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No association of estrogen receptor α and cytochrome P450c17α polymorphisms with age at menopause in a Dutch cohort

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BACKGROUND: Age at menopause is under strong genetic control. So far, genetic variations of only one gene, the *Pvu*II polymorphism of the estrogen receptor α (ERα) gene, have been shown to be associated with age at onset of menopause. This study aims to investigate whether *Pvu*II, *Xba*I and B-variant polymorphisms of the ERα gene, and the *Msp*AI polymorphism of the cytochrome P450c17α (CYP17) gene are associated with age at menopause in a Dutch cohort. METHODS: DNA was isolated from urine samples of 385 Caucasian women with natural menopause and the genotypes of the four polymorphisms were determined. A questionnaire was used for background characteristics. The genotypes of *Pvu*II, *Xba*I, *Msp*AI were obtained by PCR restriction fragment length polymorphism analysis. The B-variant was determined with an allele-specific oligonucleotide hybridization method. Two-sided *t*-tests were performed to assess the association between the four polymorphisms and menopausal age. The *Pvu*II and *Xba*I polymorphisms were analysed separately as well as in a combined score. RESULTS: The results show that none of the polymorphisms independently, nor the combined genotypes for *Pvu*II and *Xba*I, were associated with age at natural menopause. CONCLUSION: No evidence was found for a relationship between common variants of the ERα gene and the CYP17 gene with age at natural menopause.

Key words: age at menopause/association study/cytochrome P450c17α/estrogen receptor a/polymorphism

Introduction

Early menopause is related to decreased fertility (Kok *et al.*, 2003), a risk factor for osteoporosis (Kritz-Silverstein and Barrett-Connor, 1993) and cardiovascular disease (van der Schouw *et al.*, 1996) and decreases the risk of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). Studies have shown that the individual variability of age at menopause is under strong genetic control (Snieder *et al.*, 1998; Treloar *et al.*, 1998; de Bruin *et al.*, 2001). It is well conceivable that genetic variation in hormone production and metabolism as well as genetic susceptibility for general and organ-specific effects of estrogen, play an important role in determination of menopausal age. However, until now the genes involved have remained ill-defined.

A number of genetic polymorphisms causing variation in estrogen levels have been reported. Examples are variants in the estrogen receptor α gene (ERα) and a gene variant involved in estrogen biosynthesis (cytochrome P450c17α; CYP17). Since estrogen levels can modulate the release of pituitary gonadotrophins by negative and positive feedback, these gene variants can modulate the timing of menopause. Various reports of gene polymorphisms and menopausal age have been published.

Van Weel *et al.* (1999) reported that variations in the ERα were associated with onset of natural menopause and with the risk of surgical menopause in a Dutch population. Women carrying the PP genotype of the *Pvu*II restriction fragment length polymorphism (RFLP) were found to have a 1.1 year earlier onset of menopause compared to women with the pp genotype. An allele dose effect corresponding to a 0.5 year earlier onset of menopause per copy of the P allele was demonstrated. This finding could not be replicated in a Japanese population (Gorai *et al.*, 2003).

ERα is located on the long arm of chromosome 6 and several polymorphic sites in the gene are known. The *Pvu*II polymorphism is located in intron 1, ~0.4 kb upstream of exon 2. A second polymorphism, *Xba*I, is located ~50 bp apart from the *Pvu*II restriction site and a high degree of linkage disequilibrium between *Xba*I and *Pvu*II has been reported (Heimdal *et al.*, 1995; Kobayashi *et al.*, 1996). A third polymorphic site of the ERα gene is located in the B-domain. The B-variant polymorphism is caused by a G/C

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transversion in exon1 [B-wild type (B)/B-variant type (B′)] (Green *et al.*, 1986; Taylor *et al.*, 1992). It has been reported that estrogen receptors with B-variant type have decreased binding affinity to estrogen (Garcia *et al.*, 1988), although the mechanisms involved are not clearly understood.

