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Polymorphisms in the Estrogen Receptor Alpha Gene and Mammographic Density Result Study in Brazilian Women

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Abstract

The estrogen receptor (ER) is a ligand-activated transcription factor that mediates the actions of the estrogen in target tissues. Several ERα gene polymorphisms are associated with changes in the receptor expression and function. The aim of this study is to verify the hypothesis that the ERα gene polymorphisms could be associated with high mammographic density(HMD), a well known independent risk factor for breast cancer (BC) in a case-control study carried out in the city of São Paulo (SP, Brazil) from 2010 to 2013 . Two ERα gene polymorphisms named *PvuII* and *XbaI* was examined in 308 cases and 155 controls. The *PvuII* polymorphism was associated with an increased risk of having high mammographic density (HMD) post menopause, after adjustment for other risk factors, the odds ratio for pp genotypes was 1.75 (confidence interval of 95% CI 95%=1.10-2.79) compared with the genotypes PP and Pp. The *XbaI* polymorphism was also associated with a high risk of HMD, but not statistically significant, odds ratio for xx genotype was 1.31 (95% CI=0.7 to 1.9). No apparent synergistic effects of these two polymorphisms were identified. It was concluded that the Pvull polymorphism in the gene ERα was associated with an increasing chance of have HMD, a strong risk factor for BC. Thus recognizing these risk factors will be of great importance in the analyses of individual susceptibility to BC, in both the study of the response to various drugs and the prognosis.

Keywords: Breast neoplasms; Estrogen receptors; Genetic polymorphism; Mammography; Risk factors

Introduction

Estrogens affect the growth, differentiation and function of many target tissues including breast, uterus, vagina, ovary, testicles, epididymis, and prostate [1]. The biological effect of the estrogens such as growth and differentiation of normal breast tissue is mediated primarily through high affinity binding to its ER. The ERs are intra-nuclear proteins that possess a binding domain to the estrogen and a binding domain with the DNA [2]. There are two types of ERs (α and β). The ERα gene is located [3] on chromosome 6q25.1, and the ERβ gene is located on chromosome 14q22-24. Among the steroid receptors the ERα and the progesterone receptors (PR) are of special interest because their protein levels are elevated in malignant breast cells [4-6]. Both ER and PR prove to be significant prognostic factors for BC [7]. Therefore the inhibition of Erα has become a major strategy for the prevention and treatment of BC [8].

The combination of ERα gene polymorphisms and the risk of diseases, including BC have become subject of growing interest. Thus, several studies have indicated that variations in the DNA sequence of the ERα gene increase the risk of developing BC and HMD after menopause. Some studies are summarized in Tables 1 and 2.

The studies of polymorphisms related to diseases are tools that may have direct implication of great importance in the analysis of individual susceptibility to BC in the study of response to various drugs and prognosis. The ultimate goal of these strategies is to reduce the anxiety of the patients and greatly improve the approach and management of a woman with or without risk, facilitating the implementation, planning and the adoption of preventive strategies.

This article presents the results of the case-control study in the city of São Paulo, that examined the association of the polymorphisms in the genes *ERα-PvuII* and *ERα-XbaI* with the risk of high mammographic density after menopause. The associations of these polymorphisms with other risk factors for breast cancer were also evaluated.

Methods

Case-control study that included 308 women with HMD(for more than 50% density) and 155 controls(to 50% density or less) evaluated by computerized objective method [25], aged 45-65, without menstruation or hormone therapy for at least 1 year, without previous BC and ovarian cancer. Initially, the patients were selected in a subjective way by the standard ACR-BIRADS®, by a unique reader (the head) from the Institute of Radiology, *Hospital das Clinicas, Faculdade de Medicina, Universidade de São Paulo* (HC-FMUSP), São Paulo, Brazil, from January 2010 to March 2013. The selected patients were evaluated again, now objectively, by another reader as described by Boyd et al. 2013 [25]. The study was approved by the Ethics Committee for Analysis of Research Projects - CAPPesq the HC-FMUSP, and all women signed informed consent. It was characterized in the clinical

