Cyp17, Urinary Sex Steroid Levels and Breast Cancer Risk in Postmenopausal Women

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Abstract

Endogenous sex hormones play an important role in the etiology of breast cancer. Polymorphisms in genes encoding for enzymes involved in steroidogenesis may therefore play a role in breast cancer risk. Cytochrome P450c17α (Cyp17) functions at key branch points in human steroidogenesis. A T→C transition (A1 and A2 allele) in the 5′ untranslated region may be associated with increased expression of Cyp17. Using a case-cohort design, we studied the effects of the A2 allele on endogenous sex hormone levels and breast cancer risk within a large population-based cohort (n = 9,349) in the Netherlands (the DOM-cohort). Cyp17 genotype was determined in 335 incident postmenopausal breast cancer cases, which occurred after follow-up (median time to follow-up, 19 years) of the entire cohort, and in a random sample of 373 women (subcohort). Concentrations of estrone (E1), estradiol (E2), testosterone, 5α-androstane-3α, 17β-diol (3αD), and creatinine were measured in first-morning urine samples. Only among women with body mass index (BMI) < 25 kg/m² was the A2A2 genotype associated with higher levels of E1, E2, and 3αD compared with a group of women with either the A1A1 or the A1A2 genotype (e.g., geometric means of E1 in ng/mg creatinine: A2A2, 2.23; A1A1/A1A2, 1.47; P = 0.03). Adjusted breast cancer rate ratios for women with the A1A2 or A2A2 genotype compared with women with the A1A1 genotype were 0.96 (0.68-1.37) and 0.80 (0.47-1.35), respectively. These results did not differ between women with low and high BMI. In conclusion, this paper shows that women with low BMI and the A2A2 genotype had higher endogenous sex steroid levels compared with women with the A1A1 genotype. However, these increased sex steroid levels are not translated into an increased breast cancer risk in these women. (Cancer Epidemiol Biomarkers Prev 2005;14(4): 815–20)

Introduction

It has been well-established that endogenous sex hormones play an important role in the etiology of postmenopausal breast cancer (1, 2). Factors that modify the levels of these hormones might therefore play an important role in breast cancer risk as well.

Cytochrome P450c17α (Cyp17) mediates both steroid 17α-hydroxylase and 17,20-lyase activities and functions at key branch points in human steroidogenesis (3). The Cyp17 gene contains a single base pair polymorphism (T→C) in the 5′ untranslated region at −34 bp from the initiation of translation, which is hypothesized to create an SP-1-type promoter site (CCACC box; ref. 4). However, Kristensen et al. (5) found no evidence for binding of this region containing the polymorphism with human SP-1 recombinant protein. This base pair change creates a recognition site for the MspA1 restriction enzyme. Two alleles have been arbitrarily assigned: A1 (T) and A2 (C; ref. 4).

Feigelson et al. (6) were the first to describe an increased risk of (advanced) breast cancer for women carrying the A2 allele. Subsequent studies showed conflicting results (7-19).

Studies relating this polymorphism to endogenous sex steroid levels also showed conflicting results (10, 20-26).

A reason for these conflicting results might be that the effect of the A2 allele on sex steroid levels could be masked in women who have relatively high hormone levels, such as women with high body mass index (BMI). In postmenopausal women, estrogens are mainly formed through aromatization of androgens in the adipose tissue. Therefore, endogenous sex steroid levels are lower in women with low BMI (27). This hypothesis is strengthened by the fact that four out of the five studies that showed an increased risk of breast cancer for women carrying the A2 allele included Asian women (6, 14-16), who are known to have, on average, lower levels of estrogens (28, 29).

We recently showed that urinary hormonal excretion sex steroid levels are related to breast cancer risk (2). Within the same framework, we now investigate the relation between the Cyp17 MspA1 polymorphism, urinary endogenous sex steroid levels and the risk of postmenopausal breast cancer in a large population-based cohort study in the Netherlands.

