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The estrogen receptor α gene and breast cancer risk (The Netherlands)

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Abstract

Objective: In this study we aimed to investigate whether the PvuII, XbaI and B-variant polymorphisms in the estrogen receptor α gene (*ER*-α) are associated with an increased risk of breast cancer in postmenopausal women, and whether the effect of high estradiol (E2) levels on breast cancer risk is altered by these polymorphisms. The selection of these polymorphisms was based on previously published associations with osteoporosis and spontaneous abortions.
Methods: The effect of the three polymorphisms on breast cancer risk was studied using a case-cohort design nested within a large population-based cohort study (n = 9349) in the Netherlands (the DOM-cohort). In total 380 incident breast cancer cases and a subcohort of 422 women were genotyped by RFLP or ASO hybridization methods.
Results: Women with the PvuII pp genotype had a 1.5 times non significant increased risk of breast cancer (95% CI: 0.94–2.42; $p_{trend} = 0.09$) compared to women with the PP genotype. The Pp or pp genotype in combination with high E2 levels raised breast cancer risk significantly when compared to women with low E2 levels and the PP genotype (RR = 2.26; 95% CI: 1.24–4.13). This interaction was statistically significant on the multiplicative scale ($p = 0.01$). The XbaI genotype ($RR_{xx \text{ versus } XX} = 1.19$; 95% CI: 0.73–1.95) and the B′ allele ($RR_{BB'+B'B' \text{ versus } BB} = 0.87$; 95% CI: 0.56–1.33) were not associated with breast cancer risk.
Conclusion: The results of this study suggest that the PvuII polymorphism in the *ER*-α, or another mutation in linkage disequilibrium with PvuII, in combination with high E2 levels increases breast cancer risk in postmenopausal women.

Introduction

It has been well established that endogenous sex steroids play an important role in the etiology of breast cancer in postmenopausal women [1, 2]. In the breast, estrogens bind to estrogen receptors with high affinity, triggering DNA synthesis, cell division, and proliferation of the breast epithelial cells [3, 4]. Proliferating cells are susceptible to genetic errors during DNA replication, which, if uncorrected, can lead to malignancies [5]. There are two types of estrogen receptors, the estrogen receptor-α (ER-α) and the estrogen receptor-β (ER-β). The relative distribution of both receptors differs for different tissues although there is some overlap. ER-β is present in the granulose cells, developing spermatids, kidney, intestinal mucosa, lung parenchyma, bone marrow, bone, brain, endothelial cells and the prostate gland. The ER-α is primarily present in the endometrium, breast cancer cells and the ovarian stroma [4]. The ER-α has been extensively studied with respect to the prognosis of breast cancer, but its function in regulating the effect of estrogens on breast cells and its presence in normal breast epithelium suggests that the receptor might also be involved in the etiology of breast cancer [6].

The *ER*-α gene is located on chromosome 6q25.1 [7] and several polymorphisms are described. We have chosen three of these polymorphisms to evaluate in this

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study. Two polymorphisms are located in the first intron approximately 0.4 kb upstream from exon 2 and 46 bp apart. The first is a T $\rightarrow$ C transition at −397 bp before exon 2, which results in a restriction site for PvuII [8]. The second is a G $\rightarrow$ A substitution at −351 bp before exon 2, resulting in a XbaI restriction site [9]. Although there is no evidence yet of a functional effect of these polymorphisms on the expression or the function of the *ER-α*, we included them because they have been associated with breast cancer before [10–12], but also with bone mineral density and age at menopause [13–17]. Only five studies investigated the relation between the PvuII polymorphism and the risk of breast cancer of which four also included XbaI with inconclusive results [8, 10–12, 18].

A third polymorphism (a G $\rightarrow$ C substitution) is located in the B-region of the *ER-α* at codon 87 [19]. This polymorphism was selected because it has been associated with a significantly lower degree of estrogen binding [20]. The variant allele has not been found in Asian populations [21, 22]. The polymorphism has been studied in relation to breast cancer in two case–control studies [18, 23]. Only one of these studies reported a higher frequency of the B′ allele in breast cancer cases compared to healthy women [23].