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Genotype	Allele	Country	Type and size of the study	Association	OR/RR	References
ERα-397 T/C Pvull	TT	USA pre-andpostmenopausal	Only Cases/Case Control 257/140	Diagnosis in younger women		Yaich et al. [9]
	TT	Shanghai, China	Case/control 1069/1166	↑ risk for breast cancer	OR: 1.4 IC 95%: 1.1-1.8 p=0.042	Cai et al. [10]
	TT	Netherlands	Case/control 380/422	↑ risk for breast cancer	RR: 1.5 IC 95%: 0.94-2.42	Onland-Moret et al. [11]
	TT	China 25 a 55 years-old	Case/control 259/278	↑ risk of breast cancer in women with a positive family history	OR: 3.04 IC 95%: 0.73-12.67	Shen et al. [12]
	TT	Spain 26 to 86 years-old	Case/control 444/704	↑ risk of breast cancer in women with a positive family history	OR: 3.81 IC 95%: 1.25-11.6	Gonzalez-Mancha et al. [13]
	TT	Netherlands 55 yearsor more	Case/control 190/3513	↑ risk of breast cancer in women after menopause	OR: 1.4 IC 95%: 0.8-2.2	Ladd et al. 2007 [14]
	TT	USA peri-menopause 42 to 52 years-old	longitudinal cohort 451	↑ Increased mammographic density in white women	7.0%; p=0.01	Crandall et al. [15]
	TT	Brazil After-menopause	Only cases 308 women	Mammographic density>50%	TT=32.14% greater than CC=20.13%	Souza et al. [16]
	TT/TC	Netherlands and England After-menopause	Population Cohort prospective case/ control (795 with HT/781 with no HT)	↑ Increased mammographic density only in HT users	2.24% p<0.01	van Duijnhoven et al. [17]
	TC	Netherlands	Caso/controle380/422	↑ risk for BC	OR: 1.14 IC 95%: 1.00-1.32	Onland-Moret et al. [11]
	TC	China 25-55 anos	Case/control 259/278	↑ risk for BC(with family history)	OR: 2.46 IC 95%: 0.61-9.88	Shen et al. [12]
	CC	Shanghai, China 22-64 years-old	ProspectivePopulation Cohort 1459 cases	RE negative expression; worse prognosis for breast cancer	OR: 3.30 IC 95%: 1.42-7.69	Boyapatti et al. [5]
	CC	Netherlands and England After-menopause	Population Cohort prospective case/ control [795/781]	Mammographic density without changes in user HT	0.90% p=0.47	van Duijnhoven et al. [17]

Table 1: Association studies of the ERα-397 gene polymorphism *PvuII* C/T with BC and/or risk factors for disease.

history and physical examination: age at menarche and menopause, parity, age at first birth, family history of breast cancer (FHBC), smoking, alcohol intake and body mass index (BMI). Peripheral blood samples were obtained for genomic DNA extraction and determination of polymorphisms in question.

The genomic DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (*Qiagen*), following manufacturer instructions. After DNA quality and integrity evaluation were performed PCR-RFLP assay for Estrogen Receptor *PvuII* and *XbaI* polymorphism analysis as described by Herrington et al. 2002 [26]. The laboratory was blinded on the subject identification.

Hardy-Weinberg Equilibrium (H_W) was used to verify if the genotype frequencies of *PvuII and XbaI* polymorphisms in our population were in genetic equilibrium. Simple mathematical model [(p+q) 2=p2 +2 pq+q2] used to calculate genotype frequencies from allele frequencies.

Statistical analysis: The data were described using average, standard deviation (sd), absolute frequency (n) and relative frequency (%). To verify the association between qualitative variables with the mammographic density was used the chi-square (X^2). For comparison between the groups of HMDand controls as quantitative variables was used the *Kolmogorov-Smirnov Test* to verify the normality of the data, as these are not normally distributed in all groups, was used the nonparametric *Mann-Whitney Test* for comparison between groups. To verify the relationship of the variables with the occurrence of high mammographic density was used Multivariate Logistic Regression model stepwise backward. The variables that entered in the model were those presented in the bivariate analyzes values p<0.20. In all statistical tests were adopted a significance level of 5% (p<0.05).