Materials and Methods

Participants. From 1975 to 1986, women born between 1911 and 1945, and residing in Utrecht and the surrounding areas, were invited to participate in a large, population-based screening program for early detection of breast cancer (the DOM-cohort; ref. 30). All participants were asked to complete a life-style questionnaire containing questions regarding breast cancer risk factors, medical history, exogenous hormone use, and menopausal status. Also, anthropometric measurements (e.g., height and weight) were taken and women were asked to bring in a first-morning urine sample on the day of their examination. Urine samples were then stored at −20°C in 250 mL plastic polypropylene jars, without preserving agents, until analysis. Furthermore, all women had a mammogram taken at the first screening round and were then invited for mammography at regular intervals. The response rate for the initial screening examination was 72%, whereas rates for the following examinations were > 80% (30). A total of 27,718 women participated in this cohort.
Women who were naturally postmenopausal at recruitment (defined as no menstrual period for at least 12 months, after spontaneous cessation of their menses) and who had no history of breast cancer were eligible for the present study (base population: \( n = 9,349 \)). Women were followed until January 1\textsuperscript{st} 1996 from the start of the study (i.e. 1975). First through their general practitioners, and, from 1986 onwards, through the regional cancer registry. This construction was used because the regional cancer registry did not start until 1986.

We retrieved urine samples from patients with breast cancer as well as from subcohort members for hormonal measurements and DNA extraction. Urine samples were available for 377 cases and 420 subcohort members.

**Genotyping.** DNA was isolated from 100 mL urine samples as it was described earlier (32). In brief, after centrifugation, DNA was isolated from the urine pellet by protein precipitation and subsequent DNA purification with alcohol. DNA was finally resuspended in 80 \( \mu \)L Tris-EDTA [10 mmol/L Tris, 1 mmol/L EDTA (pH 7.6)].

Genotypes of the Cyp17 Msp1A polymorphism were determined with PCR followed by a RFLP assay as it was described earlier by Feigelson et al. (6). Briefly, a PCR fragment of 459 bp containing the base pair change was amplified using the following primer set: forward, 5'-CAT TCG CAC TCT GGA GTC-3' and reverse, 5'-AGG CTC TGT GGG TAC TTG-3'. PCR reactions were carried out in a final volume of 20 \( \mu \)L of 1× Perkin-Elmer buffer (Applied Biosystems, Foster City, CA), 2.5 mmol/L of each nucleotide (dATP, dCTP, dGTP, and dTTP), 2.5 mmol/L MgCl\(_2\), 0.5 \( \mu \)mol/L of both primers, 1.0 units AmpliTaq Gold polymerase (Applied Biosystems), and 2 \( \mu \)L of DNA. Thirty-five amplification cycles were carried out with denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute. An initial denaturing step of 5 minutes at 95°C and a final extension step for 10 minutes at 72°C were used. Seven microliters of PCR product were then digested with Msp1A (New England Biolabs, Beverly, MA) at 37°C overnight and separated by agarose gel electrophoresis and stained with ethidium bromide to identify base pair changes.

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 experiments were repeated and a final genotype was assessed. After repeated efforts, genotyping for Cyp17 failed in 42 breast cancer cases (11%) and 47 subcohort members (11%).

**Hormonal Assay.** For the hormonal assay, we excluded women using hormone replacement therapy or oral contraceptives at the time of urine sampling (29 cases and 34 women from the subcohort). Urine samples were sent to the IARC (Lyon, France) for hormonal measurements. One sample was lost during the hormonal analyses and was also excluded. Laboratory technicians were blinded as to the disease status of the samples. Equal numbers of samples taken from the case group and from the subcohort were analyzed together within batches of 22 urine samples. The hormone metabolites, estrone (E\(_1\)), estradiol (E\(_2\)), testosterone, and 5α-androstane-3a, 17β-diol (3αD), were measured by RIA after enzymatic hydrolysis, solid phase extraction and high-performance liquid chromatography purification of the urine samples. Results were expressed in nanograms of analyte per liter. The method used in this study has been described in detail elsewhere (33). Intra- and interassay coefficients of variation were 8.7% and 17.2% for E\(_1\), 12.2% and 14.8% for E\(_2\), 8.3% and 15.3% for testosterone, and 9.0% and 11.4% for 3αD.

Creatinine was measured in each sample by kinetic Jaffé reaction (Hitachi 717, Roche, Central Laboratory for Biochemistry, Hôpital de l’Antiquaille, Lyon, France).

**Data Analyses.** For the analyses of Cyp17 in relation to breast cancer, we included 335 breast cancer cases and 373 subcohort members. Means with its SD, median and range (for the not-normally distributed characteristics), or frequencies (where appropriate) of baseline characteristics were calculated in strata of Cyp17 genotype.

Deviations from Hardy-Weinberg equilibrium were assessed using a goodness-of-fit \( \chi^2 \) test with 1 df. We estimated rate ratios (RR) for the risk of breast cancer in women with the A1A2 or A2A2 genotypes versus women with the A1A1 genotype by calculating hazard RRs from a Cox proportional hazards model with Barlow’s weighing method (34). To adjust for the fact that we only included a random sample from the entire cohort (the subcohort) the follow-up time of the subcohort is weighted with the inverse of the sampling fraction (1/4.5). Robust SEs can then be calculated (34).