Using a case-cohort design nested within a large population-based cohort study, we investigated the relationship between the above-mentioned three polymorphisms in the *ER-α* gene and the risk of breast cancer in postmenopausal women. In a previous study, we showed that high urinary excretion levels of estradiol (E2) were associated with increased breast cancer risk [2]. In addition to studying the main effects of above polymorphisms in relevant genes, we also wanted to study the possible mediating effects of these genetic variations on the effect of endogenous E2 levels.

Methods

Subjects

Between 1975 and 1986, all women born between 1911 and 1945 living in Utrecht and surroundings were invited to participate in a large population based screening program for the early detection of breast cancer (the DOM-cohort) [24]. All participants were asked to fill in a lifestyle questionnaire containing questions regarding breast cancer risk factors, medical history, exogenous hormone use and menopausal status. In addition, anthropometric measurements (*e.g.* height, weight) were taken and women were asked to donate a first morning urine sample on the day of their mammographic examination. Urine samples were then stored at −20 °C in 250 ml plastic polypropylene jars, without preserving agents, until analysis. 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 = 9349). All women were followed until January 1st 1996 for the occurrence of breast cancer through their general practitioners and, from 1986 onwards, through linkage with the regional cancer registry. By the end of follow-up (January 1st 1996), 380 new breast cancer cases (invasive as well as ductal carcinoma *in situ* (n = 23)) were identified. Follow-up of the cohort members for the occurrence of breast cancer was shown to be largely complete (5% lost to follow-up) [25].

For this study we used a case-cohort design. The term "case-cohort design" was first mentioned by Prentice [26], who described a design which is a cross between a cohort and a case–control study, incorporating the advantages of both designs in one. Basically, all incident cases of, in our case, breast cancer are selected from an existing cohort. Subsequently, a subcohort is sampled from the entire baseline population, without regard to case status or time. In this study we sampled a subcohort of approximately 4.5% (422 women). Seventeen breast cancer cases were also members of the subcohort.

We retrieved urine samples of all breast cancer cases and subcohort members for hormonal measurements and DNA extraction. A urine sample could not be found for three breast cancer cases and two members of the subcohort.

Genotyping

To detect the PvuII and XbaI polymorphisms we used restriction fragment length polymorphism (RFLP) PCR-protocols [14, 27]. A PCR fragment of 345 bp containing the two base pair changes was generated using the following primers: forward: 5′-GAT ATC CAG GGT TAT GTG GCA-3′ and reverse 5′-AGG TGT TGC CTA TTA TAT TAA CCT TGA-3′. PCR reactions were carried out in a final volume of 20 μl containing 1X Perkin-Elmer Buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 mM MgCl2, 0.45 μM of each primer, 1.0 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) and 2 μl of DNA. DNA was amplified in 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 were used. 5 μl of PCR product was then digested with PvuII (New England Biolabs, Beverely, MA, USA) and an equal amount was digested with XbaI (New England Biolabs, Beverely, MA, USA) both overnight at 37 °C. Fragments were separated by agarose gel electrophoresis and stained with ethidium bromide to identify base pair changes. PP (CC-genotype) and XX (GG-genotype) signified the absence of restriction sites.

Genotypes of the B-variant were determined with an allele specific oligonucleotide (ASO) hybridization method [18]. 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 μl containing 1X Perkin-Elmer Buffer (Applied Biosystems, Foster City, CA, USA), 0.5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1.25 mM MgCl2, 0.6 μM of the forward primer (5′-TGT ACC TGG ACA GCA GCA AG-3′), 1.0 μM of the reverse primer (5′-CGG AGA CAC GCT GTT GAG T-3′) (Isogen Life Science, Maarssen, the Netherlands), 1.0 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) and 2 μl of DNA. The ASO used to detect the common B allele and B′ allele respectively are 5′-TCT GAG GCT GCG GCG TTC GG-3′ and 5′-TCT GAG GCT GCC GCG TTC GG-3′. The allele specific washing temperature was 58 °C for ASO B and 66.4 °C for ASO B′.