Results

The distributions of selected demographic characteristics that are the main risk factors for breast cancer are presented in Table 3. It was observed elevated risk of HMD for the main risk factors that have been reported in other previous studies [20,16].

The risk of having HMD was also elevated for younger women, lower WC, fewer pregnancies, higher age at having first birth, high number of women with a FHBC. It was not observed any apparent modification effect for other indicators of exposure to endogenous estrogens and lifestyle factors. In our study, the sample consisted of postmenopausal women, aged 45-65 years.

The allele frequencies of *ERα-PvuII and ERα-XbaI* in both groups were similar to those reported in previous studies [16,17,20,27-29].

Regarding the genotype, 21.5% of controls and 32.5% of cases were mutated homozygous (pp), and 28.5% of controls and 19.8% of cases were wild homozygous (PP), with statistically significant difference (X^2=7.42, p=0.024). OR adjusted for pp genotype was 1.75 (95% CI=1.10 to 2.79) compared with PP and Pp genotype. There were not any significant difference in allele frequency or genotype polymorphism *ERα-XbaI* between controls and cases (Table 4).

When the two ERα polymorphisms were analyzed together, no synergistic effect was consistently noted.

Additional analyzes (Multivariate Logistic Regression) were performed to evaluate the independent risk factors for HMD. From all clinical characteristics analyzed, entered only those presented in the bivariate analyzes values p<0.20 were included in the model: the *Pvull* polymorphism of the ERα gene; indicators of exposure to endogenous estrogen: age at menarche, menopause, time after menopause,

Genotype	Allele	Population	Type and size of the study	Association	OR/RR	References
ERα351 *Xbal* A/G	AA	Norway 27 to 94 years-old	Case/control 360/672	↑ risk for breast cancer after menopause	OR: 2.02 IC 95%: 0.96-4.31	Andersen et al. [18]
	AA	Korea	Case/control 205/205	↑ risk for breastcancer	OR: 2.38 IC 95%: 1.58-3.58	Shin et al. [6]
	AA	Korea	Case/control 205/205	↑ risk for breast cancer in nulliparous	RR: 4.0 IC 95%: 1.9-8.8	Shin et al. [6]
	AA	Netherlands and England After-menopause	Cohort prospective 791 with HT/781 no HT	↑mammographic density in HT users	2.20% p<0.01	van Duijnhoven et al. [17]
	AA	China		↑ risk for breastcancer	OR: 6.88 IC 95%: 0.80-59.15 p=0.079	Hu et al. [19]
	AA	China 25-55 years-old	Case/control	↑ risk for breast cancer, with positive family history	OR: 4.20 IC 95%: 0.65-27.28	Shen et al. [12]
	AA	Netherlands 55 yearsor more	Case/control 190/3513	↑ risk for breast cancer in women after menopause	OR: 1.3 IC 95%: 0.7-2.2	Ladd et al. [14]
	AA	Brazil After-menopause	Prospective 120	↑ higherbreastdensity	OR: 2.34 IC 95%:1.06-5.16 p=0.03	Ramos et al. [20]
		England	Systematic review of case-control studies	Nonsignificantdifference	P=0.06	Dunning et al. [21]
	AA	Pakistan, 15-65 years-old	Case/control 100/100	↑ of the risk of BC post menopouse	AA 45% greater than GG p<0.01	Javed et al. [22]
	AA	BrazilAfter-menoppausa	Only cases 308 women	Mammographicdensity>50%	AA 33.44% greaterthan GG 16.56%	Souza et al. [16]
Haplotyp*Xbal*-C975→G		Sweden after-menoppausa	Case/control 1556/1512	↑ risk of breast cancer in postmenopausal and obese women	OR=1.48 IC 95%: 1.17-1.88	Wedren et al. [23]
	AG	Norway 27-94 years-old	Case/control 360/672	↑ risk for breastcancer	OR: 2.00 IC 95%: 0.92-4.37	Andersen et al. [18]
	GG	USA, Caucasian greater than 65 years-old	Case/control 393/790	↓ risk for breastcancer	OR 0.82 IC 95%: 0.68-1.00 P=0.04	Wang et al. [24]
	GG	Netherlands and England After-menopause	Prospective Cohort 795 with HT/781 no HT	Mammographic density without changes in users of HT	0.65% p=0.70	van Duijnhoven et al. [17]
	A	China	Case/control 114/121	↑ risk for breastcancer	OR: 1.4 IC 95%: 1.0-1.9	Hu et al. [19]
	G	Korea	Case/control 205/205	↓ risk for breast cancer in postmenopausal	RR: 0.3 IC 95%: 0.1-0.5	Shin et al. [6]