It has been proposed that polymorphisms in the genes involved in estrogen biosynthesis (CYP17) (Dunning *et al.*, 1998; Helzlsouer *et al.*, 1998; Weston *et al.*, 1998; Bergman-Jungestrom *et al.*, 1999; Feigelson *et al.*, 1999; Haiman *et al.*, 1999b; Kristensen *et al.*, 1999; Spurdle *et al.*, 2000; Mitrunen *et al.*, 2000a; Miyoshi *et al.*, 2000; Ambrosone *et al.*, 2003; Gudmundsdottir *et al.*, 2003; Wu *et al.*, 2003), hydroxylation (cytochrome P4501a) (Huang *et al.*, 1999; Basham *et al.*, 2001; da Fonte *et al.*, 2002; Laden *et al.*, 2002; Miyoshi *et al.*, 2002, 2003; Li *et al.*, 2004), and inactivation of the reactive metabolites (catechol-O-methyltransferase) (Yaich *et al.*, 1992b; Millikan *et al.*, 1998; Thompson *et al.*, 1998; Bergman-Jungestrom and Wingren, 2001; Goodman *et al.*, 2001; Hamajima *et al.*, 2001; Mitrunen *et al.*, 2001, 2002; Yim *et al.*, 2001; Kocabas *et al.*, 2002; Miyoshi and Noguchi, 2003; Wedren *et al.*, 2003; Cheng *et al.*, 2004) may be associated with an elevated risk of breast cancer (Lavigne *et al.*, 1997; Haiman *et al.*, 1999a; Huang *et al.*, 1999), although the findings until now have been conflicting. So far, associations of these polymorphisms with age at menopause are scarce (Gorai *et al.*, 2003).

The *Msp*AI polymorphism of the CYP17 gene, located on chromosome 10, gives rise to the genotypes A1A1, A1A2 and A2A2. Two studies found that both pre-menopausal and post-menopausal women with the variant A2 allele had higher levels of circulating estrogens than those with A1 alleles (Feigelson *et al.*, 1998; Haiman *et al.*, 1999a).

In the present study, we evaluate whether polymorphisms in the ERα gene (*Pvu*II, *Xba*I and B-variant) or in the cytochrome P450c17α gene (*Msp*AI) are associated with age at natural menopause in a Dutch cohort.

Materials and methods

Participants

The study population was selected from participants in a population-based screening programme for early detection of breast cancer (the DOM project), that has been described earlier (de Waard *et al.*, 1984). During the period 1974–1986, women born between 1911 and 1945 who lived in the city of Utrecht, The Netherlands, were invited to participate in this screening programme; a total of 27 718 women attended. In this period, >95% of the women in this age group, living in Utrecht and surroundings, had the Dutch ethnicity, i.e. Caucasian (Bureau voor Statistiek Utrecht, 1979; Bestuursinformatie, 1994). Women who responded to the first invitation to participate in this study were then invited for regular screening examinations. At each visit, participants provided an overnight urine sample, which was stored at −20°C in plastic polypropylene jars without preserving agents. Furthermore, they filled out a questionnaire on health status and lifestyle, and height and weight measures were taken. A random sample of 420 women was drawn from the total of 9349 participants in the baseline cohort who were naturally post-menopausal at time of urine collection (defined as no menstrual period for ≥12 months, after spontaneous cessation of their menses). Women who reported regular use of HRT ($n = 35$) were excluded from the analysis to avoid any uncertainty on menopausal age, leaving 385 women in the study population. Information on age at menarche was not obtained until the fifth screening round. Therefore, age at menarche was only known for women participating in the fifth screening round ($n = 213$). All other variables used in this study were available for all women in the study.

Laboratory analysis

DNA isolation

DNA was isolated from 100 ml urine samples as was described earlier (van der Hel *et al.*, 2002). In brief, after centrifugation, DNA was isolated from the urine pellet. Pellets containing cells and nuclei were resuspended in a cell lysis buffer, followed by protein precipitation. DNA was precipitated by alcohol and finally resuspended in 80 μl TE (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.6).

