Polymorphisms in the Estrogen Receptor α Gene and Mammographic Density

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Abstract

The presence of the *Pvu*II or the *Xba*I polymorphism in the estrogen receptor α gene (*ESR1*, *6q25*) has been related to breast cancer risk; however, results are not fully consistent. To further elucidate this relation, we examined these polymorphisms in relation with mammographic density, a measure of dense tissue in the breast, which is strongly associated with breast cancer risk. For this study, 620 participants aged 49 to 68 years were selected from the Prospect-European Prospective Investigation into Cancer and Nutrition cohort. Blood samples, lifestyle– and medical questionnaire data and mammograms were available for these women. Genotyping was done using the TaqMan PCR assay and mammographic density was assessed using a computer-assisted method. Means of mammographic density were compared by *ESR1* genotypes and haplotypes. The percentage density was higher in women with one or two copies of the *Pvu*II *p* allele (means for *Pp* and *pp* are 37% and 36%, respectively) than in those with the *PP* genotype (32%, P_{trend} = 0.09). Women with one or two copies of the *Xba*I *x* allele had higher mean percentage density (*Xx* and *xx*, 36% and 37%, respectively) than those with the *XX* genotype (31%, P_{trend} < 0.01). Haplotype 1 (*px*) was associated with increased density, whereas haplotype 2 (*PX*) was associated with decreased density, both suggesting an allele-dose effect (P_{trend} = 0.08 and <0.01, respectively). Similar associations were found with absolute density (P_{trend} < 0.01). The findings of this study support the view that *ESR1* polymorphisms may affect breast cancer risk through differences in breast density. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2655–60)

Introduction

The appearance of the female breast on a radiographic image varies between individuals due to differences in tissue composition. On a mammogram, fat is translucent (dark) and connective and epithelial tissues are dense (light). The proportion of dense tissue in the breast is called mammographic density and high-density patterns are strongly associated with breast cancer risk (1, 2). In a study on quantitative classification of breast density and breast cancer risk, the increment in risk of breast cancer for each percentage increase in density was 2% (1).

Age, weight, menopausal status, and parity are important determinants of mammographic density but account for only a part of density variations between women (3). To explore the proportion of variation that can be explained by genetic factors, family studies were undertaken that showed a correlation between mammographic features of sisters (4). A recent study has found a strong correlation between monozygotic twin sisters and states that heritability accounts for about two thirds of variation in density (5).

In view of the important role of reproductive factors in determining mammographic density, plausible candidate genes would be those that regulate hormone synthesis, metabolism, and action of hormones, which have been investigated in a few previous studies (6-10). Another candidate gene for mammographic density, which has not been studied yet, is the gene coding for the estrogen receptor α (*ESR1*). ESR1 is a nuclear receptor that influences DNA transcription upon binding estrogens or other ligands (11). Estrogen receptor–estrogen interaction thus leads to stimulation of cell growth in various tissues, including breast epithelial tissue (12).

In the *ESR1* gene, several DNA sequence variations have been described that are of increasing interest because of their potential association with breast cancer and other hormone-related diseases. Most frequently studied are the single nucleotide polymorphisms *Pvu*II (also known as c.454-397T→C, IVS1-397 T/C, or rs2234693; where the *T* and *C* allele are often reported as the *p* and *P* allele, respectively) and *Xba*I (also known as c.454-351A→G, IVS1-351 A/G or rs9340799; where the *A* and *G* allele are often reported as the *x* and *X* allele, respectively), both located in intron 1 of the *ESR1* gene (13, 14). The space between these polymorphisms is only 45 bp and, therefore, *Pvu*II and *Xba*I are in strong linkage disequilibrium (15, 16).

We aimed to examine the relation between the *Pvu*II and *Xba*I polymorphisms and breast cancer risk by linking these *ESR1* polymorphisms to the intermediate phenotype mammographic density. The power to detect an association is greater for this quantitative trait (17). Moreover, the advantage is that the number of genetic and environmental factors influencing this intermediate phenotype is presumably smaller than the number of factors affecting the clinical end point breast cancer, which will make genetic factors easier to identify (18).