Two investigators 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.

Due to reduced quality and quantity of DNA obtained from urine, genotyping usually fails in 10–20% of the samples despite repeated efforts [28]. In this study genotyping failed in 69 cases (18%) and 83 subcohort women (20%) for PvuII, in 70 cases (19%) and 85 subcohort women (20%) for XbaI, and in 36 cases (10%) and 36 subcohort women (9%) for the B-variant.

Hormonal assay

We previously reported on the measurements of urinary sex hormones in these samples and their effect on breast cancer and details can be found there [2]. In short, for the hormonal assay we excluded women using hormone replacement therapy (HRT) or oral contraceptives (OC) at the time of urine sampling (29 cases and 34 women from the subcohort). As E2 is the hormone that primarily binds to the estrogen receptor, we hypothesized that any interaction would be most pronounced with this hormone. Amongst others, the hormone metabolite E2 was measured by radioimmunoassay (RIA) after enzymatic hydrolysis, solid phase extraction and high-performance liquid chromatography (HPLC) purification of the urine samples. Results were expressed in ng analyte per liter [29]. Intra- and interassay coefficients of variation was 12.2% and 14.8% for E2.

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

Statistical analyses

Means with its standard deviation, median and range or frequencies (where appropriate) of baseline characteristics were calculated for the different genotypes of PvuII, XbaI, and B-variant.

Deviations from Hardy–Weinberg equilibrium were assessed using a goodness-of-fit chi-square test with one degree of freedom.

We estimated rate ratios (RR) for the risk of breast cancer by calculating Hazard RR from a Cox Proportional Hazards Model with Barlow's weighting method [30]. 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 standard errors should then be calculated.

For the PvuII and XbaI genotype, women with the PP and XX genotype respectively, served as a reference category. For the B-variant we calculated the breast cancer risk for the combined group of women with either the B′B′ or the BB′ genotype compared to the BB homozygotes, because very few women were homozygous for the B′ allele.

PvuII/XbaI haplotypes were inferred from the genotype frequencies. Haplotype combinations could be unambiguously determined for all genotypes, except for the PpXx genotype. This genotype was assumed to consist of a combination of the haplotypes px and PX, since subjects homozygous for the alternative haplotype (pX) were not found in this and other populations [27]. Because it was suggested that women homozygous for pp or xx are at an increased risk of breast cancer, we defined px to be the haplotype of interest. We grouped women by allele copy number (0, 1 or 2 copies) of this haplotype and calculated RR for breast cancer for women with one or two copies of this haplotype compared to women without any copy of this haplotype.

Age at recruitment, height (cm), weight (kg), OC use (never/ever), hormonal replacement therapy (HRT) use

(in previous 12 months prior to recruitment, no/yes), family history of breast cancer (no/yes), defined as at least a mother or one sister diagnosed with breast cancer, smoking (never/ever), parity/age at first full term pregnancy (two groups: <30 years *versus* ≥30 years or nulliparous) and age at menopause, were evaluated for confounding.

Finally, we calculated adjusted RR's for the separate and combined effects of E2 (high/low) and PvuII polymorphisms (PP/Pp + pp). A *p*-value for multiplicative interaction was calculated.

Results

All genotype frequencies were in Hardy Weinberg Equilibrium in the subcohort as well as in the cases.