ER

To screen for the two polymorphisms in the ERα, we used PCR–restriction fragment length polymorphism (PCR–RFLP) protocols (Bergink *et al.*, 2003; van Meurs *et al.*, 2003). The first polymorphism is a T → C transition at −397 bp before exon 2, which results in a restriction site for *Pvu*II (Yaich *et al.*, 1992a). The second is a G → A substitution at −351 bp before exon 2, resulting in a *Xba*I restriction site (Zuppan *et al.*, 1989). A PCR fragment of 345 bp containing the two base pair changes was generated using the following primers: forward: 5′-GATATCCAGGGTTATGTGGCA-3′; and reverse: 5′-AGGTGTTGCCTATTATATTAACCTTGA-3′. PCR reactions were carried out in a final volume of 20 ml containing 1 × Perkin–Elmer Buffer (Applied Biosystems, USA), 2.5 mmol/l of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 mmol/l $MgCl_2$, 0.45 mmol/l of each primer, 1.0 IU AmpliTaq Gold polymerase (Applied Biosystems) and 2 ml of DNA. The amplification was for 35 cycles with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. An initial denaturing step of 5 min at 95°C and a final extension step for 10 min at 72°C was used. According to the manufacturer's instructions, 5 ml of PCR product was digested with *Pvu*II and an equal amount was digested with *Xba*I (New England Biolabs, USA), both at 37°C overnight and separated by agarose gel electrophoresis and stained with ethidium bromide to identify details of fragments.

A third polymorphism (a G → C substitution) is located in the B-region of the ERα at codon 87 (Taylor *et al.*, 1992). Genotypes of the B-variant were determined with an allele-specific oligonucleotide (ASO) hybridization method (Andersen *et al.*, 1994; Berkowitz *et al.*, 1994; Roest *et al.*, 1999) (Accession number: NM_000125). A PCR fragment of 143 bp was amplified with the following conditions: denaturing for 4 min at 94°C, 33 cycles of 40 s denaturing at 94°C, 40 s annealing at 53°C and 1 min extending at 72°C and a final extending step of 10 min at 72°C. PCR reactions were carried out in a final volume of 25 ml containing 1 × Perkin–Elmer Buffer (Applied Biosystems), 0.5 mmol/l of each nucleotide (dATP, dCTP, dGTP and dTTP), 1.25 mmol/l $MgCl_2$, 0.6 mmol/l of the forward primer (5′-TGTACCTGGACAGCAGCAAG-3′), 1.0 mmol/l of the reverse primer (5′-CGGAGACACGCTGTT-GAGT-3′) (Isogen Life Science, The Netherlands), 1.0 IU AmpliTaq Gold polymerase (Applied Biosystems) and 2 ml of DNA. The ASO used to detect the common B allele and B′ allele respectively are 5′-TCTGAGGCTGCGGCGTTCGG-3′ and 5′-TCTGAGGCTGCCG-CGTTCGG-3′. The PCR products were spotted on a membrane and hybridized with the oligonucleotides. The allele-specific washing temperature was 58°C for ASO B and 66.4°C for ASO B′. Wash buffer/salt concentrations: 2 × SSC 0.1% SDS. The combination of

probes used could correctly separate individuals carrying the B′ allele from BB homozygotes, but BB′ heterozygotes could not be distinguished from B′B′ homozygotes with certainty.