Materials and Methods

Study Population. Between 1993 and 1997, all women living in Utrecht and surroundings who were taking part in the regional population-based program of breast cancer screening were invited to participate in the Prospect-EPIC study (19), a Dutch cohort participating in the European Prospective Investigation into Cancer and Nutrition (20). Participants filled out lifestyle and food frequency questionnaires (21, 22) and donated a blood sample, which was fractionated into serum,

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Table 1. Breast cancer risk factors according to genotype

Breast cancer risk factors	*Pvu*II				*Xba*I			
	PP	*Pp*	*pp*		*XX*	*Xx*	*xx*	
n (%)	138 (22)	314 (51)	166 (27)		76 (12)	289 (47)	255 (41)	
	Mean (SD)	Mean (SD)	Mean (SD)	*P**	Mean (SD)	Mean (SD)	Mean (SD)	*P**
Age at mammogram (y)	54.0 (3.8)	54.0 (3.9)	54.1 (3.8)	0.97	54.0 (3.6)	54.0 (3.9)	54.1 (3.9)	0.95
Age at menopause (y; *n* = 434)†	47.2 (5.1)	47.7 (5.5)	47.4 (5.6)	0.69	47.5 (5.2)	47.5 (5.4)	47.6 (5.5)	0.98
BMI (kg/m^2)‡	25.9 (3.8)	25.5 (3.7)	25.5 (3.4)	0.43	26.4 (3.7)	25.5 (3.6)	25.4 (3.6)	0.09
No. children‡	2.3 (1.2)	2.4 (1.1)	2.7 (1.3)	<0.001	2.3 (1.4)	2.4 (1.1)	2.6 (1.3)	0.07
Age at first delivery (y; *n* = 569)§	25.2 (3.6)	25.1 (3.3)	24.8 (3.6)	0.61	25.0 (4.0)	25.1 (3.2)	24.9 (3.5)	0.82
Age at menarche (y)	13.2 (1.6)	13.4 (1.5)	13.4 (1.5)	0.65	13.1 (1.5)	13.3 (1.5)	13.4 (1.4)	0.30
	n (%)	*n* (%)	*n* (%)	*P*‖	*n* (%)	*n* (%)	*n* (%)	*P*‖
Menopausal status								
Premenopausal	30 (22)	103 (33)	47 (29)		15 (20)	95 (33)	71 (28)	
Postmenopausal	108 (78)	210 (67)	115 (71)	0.06	61 (80)	193 (67)	180 (72)	0.07
Previous oral contraceptive use								
Yes	100 (72)	222 (71)	116 (70)		56 (74)	208 (72)	176 (69)	
No	38 (28)	92 (29)	50 (30)	0.88	20 (26)	81 (28)	79 (31)	0.64
Family history of breast cancer								
Yes	23 (17)	41 (13)	15 (9)		13 (18)	39 (14)	28 (11)	
No	112 (83)	266 (87)	148 (91)	0.13	60 (82)	245 (86)	222 (89)	0.32
Alcohol consumption (g/d)								
<1.15	48 (35)	91 (29)	41 (25)		29 (38)	87 (30)	65 (26)	
1.15-9.16	43 (31)	107 (34)	60 (37)		24 (32)	96 (33)	90 (36)	
>9.16	47 (34)	116 (37)	63 (38)	0.48	23 (30)	106 (37)	98 (39)	0.32
Smoking								
Current	22 (16)	48 (15)	37 (22)		11 (14)	48 (17)	48 (19)	
Former	55 (40)	111 (35)	52 (31)		26 (34)	109 (38)	84 (33)	
Never	61 (44)	155 (49)	77 (46)	0.25	39 (51)	132 (46)	123 (48)	0.71

*ANOVA.
†Among postmenopausal women.
‡Among all women.
§Among parous women.
‖χ^2 test.

citrate plasma, RBC, and WBC, and stored at −196°C. At the end of the inclusion period, 17,357 Caucasian women were included in the study.

For the present analysis, 620 women were randomly selected from women who had never used postmenopausal hormone therapy and were not using oral contraceptives at study intake in the Prospect cohort. These women were 49 to 68 years old at mammography.

Genotyping Analysis. Genomic DNA was extracted from WBC using the QIAamp DNA Blood Mini kit (Qiagen) according to the instructions of the manufacturer. DNA yields were quantified using a fluorescent stain (PicoGreen, Molecular Probes, Inc., Eugene, OR). Mean DNA concentration was 77 ng/μL; samples were diluted to a final DNA concentration of 5 ng/μL.

