 2004). This discrepancy between in vitro and clinical studies may be explained by the fact that aromatase has direct target organ-specific effects that can alter estrogen levels in target tissues, without substantially affecting circulating estrogen concentrations (Simpson and Davis, 2001).

The fact that CYP19 activity determines the local estrogen level may also explain the discrepancy between our findings and the results of genetic studies concerning the importance of this polymorphism in the development of breast cancer. Although short CYP19 alleles have been associated with early menarche and in turn, early menarche has been related to increased risk for breast cancer, most studies support an association between long CYP19 alleles and increased risk for breast cancer (Kristensen et al., 1998; Dunning et al., 1999; Haiman et al., 2000; Miyoshi et al., 2000; Baxter et al., 2001; Ma et al., 2010). The CYP19(TTTA)n polymorphism is within an intronic region and it is not associated with gene regulation or with posttranslational expression. Thus, the biological function of this polymorphism may be due to linkage disequilibrium with a potentially functional polymorphism. The aromatase gene is known to have several tissue-specific promoters and the CYP19(TTTA)n polymorphism may be

**Discussion**

The present study supports an association between a tetranucleotide repeat polymorphism, the CYP19(TTTA)n, with age at menarche.

**Figure 2** Girls having the genotype 7/7 of the CYP19(TTTA)n polymorphism had earlier menarche compared with girls with other genotypes.

**Figure 3** Carriers of the (TTTA)11 allele had later menarche compared with carriers of the shorter alleles.

Group 1 included girls with menarche < 13 years (n = 63) and Group 2 girls with menarche ≥ 13 years (n = 67). Among girls in Group 1, 30.2% were homozygous for the allele with 7 TTTA repeats compared with 14.9% in Group 2 (P = 0.037). Furthermore, in Group 1, only 1.6% were carriers of the allele with 11 TTTA repeats (all homozygous) compared with 14.9% in Group 2 having this allele (P = 0.009). No difference in the distribution of the other genotypes was found.

The difference in the distribution of 7 and 11 TTTA allele was more evident when girls with early menarche (< 12 years, 25th percentile) were compared with girls with late menarche (> 13.75 years, 75th percentile). It was found that 31% of the girls with early menarche were homozygous for the (TTTA)7 allele compared with 6.9% among girls with late menarche (P = 0.018). In addition, none of the girls with early menarche was carrying the (TTTA)11 allele, whereas 24.1% of girls with late menarche had this allele (P = 0.001).
related to the differential aromatase expression through activation of these tissue-specific promoters (Bulun et al., 2003).

The findings of the present study support an association between short CYP19 alleles and early menarche. This may be explained by the fact that short CYP19 alleles are related to low aromatase activity which may alter the androgen to estrogen ratio in favor of androgens. We have previously reported that the CYP19(TTTA)$_n$ polymorphism may act as a genetic modifier of the hyperandrogenic phenotype of PCOS, by showing that women with high androgens were more frequently carriers of short (TTTA)$_n$ alleles (Xita et al., 2010). Androgens are thought to play a role in disinhibition of the GnRH pulse generator as evidenced by the priming of central axis activation and induction of gonadarche by androgen treatment in adolescent boys with constitutional delay of puberty (Nader, 2007). Moreover, it has been shown that gonadarche in its earliest phase starts in an androgen-dominant state and, during pubertal development, the hormonal milieu is changed to estrogenic (Ankaberg and Norjavaara, 1999). The critical point in pubertal development could be the transitional stage from the early pubertal androgen-dominated state to the estrogenic state later in puberty and this may represent the onset of menstrual cycling. Thus, the CYP19(TTTA)$_n$ polymorphism may affect this critical point by altering the androgen-to-estrogen ratio.

In conclusion, this study provides evidence for a genetic contribution of the CYP19 gene to the age at menarche; however, further studies are necessary to confirm these results.

Authors’ roles

The authors declare the following individual contribution to the paper. N. X.: substantial contribution to conception and design, drafting the article and final approval of the version of the published. A.C.: substantial contribution to data analysis and interpretation of the data, revising the article critically for important intellectual content and final approval of the version to be published. I. S.: substantial contribution to conception and design, acquisition of the data, revising the article critically for important intellectual content and final approval of the version to be published. Ch. Z.: substantial contribution to conception and design, acquisition of the data, revising the article critically for important intellectual content and final approval of the version to be published. A.T.: substantial contribution to conception and design, interpretation of the data, revising the article critically for important intellectual content and final approval of the version to be published.

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