Age at recruitment, height (cm), weight (kg), oral contraceptive use (never/ever), hormone replacement therapy use (in the 12 months prior to recruitment, no/yes), family history of breast cancer (no/yes), parity/age at first full-term pregnancy (two groups: <30 years and nulliparous + ≥30 years), and age at menopause, were evaluated for confounding.

Effect modification of Cyp17 genotype by BMI was examined by calculating RR in combined categories of Cyp17 and BMI (two categories: <25 and ≥25 kg/m\(^2\)). Based on the results of Cyp17 in relation to breast cancer risk, we combined the A1A1 allele with the A1A2 allele. The A1A1 + A1A2 allele combined with the <25 kg/m\(^2\) category was used as a reference category.

To evaluate the effect of the Cyp17 genotype on urinary endogenous sex steroid levels, we calculated age-adjusted geometric mean levels with 95% confidence intervals (CI) of the log-transformed creatinine-adjusted levels of E\(_1\), E\(_2\), testosterone, and 3αD for all women from the subcohort with the A1A1/A1A2 allele and with the A2A2 allele. Women were classified in tertiles of BMI (<25, 25-26, >26 kg/m\(^2\)). Subsequently, the highest two tertiles of BMI were combined in one category. The age-adjusted geometric mean hormone levels were calculated for the entire subcohort as well as stratified according to the two abovementioned categories of BMI. Complete data on hormones and genotypes was available for 338 subcohort women. In this subcohort, 13 women developed breast cancer during follow-up.

Cox proportional hazards models were done using the SAS macro (SAS 8.2) described by Barlow et al. (34). For all other statistical analyses, the Statistical Package for Social Sciences (SPSS 11) was used.

**Results**

The Cyp17 genotype distribution among women in the subcohort was in Hardy-Weinberg equilibrium (\( \chi^2 = 1.73; \) df = 1; \( P = 0.19 \)).

Table 1 presents general characteristics according to Cyp17 genotypes in the subcohort. Women with the A2A2 genotype seem to be heavier (A2A2 versus A1A1, 69.5 versus 67.8 kg), reported twice as often a positive family history of breast cancer (A2A2 versus A1A1, 12% versus 6%) and were less likely to have ever used either oral contraceptives (A2A2 versus A1A1, 2.1% versus 4.4%) or hormonal replacement therapy (A2A2 versus A1A1, 0% versus 11.6%). Smoking was also more prevalent in women with the A2A2 genotype (A2A2 versus A1A1, 34% versus 25.3%).

**Breast Cancer Risk.** Table 2 shows the breast cancer incidence rates (per 1,000 person-years) for each genotype.
**Table 1. Baseline characteristics of the subcohort by CYP17 genotype**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1/A1 166 (44.5%)</td>
</tr>
<tr>
<td>Person-years of follow-up</td>
<td>59,862</td>
</tr>
<tr>
<td>Age at recruitment, median (range)</td>
<td>57 (40-65)</td>
</tr>
<tr>
<td>Height, mean (SD)</td>
<td>162.1 (5.7)</td>
</tr>
<tr>
<td>Weight, mean (SD)</td>
<td>67.8 (10.1)</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
</tr>
<tr>
<td>&lt;25 kg/m²</td>
<td>60 (36.1%)</td>
</tr>
<tr>
<td>≥25 kg/m²</td>
<td>106 (63.9%)</td>
</tr>
<tr>
<td>Age at menarche, mean (SD)</td>
<td>13.4 (1.5)</td>
</tr>
<tr>
<td>Nulliparity (%)</td>
<td>21.7</td>
</tr>
<tr>
<td>Age at first full-term pregnancy, mean (range)</td>
<td>27.0 (18-53)</td>
</tr>
<tr>
<td>Age at menopause, median (range)</td>
<td>50 (34-58)</td>
</tr>
<tr>
<td>Family history of breast cancer (%)</td>
<td>6.2</td>
</tr>
<tr>
<td>Oral contraceptive use (%)</td>
<td>4.4</td>
</tr>
<tr>
<td>Hormonal replacement therapy (%) current use)</td>
<td>11.6</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>25.3</td>
</tr>
</tbody>
</table>

*Age at menarche was missing for 53.2% (n = 237).
†At least a mother or one sister with breast cancer.