Genotyping was done using the TaqMan PCR assay (23) with fluorescent minor groove binding probes (24). The PCR reaction was done in 384-well plates, each well containing 5.0 ng DNA, 2.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.0625 μL of probe and primer solution (Assays-on-Demand, Applied Biosystems), and 2.4375 μL distilled water. PCR was initiated at 95°C for 10 minutes, followed by 40 cycles of 92°C for 15 seconds and 60°C for 60 seconds. After PCR, fluorescence was measured in an ABI 7900 HT Sequence Detector (Applied Biosystems). Samples were classified as *PP*, *Pp*, or *pp* (representing the *CC*, *CT*, and *TT* genotypes of *PvuII*, respectively) and as *XX*, *Xx*, or *xx* (representing the *GG*, *GA*, and *AA* genotypes of *XbaI*, respectively). For 22 samples with initial missing genotypes, the assay was repeated. For the *Pvu*II genotype, 2 of 620 samples were undetermined after repeating the PCR. All participants could be genotyped for the *Xba*I polymorphism. The genotype data for each of the two polymorphisms were taken to infer the haplotypes of the *Pvu*II and *Xba*I polymorphisms by using the PHASE software, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (25). Subsequently, haplotypes were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population (1 = *px*, 2 = *PX*, 3 = *Px*, and 4 = *pX*) and subjects were classified as having 0, 1, or 2 copies of these haplotypes.

Mammographic Density Analysis. Mammographic density was assessed using the mediolateral oblique mammogram, which is the routine view for breast cancer screening in the Netherlands. In the past, it has been observed that the proportions of mammographic density on craniocaudal views and mediolateral oblique views and on left and right views show very strong correlation and that representative information on mammographic density is provided in a single view (26). For each study subject, the mammogram taken closest to the date of recruitment was collected and mammographic density was assessed on the left view for all women.

After digitizing the films using a laser film scanner (Lumiscan 50, Lumisys/Eastman Kodak Co., Rochester, NY), mammographic density was quantified using a computer-assisted method based on gray levels in the digitized mammogram (27). For each image, the reader first sets a threshold to determine the outside edge of the breast to discriminate between the dark area outside the breast and the lighter area within the breast. Another threshold is set to determine the area of dense tissue within the breast, which is the lightest tissue visible on the mammogram. The computer then determines the amount of pixels within the total breast area and within the dense area and calculates the percentage of dense tissue in the breast, which is the dense area divided by the total breast area multiplied by 100. In literature, the percentage of dense tissue, which is a relative measure of dense tissue, is mostly used. It may, however, be more relevant

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to study the absolute amount of dense tissue, which consists of connective and epithelial tissue and is regarded as the target tissue for breast cancer. We, therefore, present results on both relative and absolute measures of breast density. The absolute measure of dense tissue was calculated by multiplying the amount of pixels within the dense area with the area of one pixel, which was 0.0256 mm^2.

All films were read by one observer (F.J.B. van Duijnhoven) in sets of 70 images composed of randomly ordered films. To assess the reliability of the computer-assisted method, a library set of 70 images was made, which consisted of randomly chosen films that were not included in our study. This library set was read before the first set, after the last set, and at three time points between sets, which were blinded for the reader. The images in the library set were randomly ordered every time they were read to prevent the observer from recognizing this set. This computer-assisted method to determine mammographic density has proved to be very reliable (27) and, in this study, an average intraclass correlation coefficient of 0.87 (range 0.82-0.90) for dense area and 0.93 (range 0.91-0.95) for percentage density was reached between repeated readings.

Data Analysis. Means with SDs or frequencies (where appropriate) of breast cancer risk factors were calculated for the different genotypes of *Pvu*II and *Xba*I. Differences were tested by ANOVA or χ^2 analysis. Menopausal status at the time of the mammogram was divided in premenopausal or postmenopausal status, where postmenopausal was defined as at least 12 consecutive months of amenorrhea. Family history of breast cancer was defined as having at least a mother or a sister diagnosed with breast cancer. Current alcohol intake was defined as grams of ethanol per day and was categorized in tertiles.

Analysis of percentage mammographic density by breast cancer risk factors was done by ANOVA.