There were no differences between the women from the subcohort and the women in the whole cohort with regard to the general characteristics (Table 1). This indicates that sampling of the subcohort was indeed random. Compared to women from the subcohort, breast cancer cases had more often a body mass index (BMI) above 25 kg/m^2 (69.5% *versus* 63.3% in the subcohort). Furthermore, breast cancer cases also had more often a positive family history of breast cancer (15.1% *versus* 7.0% in the subcohort; Table 1). The mean age at diagnosis of the cases was 67.3 years (sd = 7.4).

Women with the pp genotype had a 1.5 times increased risk of breast cancer compared to women with the PP genotype (95% CI: 0.94–2.42) (Table 2). This was statistically non-significant. There was a trend between the number of *p* alleles and breast cancer risk of borderline statistical significance ($p = 0.09$). Risk of breast cancer was not increased for women with the xx genotype compared to the XX genotype (RR = 1.19; 95% CI: 0.73–1.95). Table 3 shows that the PvuII and the XbaI polymorphisms are in strong linkage disequilibrium ($r^2 = 0.60$) [31]. The combination of the PvuII and XbaI genotypes into haplotypes showed that women with two copies of the px haplotype had an approximately 1.5 times increased risk of breast cancer (95% CI: 0.91–2.36), similar to the risk of women with the pp genotype, independent of Xba1 genotype. Again, there was a non-significant tendency of a trend for the number of copies of the px haplotype and the risk of breast cancer ($p = 0.11$).

Table 1. Baseline characteristics according to PvuII, XbaI or B-variant genotype among the subcohort

Characteristic	Whole cohort	Subcohort	Breast cancer cases
N	9349	420	377
PvuII			
PP		96 (22.9%)	69 (18.3%)
Pp		153 (36.4%)	150 (39.8%)
pp		88 (21.0%)	89 (23.6%)
Missing		83 (19.8%)	69 (18.3%)
XbaI			
XX		61 (14.5%)	55 (14.6%)
Xx		151 (36.0%)	130 (34.5%)
xx		123 (29.3%)	122 (32.4%)
Missing		85 (20.2%)	70 (18.6%)
B-variant			
BB		316 (75.2%)	290 (76.9%)
BB′ + B′B′		68 (16.2%)	51 (13.5%)
Missing		36 (8.6%)	36 (9.5%)
Age at enrolment, mean (SD)	56.2 (5.4)	57.4 (4.7)	57.3 (4.5)
Height (cm), mean (SD)	162.3 (6.6)	162.0 (6.1)	162.6 (6.0)
Weight (kg), mean (SD)	68.5 (10.8)	68.4 (10.9)	70.5 (11.5)
BMI			
≤25 kg/m^2	37.8%	36.7%	30.5%
>25 kg/m^2	62.1%	63.3%	69.5%
Nulliparity, %	18.5%	20.5%	19.1%
Age at first full term pregnancy, median (IQR)	27 (24–30)	27 (24–30)	27 (24–30)
Age at menopause, median (IQR)	50 (47–52)	50 (48–52)	50 (48–52)
Family history of breast cancer[a], % yes	8.0%	7.0%	15.1%
OC use, % ever	4.7%	3.8%	5.3%
HRT, % current use	7.2%	8.4%	7.2%
Smoking, % ever	31.1%	26.4%	26.4%

[a] At least a mother or one sister with breast cancer.