**Discussion**

In this large population-based study with ~138,000 years of follow-up and 377 cases of breast cancer, we observed increased urinary levels of E₁ and E₂ for women with the A2A2 genotype among women with low BMI (<25 kg/m²). However, we found no evidence that the *Cyp17 A2A2* genotype increases the risk of breast cancer in postmenopausal women.

To fully appreciate the findings of this study, some strengths and limitations need to be addressed. In this study, we were able to investigate the effect of the *MspA1* polymorphism on sex steroid levels as well as on breast cancer risk in the same women.

**Table 2. Breast cancer RR in relation to Cyp17 genotype**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n cases</th>
<th>Person-years follow-upa</th>
<th>Incidenceb</th>
<th>RR unadjusted (95% CI)</th>
<th>RR adjusted (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1A1</td>
<td>151</td>
<td>61,379.12</td>
<td>2.46</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>A1A2</td>
<td>140</td>
<td>58,129.45</td>
<td>2.41</td>
<td>0.96 (0.69-1.33)</td>
<td>0.96 (0.68-1.37)</td>
</tr>
<tr>
<td>A2A2</td>
<td>44</td>
<td>18,478.11</td>
<td>2.38</td>
<td>0.97 (0.61-1.53)</td>
<td>0.80 (0.47-1.35)</td>
</tr>
</tbody>
</table>

P tend = 0.83
P trend = 0.44

*Follow-up for subcohort controls is weighted with 1/α = 22.28 (α = sampling fraction of 4.5%).

†Per 1,000 person-years, unadjusted.

‡Adjusted for age at recruitment, BMI (kg/m²) parity/age at first full-term pregnancy (<30 years, nulliparous + ≥30 years), age at menopause, oral contraceptives use (never/ever), hormonal replacement therapy use, (never/ever), smoking (never/ever), and familial breast cancer (yes/no).
Table 3. Association between Cyp17 genotype, BMI, and breast cancer risk

<table>
<thead>
<tr>
<th>Cyp17</th>
<th>BMI (kg/m²)</th>
<th>n cases</th>
<th>Person-years follow-up</th>
<th>HR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1A1 + A1A2</td>
<td>&lt;25</td>
<td>93</td>
<td>45,295</td>
<td>1.0</td>
</tr>
<tr>
<td>A1A1 + A1A2</td>
<td>≥25</td>
<td>198</td>
<td>74,214</td>
<td>1.27 (0.86-1.88)</td>
</tr>
<tr>
<td>A2A2</td>
<td>&lt;25</td>
<td>12</td>
<td>5,642</td>
<td>0.84 (0.34-2.07)</td>
</tr>
<tr>
<td>A2A2</td>
<td>≥25</td>
<td>32</td>
<td>12,836</td>
<td>1.08 (0.60-1.93)</td>
</tr>
</tbody>
</table>

*Adjusted for age at recruitment, parity/age at first full-term pregnancy (<30 years, nulliparous + ≥30 years), age at menopause, oral contraceptives use (never/ever), hormonal replacement therapy use, (never/ever), smoking (never/ever), and familial breast cancer (yes/no).

Genotyping failed in 11% of the samples, probably due to low amounts of DNA (32). However, women from the subcohort for whom genotyping failed were very similar to the successfully genotyped subcohort women with respect to the general characteristics. Furthermore, the percentage of women for whom genotyping failed was the same in both cases and the subcohort. Therefore, the high percentage of failed genotyping would probably not have affected the estimates found in our study.

We also believe that misclassification did not affect our study results. Genotyping was done by two independent readers who were blinded to the disease status. Also, the genotype distribution in this study is comparable to that found in other studies among Caucasian women (7-11, 13, 15, 17, 18). Metabolized amounts of hormones were measured in first-morning urine samples. The reproducibility of these measurements is very high. In a random sample of 45 postmenopausal women from our cohort, intraclass correlation coefficients for sample replicates were all >0.93 for all four hormone metabolites, indicating good reproducibility from a laboratory error perspective. The intraclass correlations over time also indicated reasonable stability in the women's urinary hormone levels over several years (33).