Deviations from Hardy-Weinberg equilibrium were assessed using a goodness-of-fit χ^2 test with 1 degree of freedom. The observed number of women for all possible combinations of both *Pvu*II and *Xba*I genotypes was compared with the expected number of women with these combinations of genotypes, which was based on the independent frequencies of the *Pvu*II and *Xba*I genotypes. Differences were tested by χ^2 analysis. The allele and haplotype frequencies were used to calculate the linkage disequilibrium coefficient (D'; ref. 28). When D' is 1, the two alleles are completely linked and when D' is -1, the two alleles exclude each other completely.

Mean percentage mammographic density was compared between women with different genotypes and haplotypes by linear regression analysis and P values for linear trend were calculated. Covariates included in the adjusted model were age (continuous), body mass index (BMI; continuous), age at menarche (continuous), parity/age at first full-term pregnancy (three groups: nulliparous, <26 and ≥26 years), menopausal status/age at menopause (three groups: premenopausal, <49 and ≥49 years), family history of breast cancer (no/yes), previous oral contraceptive use (no/yes), smoking (three groups: current, former, and never), and alcohol consumption (tertiles). Besides the proportion of mammographically dense tissue, absolute amounts of dense tissue were assessed as well and compared between different genotype and haplotype groups. The distributions of the absolute amount of dense tissue were skewed and were, therefore, LN-transformed to approach normality. After back-transformation, geometric means were presented. The covariates in this adjusted model were the same as those in the adjusted model for percentage density.

All analyses were done with SPSS version 11.

Results

In Table 1, breast cancer risk factors are listed according to genotypes of *Pvu*II and *Xba*I. Mean BMI seemed to be slightly higher for women with the *PP* or *XX* genotype compared with the other genotypes. The mean number of children and mean age at menarche was slightly higher for women with the *pp* or *xx* genotype compared with the other genotypes. Previous use of oral contraceptives and having a family history of breast cancer were more frequent and consumption of alcohol was less frequent in women with the *PP* or *XX* genotypes compared with *Pp* and *pp* or *Xx* and *xx* genotypes.

Mean percentage density was assessed according to several breast cancer risk factors and is shown in Table 2. Mean mammographic density was highest (42.2%) in women aged 50 years or younger and declined from 37.0% in the 51 to 53 age group to 33.2% in the 54 to 56 age group and 30.7% in women aged 57 years or older ($P < 0.01$). Women who were premenopausal had a higher mammographic density (41.5%) than women who were postmenopausal (33.2%; $P < 0.01$). Mean percentage density was 43.8% for women in the lowest BMI category and 27.7% for women in the highest BMI category ($P < 0.01$), which is probably due to a greater amount of fat in the breast that results in a lower percentage of dense tissue. Parity also had a clear effect on mammographic density because women who had children had a lower percentage of dense breast tissue (34.9%) than women who never had children (44.1%; $P < 0.01$).

Genotype distributions of *Pvu*II and *Xba*I were similar to other Caucasian populations (15, 29) and were in Hardy-Weinberg equilibrium (P values were 0.65 and 0.67, respectively). In Table 3, the combinations of *Pvu*II and *Xba*I

Table 2. Percentage mammographic density by breast cancer risk factors

Breast cancer risk factors	*n*	Mean percentage density (95% CI)	*P**
Age at mammogram (y)			
≤50	135	42.2 (39.5-44.9)	
51-53	167	37.0 (34.4-39.6)	
54-56	173	33.2 (30.9-35.6)	
≥57	143	30.7 (28.5-32.8)	<0.01
Menopausal status/age at menopause (y)			
Premenopausal	181	41.5 (39.2-43.9)	
<49	201	33.3 (31.0-35.6)	
≥49	231	33.1 (31.2-35.0)	<0.01
Previous oral contraceptive use			
Yes	440	35.8 (34.4-37.3)	
No	178	35.1 (32.6-37.6)	0.59
BMI (kg/m^2)			
≤23	179	43.8 (41.5-46.2)	
24	98	38.5 (35.3-41.7)	
25-27	185	32.6 (30.6-34.7)	
≥28	154	27.7 (25.5-29.8)	<0.01
Parity/age at first full-term pregnancy			
Nulliparous	50	44.1 (38.9-49.2)	
<26	336	34.8 (33.1-36.6)	
≥26	231	35.0 (33.0-36.9)	<0.01
Age at menarche (y)			
≤12	180	36.3 (33.8-38.9)	
13	175	34.5 (32.4-36.7)	
≥14	253	35.8 (33.7-37.8)	0.55
Family history of breast cancer			
Yes	79	35.8 (32.6-39.0)	
No	526	35.4 (34.0-36.8)	0.83
Alcohol consumption (g/d)			
<1.15	181	33.8 (31.4-36.1)	
1.15-9.16	210	36.0 (33.9-38.2)	
>9.16	225	36.8 (34.7-38.8)	0.16
Smoking			
Current	106	35.0 (32.0-38.0)	
Former	218	36.3 (34.0-38.6)	
Never	294	35.3 (33.6-37.1)	0.73