Table 2. Breast cancer RRs in relation to PvuII, XbaI and B-variant genotype

Genotype	Cases	Person years[a]	RR unadjusted (95% CI)	RR[b] adjusted (95% CI)
PvuII				
PP	69	35,275	1.0	1.0
Pp	150	57,948	1.32 (0.90–1.95)	1.28 (0.84–1.95)
pp	89	31,805	1.43 (0.93–2.22)	1.50 (0.94–2.42)
		p_{trend}	0.11	0.09
PP	69	35,275	1.0	1.0
Pp OR pp	239	89,753	1.36 (0.95–1.96)	1.35 (0.91–2.01)
XbaI				
XX	55	23,078	1.0	1.0
Xx	130	55,447	0.97 (0.62–1.52)	0.97 (0.60–1.57)
xx	122	45,482	1.11 (0.71–1.75)	1.19 (0.73–1.95)
		p_{trend}	0.55	0.39
XX	55	23,078	1.0	1.0
Xx OR xx	252	100,929	1.04 (0.69–1.57)	1.07 (0.68–1.67)
Haplo1 (px)				
0 copies	72	35,741	1.0	1.0
1 copy	144	57,003	1.24 (0.84–1.82)	1.22 (0.80–1.87)
2 copies	87	30,977	1.38 (0.89–2.14)	1.47 (0.91–2.36)
		p_{trend}	0.15	0.11
B-Variant				
BB	290	118,381	1.0	1.0
BB′ + B′B′	51	24,810	0.84 (0.56–1.25)	0.87 (0.56–1.33)

[a] Follow-up for subcohort controls is weighted with $1/\alpha = 22.28$ (α = sampling fraction).
[b] Adjusted for: Age at recruitment, BMI (kg/m^2), parity/age at first full term pregnancy (<30 years, nulliparous + ≥30 years), age at menopause, OC use (never/ever), HRT use, (never/ever), smoking (never/ever) and familial breast cancer (yes/no).

Women carrying the B′ allele, did not have an increased risk of breast cancer (RR = 0.87; 95% CI: 0.56–1.33).

As only the PvuII genotype showed a possible effect on breast cancer risk, the interaction with E2 was calculated only for this genotype. We found a statistically significant interaction ($p = 0.01$) between the level of E2 and the PvuII polymorphism on breast cancer risk. The effect of E2 on breast cancer risk is more pronounced among women with the Pp or pp genotype (RR = 2.26; 95% CI: 1.24-4.13; Table 4).

Discussion

Women homozygous for the PvuII polymorphism in the *ER-α* gene appear to have a 1.5 times increased risk for breast cancer, although the results did not reach statistical significance at the 0.05 level. Furthermore, the increased risk of breast cancer for women with high E2 levels is more pronounced among women with the Pp and pp genotype.

It is unlikely that misclassification of genotypes could explain this effect. Any misclassification is likely to be random as genotyping was done by two independent readers who were blinded to the disease status. Non-differential misclassification would typically bias the results towards no effect. Also, the genotype frequencies of PvuII and XbaI in this study are comparable to those found in other studies among Caucasian women [8, 12, 17, 32, 33].

Five studies have investigated the PvuII polymorphism in the *ER-α* gene in relation to breast cancer risk [8, 10, 11, 18, 34]. Cai *et al.* found a 1.4 times increased

Table 3. Classification of the women in the study according to both PvuII and XbaI genotypes

	PP	Pp	pp
XX	104	5	0
Xx	51	222	2
xx	5	64	169

Table 4. Breast cancer risk ratio's in relation to levels of estradiol (E2) and the estrogen receptor *PvuII* genotype

E2 levels	Genotype	Cases	Person years	RR (95% CI)	$p_{interaction}$
E2low	PP	30	20,124	1.0	
E2high	PP	33	12,313	1.73 (0.81–3.69)	
E2low	Pp + pp	100	46,365	1.39 (0.77–2.48)	
E2high	Pp + pp	122	36,216	2.26 (1.24–4.13)	0.011

risk (95% CI: 1.1–1.8) of breast cancer for Asian women with the pp genotype compared to the PP genotype [10]. Another Asian study found non-significant reductions in risk for women heterozygous or homozygous for p [11]. Three studies were done in Caucasian populations. Two of these studies could not demonstrate an increased risk for women with the pp genotype [8, 18]. The third also found an increased risk of breast cancer in women with the pp genotype, although not statistically significant. However, when combining the PvuII polymorphism with the 975C → G polymorphism into a haplotype, a statistically significant increased risk of breast cancer was found for women with the TC haplotype. The OR for this haplotype was in the same direction as the OR from the single locus analysis of PvuII when comparing the TT allele to the CC allele (pp *versus* PP), but smaller [34].