Several investigators have studied the relationship between Cyp17 genotype and breast cancer risk with varying results (6-19). Feigelson was the first to publish data of an increased risk of advanced breast cancer in women with the A2A2 genotype (6). Only 4 of a total of 14 studies confirmed this result (11, 14-16). Eight studies investigated this relationship in postmenopausal women and only one study (in Japanese women) showed an increased risk of breast cancer in women with the A2A2 genotype (14). Most of these studies, however, were limited by small sample sizes (6, 8, 9, 11, 12, 14, 18, 19). A recent metaanalysis also showed no increased risk of breast cancer in women with the A2A2 genotype either overall (OR, 1.05; 95% CI, 0.87-121) or in postmenopausal women (OR, 1.14; 95% CI, 0.75-1.74; ref. 35).

These discrepancies might be the result of the differential effect of the genotype in women with high or low BMI. In postmenopausal women, estrogens are mainly formed in adipose tissue through aromatization of testosterone. Due to the higher background levels of sex steroids in women with high BMI, marginally increased levels due to the MspA1 polymorphism in Cyp17 might not have a large influence, whereas in women with low BMI, the effect of this polymorphism might be more pronounced. Feigelson et al. (16) indeed found that breast cancer risk associated with the MspA1 polymorphism was most pronounced in lean women. Only few women in our study had both the A2A2 genotype and a BMI lower than 25 kg/m² (14 women in the subcohort and 12 cases). The small sample size especially in this stratum of BMI could explain the fact that we were not able to detect a relationship between the genotype and breast cancer risk in this subgroup. However, we were able to detect an effect of the genotype on the phenotype in this group. Furthermore, in our study BMI may have changed during follow-up. If so, this is likely to have led to an underestimation of the interaction effect of BMI. Since we have only updated covariate information for a small portion of the women in the study, we were not able to examine this effect.

Although we did not find the MspA1 polymorphism to be associated with an increased risk of breast cancer among women with low BMI, we did find this polymorphism to be related to increased levels of E₁ and E₂ and decreased levels of 3αD, among these women.

The relationship between MspA1 genotype and sex steroid levels was measured in three other studies (10, 36, 37). Although it is not exactly clear how levels of metabolized hormones in...
urine correlate with serum hormone levels, in the two studies by Haiman et al., elevated levels of E1 and E2 were found in women with the A2A2 genotype. This is comparable to what we found in our study. The third study could not confirm this result. However, their analysis included cases of osteoporosis and controls together, instead of just the control population (37).

No other study investigated the effect of this polymorphism on 3αD, which is a metabolite of testosterone. As none of the studies showed an effect of the MspA1 genotype on testosterone levels, it is not to be expected that levels of metabolites of testosterone will be affected. We were not able to come up with a logical explanation as to why 3αD levels would be decreased in these women.

Although increased, the amount of increase in levels of E1 and E2 for women with the A2A2 genotype is not large. In the same study population, we showed that women with estrogen levels in the highest quartiles (E1 $>2.50$ ng/mg; E2 $>0.77$ ng/mg) had a 2.5 and 1.5 times increased risk of breast cancer, respectively (2). The geometric mean E1 and E2 levels of women with the A2A2 genotype and low BMI were 2.23 and 0.68 ng/mg, respectively, which corresponds to the third quartile in our previous study. The geometric mean levels in women with the A1A1 or the A1A2 genotype were 1.47 ng/mg for E1 and 0.47 ng/mg for E2, which corresponds to the second quartile in our previous study. An increase in estrogen levels from the second to the third quartile hardly increased breast cancer risk (2). Also, a recent reanalysis of nine prospective studies into endogenous sex steroids and postmenopausal breast cancer showed that a doubling of E1 and E2 levels was associated with a 1.29 and 1.45 times increased risk, respectively (1). It may therefore not be surprising that the small, but significant, increase in hormone levels caused by this polymorphism, could not be translated into an increased breast cancer risk.

Furthermore, steroidogenesis is a complex process in which many enzymes are involved. Moreover, the effect of estrogens also depends on the function of the estrogen receptor. Variants in the proteins involved in steroidogenesis as well as in the estrogen receptor may change the exposure of the breast to estrogens. Also, levels of sex steroids are dependent on several environmental determinants, such as alcohol and physical activity (38, 39). Therefore, the effect of Cyp17 alone on estrogen levels might not be large enough to have an effect on breast cancer risk. However, in combination with other determinants of sex steroid levels, the additional effect of Cyp17 might be important. In the future, it might therefore be desirable to design studies that investigate the influence of polymorphisms in Cyp17 in a polygenic model in which environmental determinants of sex steroid levels are also taken into account (40).

In summary, the results of our study do not support the relation between the A2A2 genotype and breast cancer risk. However, we did find evidence for the role of the MspA1 polymorphism in steroidogenesis.

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References

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