*ANOVA.

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Table 3. Distribution of women according to *Pvu*II and *Xba*I genotypes

	*Xba*I		
	XX	*Xx*	*xx*
*Pvu*II			
PP	74 (17)	53 (65)	11 (57)
Pp	1 (38)	236 (147)	77 (129)
pp	0 (20)	0 (78)	166 (68)

NOTE: Results are expressed as no. observed (no. expected). $P < 0.01$, χ^2 analysis.

genotypes are listed. Combinations *PP/XX* and *pp/xx* were frequent, and χ^2 analysis showed that the observed numbers of the combined genotypes were not equal to the expected numbers as based on their independent frequencies ($P < 0.01$). The linkage disequilibrium analysis showed that D' was 0.996 between the *p* allele and the *x* allele, which shows that the polymorphisms are in strong linkage disequilibrium. After separate genotyping of *Pvu*II and *Xba*I, haplotypes were reconstructed. The frequencies were 52.2% for haplotype 1 (*px*), 35.4% for haplotype 2 (*PX*), 12.3% for haplotype 3 (*Px*), and 0.1% for haplotype 4 (*pX*), which are similar to haplotype frequencies in other Caucasian populations (15, 30).

In Table 4, mammographic densities are given according to *Pvu*II and *Xba*I genotypes and to the number of copies of haplotypes 1 and 2. The unadjusted mean mammographic density was higher in women with the *Pp* or *pp* genotype (36.8% and 35.8%, respectively) than in those with the *PP* genotype (32.4%, $P_{\text{trend}} = 0.09$). Similarly, the unadjusted mean mammographic density was higher in women with the *Xx* or *xx* genotype (35.6% and 37.0%, respectively) than those with the *XX* genotype (31.0%, $P_{\text{trend}} < 0.01$). These results suggested that the *p* and *x* alleles were associated with higher breast densities. Therefore, haplotype 1 was taken as a ''risk allele'' for higher mammographic density in further analysis and the allele-dose effect was assessed by grouping individuals by the number of copies of haplotype 1. A similar strategy was taken for the analysis of haplotype 2 because opposite trends were expected. Mean mammographic density increased from 32.3% for no copies of haplotype 1 to 36.8% and 35.8% for one and two copies of haplotype 1, respectively ($P_{\text{trend}} = 0.08$). On the contrary, mean mammographic density decreased from 37.0% for no copies of haplotype 2 to 35.6% and 30.5% for one and two copies of haplotype 2, respectively ($P_{\text{trend}} < 0.01$). The adjusted values for mean percentage density were comparable with the unadjusted values.

In Table 5, the absolute amount of dense breast tissue is presented by genotype, represented as the amount of cm^2 on the digitized mammogram. Consistent with the results of percentage density, women with the *pp* or *xx* genotype, or two copies of haplotype 1, were found to have a larger area and women with two copies of haplotype 2 were found to have a smaller area of dense tissue on their mammogram ($P_{\text{trend}} < 0.01$), whereas total breast areas were similar in each genotype group (data not shown). The adjusted values for mean absolute density were comparable with the unadjusted values.

Discussion

In this study, the separate analyses of *Pvu*II and *Xba*I polymorphisms show that the *p* and *x* alleles are associated with higher mammographic densities. The aggregated haplotype 1 shows an allele-dose effect in which percentage density increases with an increasing number of haplotype 1 copies, whereas haplotype 2 shows an opposite trend.