CYP17

The CYP17 gene contains a single nucleotide polymorphism, T → C, in the 5′ untranslated region at −34 bp from the initiation of translation. The base pair change creates a recognition site for the *MspA1* restriction enzyme. Two alleles have been arbitrarily assigned: A1 (T) and A2 (C) (Carey *et al.*, 1994). Genotypes of CYP17 were assessed by PCR–RFLP (Feigelson *et al.*, 1998) (Accession number: M31146). A PCR fragment of 459 bp containing the base pair change was generated using the following primer set: forward: 5′-CATTCGCACTCTGGAGTC-3′; and reverse: 5′-AGGCTCTTGGGGTACTTG-3′. PCR reactions were carried out in a final volume of 20 ml of 1 × Perkin–Elmer Buffer (Applied Biosystems), 2.4 mmol/l of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.4 mmol/l $MgCl_2$, 0.5 mmol/l of both primers, 1.0 IU AmpliTaq Gold polymerase (Applied Biosystems) and 2 ml of DNA. Thirty-five amplification cycles were carried out with denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. An initial denaturing step of 5 min at 95°C and a final extension step for 10 min at 72°C was used. Seven millilitres of PCR product was then digested with *Msp*AI (New England Biolabs, USA) at 37°C overnight and separated by agarose gel electrophoresis and stained with ethidium bromide to identify details of fragments.

Genotyping

Two individuals assessed the genotypes independently from each other. In case of sample failure or if there was disagreement between the observers without reaching a consensus, the PCR and RFLP/ASO were repeated and a final genotype was assessed. After repeated efforts, genotyping did not succeed in 74 samples (19.2%) for *Pvu*II, in 76 samples (19.7%) for *Xba*I, in 31 samples (8.1%) for B-variant and in 44 samples for CYP17 (11.4%), probably because of low amounts of DNA (van der Hel *et al.*, 2002).

Statistical analysis

Deviations from Hardy–Weinberg equilibrium were assessed using a goodness-of-fit χ^2-test with one degree of freedom. The two RFLP of ERα were analysed separately as well as in a combined score.

The following potential confounders were considered: smoking (ever/never), oral contraceptive (ever/never) use, socio-economic status (low/high), parity (nulliparous/parous), body mass index (BMI, kg/m^2), age at menarche (years).

Although for the *Pvu*II polymorphism an allele dose effect was demonstrated on age at menopause in an earlier study (Weel *et al.*, 1999), we tested between the homozygous wild type and homozygous mutant genotypes. This avoids making assumptions about the underlying genetic model. An exception was made for the B-variant where the BB′ genotype and B′B′ genotype were collapsed into one category. Two-sided *t*-tests were performed to assess the association between the polymorphisms and age at menopause. All analyses were performed using SPSS (version 11.0).

Results

The mean age at menopause for the study population was 49.5 years (SD 4.0 years). Table I shows some general characteristics of the study population. In Tables IIa and IIb, determinants of age at menopause are presented. Smoking and nulliparity were associated with an earlier age at menopause ($P < 0.001$ and $P = 0.02$ respectively), whereas obesity was associated with a later age at menopause ($P = 0.001$). The proportion of smoking women and nulliparous women did not differ across the genotypes in any of the polymorphisms. BMI was not significantly different in the homozygous mutant genotype compared to the homozygous wild type genotype in all polymorphisms.

For all polymorphisms assessed, the distribution of each genotype followed Hardy–Weinberg equilibrium, which indicates that no selection has occurred among genotypes. No differences in mean age at menopause were found for the *Pvu*II and *Xba*I genotypes ($P = 0.63$ and $P = 0.96$ respectively; Table III).

Age at menopause was not associated with B-variant genotype of ERα ($P = 0.28$). In addition, for the *Msp*AI

Table I. Characteristics of the study population ($n = 385$)

Characteristics	*n*	Mean (SD) or %
Age (years) at menopause	385	49.5 (4.0)
Age at intake	385	58.0 (4.6)
Years since menopause	385	8.5 (5.2)
Age at menarche	213	13.5 (1.5)
Height (cm)	385	162.0 (6.1)
Weight (kg)	385	68.4 (11.1)
Body mass index (kg/m^2)	385	26.1 (4.2)
Parity	305	3.2 (1.9)
Age at first full-term pregnancy	305	27.1 (4.5)
Nulliparous women	80	20.8
Ever smokers	100	26.0
Ever oral contraceptive users	13[a]	3.4
Low socio-economic status	263	68.3

[a]No information available for 22 women.