Table 2: Association studies of the ERα-351 *Xbal* A/G gene polymorphism with BC and/or risk factors for disease.

		Controls		HMD			
Quantitativevariables	**Average**	**Dp**		**Average**	**Dp**	**Z**	**P**
Age		58.16	4.61	56.31	5.42	3.40	0.001*
Wast circunference	95.06	11.13		89.47	10.83	5.04	<0.001*
Number of Pregnancies	3.63	2.56		2.46	1.83	4.62	<0.001*
Number of Births		2.84	1.98	1.99	1.54	4.37	<0.001*
Number of Abortions		0.79	1.20	0.48	0.87	2.93	0.003*
Date of last menstruation		46.83	6.26	46.45	6.33	0.49	0.621
Age at first birth		21.88	5.16	24.04	6.04	3.61	<0.001*
Menarche		12.87	1.78	13.17	1.78	1.66	0. 096
Time after menopause		11.33	6.42	9.85	7.38	2.69	0.007*
Qualitative variables	**n**	**%**		**n**	**%**	***x²***	**P**
BMI	Normal/overweight	81	52.3	195	63.3	5.23	0.022*
	Obese	74	47.7	113	36.7		
Smoke	No	140	90.3	264	85.7	1.97	0.161
	Yes	15	9.7	44	14.3		
Alcohol intake	No	137	88.4	258	83.8	1.76	0.185
	Yes	18	11.6	50	16.2		
Metabolic syndrome	No	104	67.1	220	71.4	0.92	0.337
	Yes	51	32.9	88	28.6		

Quantitative variables *Statistically significant difference [p<0.05]
Nonparametric Mann-Whitney test
*Qualitative variables: statistically significant difference [p<0.05]
Chi-square test

Table 3: Comparison between the two groups of Mammographic Density: HMD and controls in quantitative and qualitative variables.

High mammographic density	*PvuII*				
	Pp	Pp	PP	Total	
No	72	31	41	144	X^2=7.42
	50.0%	21.5%	28.5%	100.0%	p=0.024*
Yes	147	100	61	308	OR=1.75
	47.7%	32.5%	19.8%	100.0%	IC 95%=1.10-2.79
Total	219	131	102	452	
	48.5%	29.0%	22.6%	100.0%	
	XbaI				
	Xx	Xx	XX	Total	
No	74	40	30	144	X^2=2.03
	51.4%	27.8%	20.8%	100.0%	p=0.362
Yes	154	103	51	308	OR=1.31
	50.0%	33.4%	16.6%	100.0%	IC 95%=0.85-2.02
Total	228	143	81	452	
	50.4%	31.6%	17.9%	100.0%	

Table 4: Association between mammographic densities and polymorphisms *PvuII and XbaI*.

number of pregnancies, age at first birth, number of births, number of abortions, FHBC, BMI, waist circumference (WC), and the variables that characterize the sample (age, race, alcohol intake, smoking). The risk associated with the p allele of the *PvuII* was high in all strata. It was observed an increased risk of having HMD for women with younger age, smaller WC, fewer pregnancies, old age at having the first birth, the higher number of women with a FHBC. There was no apparent modification effect for the other indicators of exposure to endogenous estrogens.

Likewise, it was performed the association between the x allele of the *XbaI* and the same independent variables that characterize the sample, but no results confirmed the association.