In addition to the association with percentage density, which is a relative measure of dense breast tissue, the *Pvu*II and *Xba*I polymorphisms and haplotypes 1 and 2 were also, and even more clearly, associated with the absolute amount of dense breast tissue. This may reflect the target tissue for breast cancer better (31, 32).

It is not yet entirely clear whether the studied polymorphisms are functional variations in the *ESR1* gene or markers for a functional site elsewhere in the gene. The polymorphisms are located in intron 1 of the gene. Introns do not provide the protein code but can play a role in the production of mRNA. A recent study showed that the *P* allele produces a potential binding site for transcription factor B-myb, which suggests that presence of this allele might either amplify ESR1 transcription or produce ESR1 isoforms that have different properties than the full-length gene product (33, 34). Another study, however, showed a slightly enhanced transcription activity in *ESR1* sequences containing the *Pvu*II and *Xba*I polymorphisms, where enhanced activity was highest in the fragment containing haplotype 1, followed by haplotypes 3 and 4 (35). Although these two studies contradict each other, they indicate a possible role of intron 1 sequences in the production of ESR1. However, it is possible that another polymorphic

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Table 4. Percentage density according to genotype and haplotype

	Unadjusted			Adjusted		
	n	Mean percentage density (95% CI)	P_{trend}*	*n*	Mean percentage density (95% CI)	P_{trend}†
*Pvu*II						
PP	137	32.4 (29.7-35.1)		131	33.1 (30.7-35.5)	
Pp	314	36.8 (35.0-38.5)		297	36.0 (34.4-37.5)	
pp	165	35.8 (33.4-38.2)	0.09	155	36.2 (34.0-38.4)	0.08
*Xba*I						
XX	75	31.0 (27.4-34.6)		71	31.8 (28.6-35.1)	
Xx	289	35.6 (33.8-37.5)		275	35.2 (33.6-36.8)	
xx	254	37.0 (35.0-38.9)	<0.01	239	36.9 (35.1-38.6)	<0.01
Copies of haplotype 1						
0	138	32.3 (29.7-35.0)		132	33.2 (30.8-35.6)	
1	313	36.8 (35.0-38.6)		296	36.0 (34.4-37.5)	
2	165	35.8 (33.4-38.2)	0.08	155	36.2 (34.0-38.4)	0.08
Copies of haplotype 2						
0	253	37.0 (35.0-38.9)		238	36.9 (35.1-38.6)	
1	290	35.6 (33.7-37.4)		276	35.2 (33.5-36.8)	
2	73	30.5 (26.8-34.1)	<0.01	69	31.2 (27.9-34.5)	<0.01

*Linear trend, no covariates.

†Linear trend, covariates age (continuous), BMI (continuous), age at menarche (continuous), parity/age at first full-term pregnancy (three groups: nulliparous, <26 years, and ≥26 years), menopausal status (three groups: premenopausal, <49 years, and ≥49 years), family history of breast cancer (no/yes), previous oral contraceptive use (no/yes), smoking (three groups: current, former, and never), and alcohol consumption (tertiles).

Table 5. Absolute density according to genotype and haplotype

	Unadjusted			Adjusted		
	n	Mean* absolute density (95% CI)	P_{trend}†	n	Mean* absolute density (95% CI)	P_{trend}‡
*Pvu*II						
PP	137	35.1 (32.3-38.2)		131	35.0 (32.2-38.2)	
Pp	314	40.7 (38.5-42.9)		297	40.0 (37.8-42.3)	
pp	165	41.4 (38.4-44.7)	<0.01	155	42.1 (38.9-45.5)	<0.01
*Xba*I						
XX	75	34.3 (30.6-38.5)		71	33.6 (29.9-37.8)	
Xx	289	39.5 (37.3-41.9)		275	39.1 (36.9-41.5)	
xx	254	41.4 (38.9-44.0)	<0.01	239	41.6 (39.1-44.3)	<0.01
Copies of haplotype 1						
0	138	35.1 (32.3-38.2)		132	35.1 (32.2-38.2)	
1	313	40.7 (38.5-43.0)		296	40.0 (37.8-42.3)	
2	165	41.4 (38.4-44.7)	<0.01	155	42.1 (38.9-45.5)	<0.01
Copies of haplotype 2						
0	253	41.4 (38.9-44.0)		238	41.6 (39.1-44.4)	
1	290	39.5 (37.3-41.8)		276	39.1 (36.9-41.5)	
2	73	34.0 (30.3-38.2)	<0.01	69	33.3 (29.5-37.5)	<0.01