Table IIa. Determinants of menopausal age, categorical variables

Categorical variables	*n*	Mean (SD)	*P*[a]
Smoking			
Never	285	50.1 (3.7)	
Ever	100	48.9 (4.6)	<0.001
Oral contraceptive use			
Never	350	49.8 (3.9)	
Ever	13	50.1 (1.5)	0.26
Socio-economic status			
Low	263	49.6 (4.0)	
High	122	49.3 (4.1)	0.59
Nulliparity			
Yes	80	48.5 (4.4)	
No	305	49.7 (3.9)	0.02
Body mass index (kg/m^2)			
Normal weight (19–25)	180	48.8 (4.4)	
Obesity (≥26)	200	50.2 (3.5)	0.001

[a]Two-sided *t*-test.

Table IIb. Determinants of menopausal age, continuous variables

Continuous variables	*n*	*B*[c]	*P*[a]
Age (years) at menarche	213	0.04	0.85
Body mass index	385	0.14	0.003[b]

[a]Linear regression analysis.
[b]Adjusted for smoking.
[c]Regression coefficient.

Table III. Age (years) at menopause according to genotype

Polymorphism	Mean (SD)	P[a]
Pvu II		
pp ($n = 82$)	49.4 (4.2)	
Pp ($n = 141$)	49.3 (3.9)	
PP ($n = 88$)	49.8 (4.4)	0.63
Xba I		
xx ($n = 116$)	49.5 (4.3)	
Xx ($n = 139$)	49.4 (3.9)	
XX ($n = 54$)	49.6 (4.4)	0.96
B-variant		
BB ($n = 287$)	49.5 (4.1)	
BB′+B′B′ ($n = 67$)	50.1 (3.7)	0.28[b]
CYP17		
A1A1 ($n = 147$)	49.7 (4.1)	
A1A2 ($n = 144$)	49.3 (4.0)	
A2A2 ($n = 50$)	49.6 (3.9)	0.51

[a]Analysis of variance.
[b]Two sided *t*-test: BB versus BB′+B′B′.

polymorphism in the CYP17 gene, no association was found with age at menopause ($P = 0.51$). Adjustment for smoking, BMI and nulliparity did not materially change any of the results and therefore crude results are given.

Combination of the two RFLP of ERα genotypes in all subjects resulted in identification of eight genotypes: PPXX ($n = 51$, 16.6%), PPXx ($n = 34$, 11.1%), PPxx ($n = 3$, 1.0%), PpXX ($n = 2$, 0.7%), PpXx ($n = 103$, 33.6%), Ppxx ($n = 33$, 10.7%), ppXx ($n = 1$, 0.3%) and ppxx ($n = 80$, 26.1%). The genotype ppXX was not detected in this study population. No significant differences in age at menopause were found for the combined genotypes.

Discussion

We found no evidence for a consistent relationship between common variants of the ERα gene (*Pvu*II, *Xba*I and B-variant) or the CYP17 gene (*Msp*AI) with age at natural menopause.

Several limitations need to be considered when interpreting the results. It should be noted that genotype assessment did not succeed in 19.2% of the *Pvu*II genotypes, in 19.7% of the *Xba*I genotypes, in 8.1% of the B-variant genotypes and in 11.4% of the *Msp*AI genotypes. This is mainly because it is difficult to obtain sufficient DNA from urine samples (van der Hel *et al.*, 2002), which are the only biological samples we have collected for this cohort of women recruited in 1974. It is, however, unlikely that this was non-random and affected the findings of this study for the following reasons. First, there were no differences in characteristics between subjects for whom genotyping succeeded and subjects for whom genotyping failed for two or more polymorphisms. Secondly, genotypes were assessed by researchers blinded for the outcome, age at menopause. Furthermore, the *Pvu*II (Gennari *et al.*, 1998; Weel *et al.*, 1999), B-variant (Schmutzler *et al.*, 1991; Lehrer *et al.*, 1993) and CYP17 (Haiman *et al.*, 1999a; Mitrunen *et al.*, 2000b) genotype distributions in our study are comparable to those found in other populations, which strongly argues against selective failure of certain genotypes.