Discussion

The association of ERα gene polymorphisms with the risk of BC draws attention because the ER functions as a regulator of the hormone dependent transcription, which plays a significant role in the development of BC [10]. Indeed, approximately two thirds of BC expresses the estrogen receptor alpha. Epidemiologic studies also correlate steroid hormones to changes the MD, and have examined whether variations in genes that regulate the biosynthesis and hormonal metabolism could explain individual differences in MD. The ER gene, located in the long arm of chromosome 6q25, has been associated to HMD in many studies, due to its importance in the development, progression and prognosis of BC. Several polymorphisms of ERα gene have been reported, with the *PvuII and XbaI* – located in intron 1 of the ERα gene with 50 base pairs between them – being the most studied. Several diseases, including BC [6,9,10,18,30], endometrial cancer [31], Alzheimer's disease [32], obesity [33,34], endometriosis [35], leiomyomas [36] and bone mineral density [37], have been evaluated for a possible connection with ERα gene polymorphisms.

Our findings revealed a statistically significant difference between the two groups of mammographic density as the main risk factors for breast cancer that has been reported in previous studies. The risk of having HMD was increased in younger women, with a lower waist circumference, fewer pregnancies, a higher age at the first birth, and a high number of women with a family history of breast cancer. We observed that for the increase of 1 year in age, the probability of being classified as HMD as compared to controls, decreased 6.8%. The groups of women with HMD were 56.31 years old in average, while in the controls group the average age was 58.16. This relationship was statistically significant (p<0.001), indicating that younger women are most likely to have HMD. These findings are in agreement with the literature, showing decrease in breast density patterns with increasing age (Stomper et al. 1996 and Boyd et al. 2007). Among the avoidable risk factors throughout life, obesity/overweight are the most important ones, since they are the main determinants of the estrogen level in women after menopause [34] and, exposure to estrogens during life is a well established factor of increased risk for BC. According to recent studies, there is a positive relationship between BMI and breast cancer, especially in postmenopausal women [38-40]. Nevertheless, women with high BMI are less likely to have HMD, even though the risk for BC development increases in women with HBD and who are obese (obese, OR=1.7: obese HBD, OR=2.01) [41]. It is also suggested that for every 5 kg of weight gain in adult life, the risk of developing BC [42] increases by 8%. Our data showed a high prevalence of obesity, but it was predominant in the control group (47.7%). Obesity was highly prevalence in the HMD group (36.7%), a surprising and worrying finding that deserves attention because of women with HBD, a factor considered as a high risk for developing BC (RR>4.0).

Our findings revealed a statistically significant difference between the two groups of mammographic density also with respect to the distribution of the *PvuII* polymorphism genotype (p=0.024). It was found that women with two mutated alleles (homozygous mutant) had 76% greater chances of being diagnosed with HMD, compared to those with one or two normal alleles for this polymorphism (Table 4). The risk associated with the p allele of *PvuII* was shown to be an independent risk for other indicators of high exposure to endogenous estrogens. It was not observed any apparent effect modification for other indicators of exposure to endogenous estrogens and lifestyle factors. Similarly, van Duijnhoven et al. [17] studied the influence of ERα and HT on the MD, reporting an association between increased MD and the presence of the mutated genotype ERα-397 (OR=2.24) in women using HT when compared to those with wild genotype and not HT users. Parl et al. [30] found that the pp genotype of the *PvuII*SNPs was higher in women with a diagnosis of BC at a younger age. Yaich et al. [9] examined the *PvuII* polymorphism in tumor tissue of 257 women with primary breast cancer and compared it to peripheral blood collected from 140 controls not affected by the disease. Women with BC and pp genotype had a diagnosis of BC at an earlier age compared with those with PP or Pp genotypes of the group that had cancer.