*Geometric mean; represented as cm^2 on the digitized mammogram.
†Linear trend, no covariates.
‡Linear trend, covariates age (continuous), BMI (continuous), age at menarche (continuous), parity/age at first full-term pregnancy (three groups: nulliparous, <26 years, and ≥26 years), menopausal status (three groups: premenopausal, <49 years, and ≥49 years), family history of breast cancer (no/yes), previous oral contraceptive use (no/yes), smoking (three groups: current, former, and never), and alcohol consumption (tertiles).

site linked to the ones studied here is the true functional sequence variation. The only way to clarify this is to identify all polymorphisms in this region and determine linkage disequilibrium between these polymorphisms to construct haplotype blocks. Once association studies have shown which haplotype carries the risk allele, functional analyses should be done to determine which of the variants in that haplotype truly contributes to the phenotype of interest.

Four studies have been published that investigated the *Pvu*II as well as the *Xba*I polymorphism in relation to breast cancer risk (13, 36-38). One study reported the *p* allele of the *Pvu*II polymorphism to be significantly associated with breast cancer risk (36); one showed a nonsignificantly elevated risk for the *p* allele (38). In two studies, the *x* allele of the *Xba*I polymorphism was significantly associated with breast cancer risk (13, 37); one showed a nonsignificantly elevated risk (36). Recently, our group (39) also found an increased breast cancer risk related to the *p* allele, which was borderline significant, and a nonsignificantly elevated risk for the *x* allele.

Onland-Moret et al. (39) combined all results from literature for the *Pvu*II polymorphism in relation to breast cancer risk. The pooled effect was estimated by abstracting odds ratios direct from the published articles or by calculating odds ratios from the data presented in the article. The pooled estimate was assessed using the precision weighted procedure described by Greenland (40). The overall odds ratio of the studies combined was 1.14 [95% confidence interval (95% CI), 1.00-1.32] for the *Pp* genotype and 1.23 (1.08-1.43) for the *pp* genotype. When we estimated the pooled effect for the *Xba*I polymorphism in relation with breast cancer from all results in literature using the same method, the overall odds ratio was 1.01 (95% CI, 0.82-1.23) for the *Xx* genotype and 1.17 (0.96-1.44) for the *xx* genotype. In the study presented here, the observed variations in dense tissue between different *ESR1* genotypes were small, indicating indeed that only very modest variations in breast cancer risk may be expected. The fact that the effects are small and that the populations investigated in previous studies vary in genotype frequencies and other characteristics may explain why some studies did not show a relation between these polymorphisms and breast cancer risk. However, the overall effect of all studies combined strongly suggests that the *p* allele and possibly also the *x* allele is involved in breast cancer risk.

The results of this study are consistent with most studies evaluating relations between these polymorphisms and breast cancer risk and indicate that *ESR1* has a modest role in the variation of dense breast tissue and, therefore, the risk of breast cancer.

Several other polymorphisms have been investigated as genetic determinants of mammographic density (6-10, 41). The polymorphisms that were found to be associated with mammographic density were located in genes that are involved in hormone synthesis (*3HSDB1* gene; ref. 6), hormone metabolism (*COMT* and *UGT1A1* genes; refs. 7, 8), action of hormones (*AIB1* gene; ref. 7), or action of growth factors (*IGFBP3* gene; ref. 41). By using the intermediate phenotype mammographic density, these studies and ours have been able to identify polymorphisms that most probably play a modest role in breast cancer risk. To use the intermediate phenotype as an end point seems to be a powerful approach to determine genetic factors of a clinical end point (17, 18). Although each polymorphism by itself has only a small to modest effect, they may play an important role when combined because it is very likely that many genes and exposures act together in the development of dense breast tissue and thus breast cancer risk. Therefore, genetic determinants of mammographic density may be useful in building multigenic models for predicting breast cancer risk. In this way, subgroups of women may be identified that are at high risk for breast cancer and could benefit from intensified screening or (chemo)preventive strategies.

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