Age at menopause was self-reported and determined retrospectively, which has been shown to be susceptible to bias (den Tonhelaar, 1997; Hahn *et al.*, 1997). Nevertheless, it seems unlikely that misclassification due to recall bias is different across genotypes.

The strength of this study is that the study population was a random sample from a population-based cohort, therefore excluding sources of selection bias.

Candidate gene studies can be seen as a useful step in exploring potential causal pathways between genetic determinants and complex traits, such as age at menopause. We reported an association of the factor V Leiden mutation with earlier age at menopause (Van Asselt *et al.*, 2003), an effect possibly enhanced by smoking.

Weel *et al.* were the first to establish an association between an ERα gene polymorphism and age at menopause. Apart from the current study, the association was also absent in a Japanese population (Gorai *et al.*, 2003). Previously, we reported that fertility problems, reduced parity, nulliparity and early menopause may all be related phenotypes of menopausal age as being an expression of accelerated ovarian ageing (Kok *et al.*, 2003). Finding an association between ERα gene polymorphism and related phenotypes as reduced parity and nulliparity, could be considered as supportive evidence for the association between the ERα gene polymorphism and menopausal age. However, the data presented by Weel *et al.* showed no association between the *Pvu*II polymorphism and nulliparity or reduced parity. Discrepant findings do not necessarily indicate that the candidate gene approach is unreliable or that associations found are spurious, but does indicate caution in both design and interpretation of such studies (Colhoun *et al.*, 2003).

Gene variants might be associated with different relative risks in different populations and then the non-replication might result from real biological differences. This could well be expected comparing the studies of Weel *et al.* (1999) and Gorai *et al.* (2003), studying Caucasian and Asian populations respectively. However, this does not explain the difference between the population studied by Weel *et al.* and the present study.

Complex traits are aetiologically heterogeneous as they are the result of multiple genetic and environmental components. Non-replication might be due to the small magnitude of relative risks that are likely to be detected in candidate gene studies of complex traits. Furthermore, confounding, bias and misclassification are more likely to obscure small to moderate relative risks than larger relative risks (Tabor *et al.*, 2002).

The choice of the polymorphisms could also be an explanation of non-replication. If polymorphisms are not likely to affect the function of the protein encoded for, finding an association is based on the polymorphism being in linkage disequilibrium with a 'causal' variant. If this linkage disequilibrium does not exist or varies between different study populations, different studies might have different results for polymorphisms of the same gene. For CYP17, it was suggested that the increase in serum estradiol and progesterone levels accompanying carriage of the CYP17 A2 allele resulted from higher activity of the enzyme through

up-regulation of transcriptional activation. However, this could not be found in experimental studies (Nedelcheva *et al.*, 1999; Lin *et al.*, 2001), indicating that the polymorphism may be in linkage disequilibrium with another functional polymorphism or that hormone levels are affected through another mechanism.

Other potential candidate genes for menopausal age may be found among those associated with premature ovarian failure (POF). The FSH axis is of importance in ovarian function and some gene variants involved in abnormal FSH responses have indeed been shown to be associated with POF. Examples are the FSH-receptor gene (Aittomaki *et al.*, 1995; Touraine *et al.*, 1999) and genes coding for inhibin (Shelling *et al.*, 2000; Dixit *et al.*, 2004). However, an earlier study showed no linkage of the FSH-receptor gene with menopausal age (Kok *et al.*, 2004). Other pathways should be explored for potential candidate genes.

In conclusion, no association of ERα and CYP17 polymorphisms with age at natural menopause was found in a population-based sample of Dutch women.

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