Investigating the genotype distribution of the *XbaI* polymorphism,

Independent variables	B	Standard error B	OR	OR [IC 95%]		p
Constant	7.330	1.715				
Age	-0.070	0.025	0.932	0.888	0.979	0.005
WC	-0.038	0.011	0.963	0.943	0.984	0.001
Number of pregnancy	-0.186	0.069	0.830	0.725	0.950	0.007
Age at first birth	0.052	0.025	1.053	1.004	1.106	0.035
FHBC	0.707	0.335	2.028	1.052	3.909	0.035
Mutated PvuII	0.562	0.290	1.754	0.993	3.099	0.053

Table 5: Multiple logistic regression, stepwise backward method, where the dependent variable in the HMD and independent variables are: Age, BC, Number of Pregnancy, Age at first birth, HFBC, Mutated PvuII.

the authors found that women with two mutated alleles (homozygous mutant) were 30% more likely (OR=1.31) of having HMD, though this difference was not significant (p=0.36). On the other hand, Andersen et al. [18], while investigating the allelic frequency of the *PvuII* and *XbaISNPs* in 360 cases of the BC and 672 controls, found significant differences only for the *XbaI* polymorphism. The x allele frequency among women with BC was 40% higher compared to that of the controls. In a case-control study conducted in South Korea by Shin et al. [6], the OR associated with the xx genotype of *Xbal* was 2.38 compared to the XX genotype. In that same vein, Cai et al. [10], in a large population study, found a significant association between ~~of~~ the *PvuII* polymorphism and BC (OR=1.4). For *XbaISNPs*, the association of risk was 1.3 and only for women after menopause.

The molecular mechanisms through which these polymorphisms alter the receptor activity are not clear because *PvuII and XbaI* are located in an intronic, and apparently nonfunctional, region of the gene. Possible explanations include: (a) the existence of a functional combination between polymorphic alleles, where the two markers in combination would alter the gene function, as well as the RNAm stability [23]. Thus, this study investigated if the combination of *PvuII and XbaI* polymorphisms increased the risk of having HMD, and did not observe any synergistic effect between them; (b) Another explanation would be that polymorphisms in intron could have a Linkage Disequilibrium (LD) with the *exon* which would affect the function of the ER as a whole [37]; in this regard, it has been investigated whether polymorphisms in intron 1 are in LD with repeat polymorphism *(GT)n* located at 6627 bp after the beginning of the transcription site of the *exon* 1 and to 144 kb before the *exon* 2; (c) Growth factors and their signaling molecules are important for cancer growth and its progression. There is considerable cross-talk between ER and growth factors such as insulin and IGF1 (growth factors like insulin), and the family of epidermal growth factors [43].

The allelic and genotypic frequencies obtained for the *ERα-397 PvuII* polymorphism (P=46.8%, p=53.2%) were similar to those found in other studies in which this polymorphism was correlated with MD or BC [11,13,17,19,27,29]. The genotype distribution was in Hardy-Weinberg equilibrium with the following frequencies: pp genotype 29.0%, Pp 48.5% and PP 22.6% (Table 4).

The allele frequencies for the *Xbal* polymorphism (x=56.9%, X=43.1%) were lower when compared to two other studies conducted in Brazil [20,27], but they were in accordance with the frequency found by van Duijnhoven et al. [17], Molvarec et al. [28] and Hsieh et al. [29]. The genotype distribution for *ERα-351 XbaI* was also in Hardy-Weinberg equilibrium with the following frequencies: xx genotype 31.6%, Xx 50.4% and XX 17.9% (Table 4).

In relation to the independent variables that showed associationwith MD (age, race, WC, menarche, menopause, time after menopause, number of pregnancies, age atthe first birth, number of births, number of abortions, FH, BMI, alcohol intake, smoking and *PvuII and XbaI* genotypes), after performing a multiple logistic regression, only the clinical factors (age, WC, number of pregnancies, age at the 1st birth, FH and *PvuII* polymorphism in the ERα) showed to be independent risk factors for HMD (p<0.05).

In summary, the present case-control study concluded that the *PvuII* polymorphism in the gene ERα was associated with an increased chance of having HMD, a factor of high risk for BC. Thus, recognizing these risk factors will be of great importance in the analyses of individual susceptibility to BC, in both the study of the response to various drugs (for example HT) and the prognosis (Table 5).

Footnote

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