We combined the results of four of the five studies on PvuII with the results of our study into a combined Odds Ratio (OR) (Figure 1). To estimate the pooled effect we abstracted OR direct from the published papers [10, 11], or we calculated OR from the data presented in the paper [8, 34]. The fifth study on the PvuII polymorphism did not present either ORs or raw data and could, therefore, not be included. The pooled estimate was calculated using the precision weighted procedure described by Greenland [35]. Although design (case control; case-cohort), effect measurements (OR; RR), and populations (Asian; Caucasian) differ, we believe that that there is strong evidence for the involvement of the PvuII genotype in breast cancer risk. The overall OR of the studies combined is 1.14 (95% CI: 1.00–1.32) for the Pp genotype and 1.23 (1.08–1.43) for the pp genotype.

We found a significant interaction between E2 levels and the PvuII genotype. Women with high E2 levels and the Pp or pp genotype had a 2.3 times increased risk of breast cancer compared to women with low E2 levels and the PP genotype. To our knowledge there are no other studies investigating the interaction between this polymorphism, E2 levels and the risk of breast cancer. However, there are two studies that investigate the interaction between the PvuII or XbaI polymorphism and breast cancer risk factors, such as alcohol, ages at menarche, menopause and first full term pregnancy, parity, BMI, *etc*. [10, 11]. These risk factors might be interpreted as surrogate markers of E2 levels. Shin *et al*. found that breast cancer risk was highest among women with the xx genotype, who were nulliparous or had a late age at first full term pregnancy, compared to women carrying a X allele and an early age at first full term pregnancy, although this interaction was not statistically significant [11]. They also found a suggestion for interaction between the XbaI genotype and alcohol consumption, again not statistically significant [11]. However, Cai *et al*. investigated the interaction with several breast cancer risk factors and the PvuII genotype

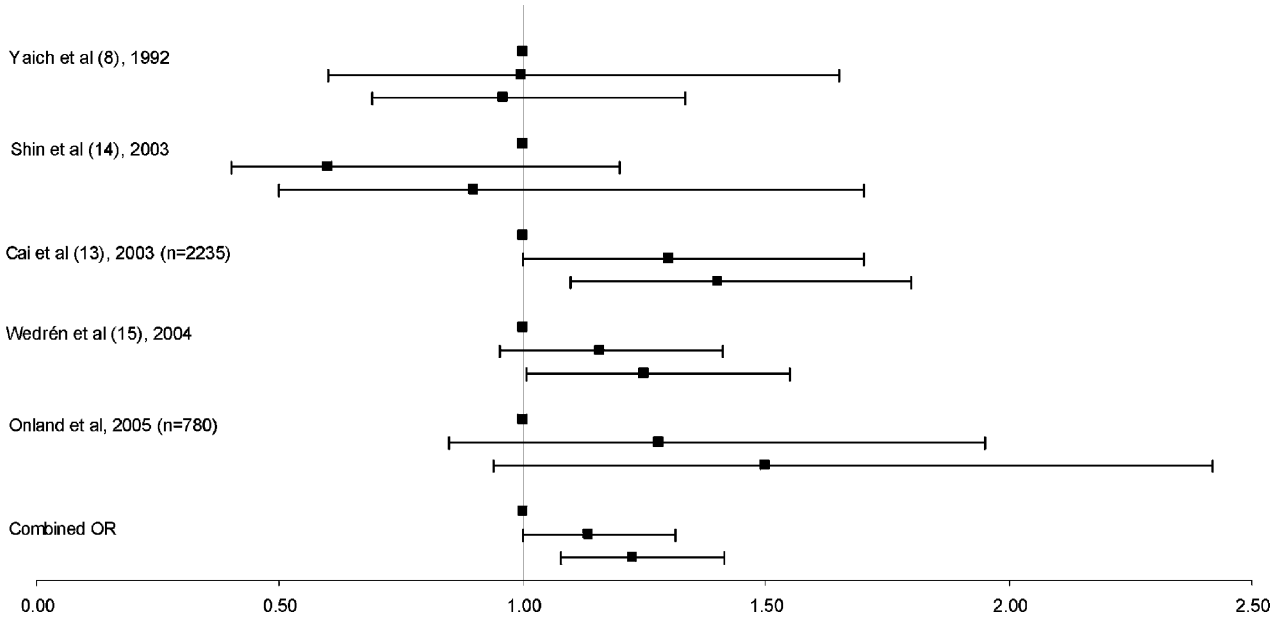

Fig. 1. Individual and combined OR [8,10,11,12] or RR (present study) and 95% CI for the PvuII genotypes, PP (reference group, OR = 1), Pp and pp and breast cancer risk. Corresponding ORs and 95% confidence intervals: Yaich *et al*. Pp: 1.0 (0.60–1.65) and pp: 0.96 (0.69–1.33); Shin *et al*.: Pp: 0.60 (0.40–1.20) and pp: 0.90 (0.50–1.70); Cai *et al*. Pp: 1.30 (1.10–1.80) and pp: 1.40 (1.10–1.80); Wedrén *et al*. Pp: 1.16 (0.95–1.41) and pp: 1.25 (1.01–1.55); Onland *et al*.: Pp: 1.28 (0.85–1.95) and pp: 1.50 (0.94–2.42); Combined OR: Pp: 1.14 (1.00–1.32) and pp: 1.23 (1.08–1.42).

and found no evidence at all for any interaction [10]. Possibly, these breast cancer risk factors do not explain a woman's exposure to E2 well enough to pick up a possible interaction. If indeed this polymorphism in itself or another functional variant linked to this one affects the function or concentration of the ER-α, it seems plausible that increased levels of E2, which binds to this receptor, can have a stronger adverse effect on breast epithelial cells in the presence of this polymorphism.

In this study we demonstrated an effect for the PvuII polymorphism, but not for the XbaI polymorphism. Others too, found different results for these two polymorphisms [10, 18]. The PvuII and XbaI polymorphisms are very strong linkage disequilibrium with each other, but also with the TA-repeat [14, 36]. This may have important implications. If only one of these polymorphisms, or a polymorphism close by, is functional, a differential degree of linkage disequilibrium among different populations may partly explain discrepancies in results of these polymorphisms on breast cancer risk [36].

Recently, Herrington *et al*. indeed found evidence for a functional effect of the PvuII polymorphism. They showed that the C allele (P-allele) produces a potential binding site for myb transcription factors [37]. Women with the P-allele had, therefore, higher transcription of the *ER-α* gene compared to women with the p allele [37], although it is not entirely clear how this affects the estrogen receptor. This additional myb binding site in the first intron might either amplify ER-α transcription or produce ER-α isoforms that have different properties than the full-length gene product. Therefore, it cannot easily be predicted how the mutation affects breast cancer risk. Furthermore, Žofková *et al*. showed that the p allele was associated with increased levels of androstenedione [32], which would suggest an increased risk for breast cancer for women carrying this allele.

In our study women with the PP or XX genotype were heavier and smoked less often compared to women with the pp or xx genotype. Probably this difference is due to chance. Furthermore, women with the B′ allele were more often nulliparous compared to women homozygous for the BB genotype, which could be due to the fact the the B′ allele has been associated with a higher risk of spontaneous abortions [38].

Most likely our results were not influenced by these differences, as unadjusted and adjusted relative risks were quite similar.

In conclusion this study suggests that the estrogen receptor α plays a role in the etiology of breast cancer. Most likely, either the PvuII RFLP or a functional mutation close to this polymorphism, in combination with elevated E2 levels, is responsible for increased breast cancer risk. Functional studies of this and other polymorphisms in the *ER-α* gene are, therefore, needed.

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