

Mammographic density and estrogen receptor α gene polymorphism in Javanese women

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ABSTRACT

Estrogen plays important roles in breast cancer as it binds its receptor in breast tissue. The most studied variants in estrogen receptor α encoded by *ESR1* gene are the *ESR1 PvuII* and *XbaI* polymorphisms, which were associated with lower sensitivity to estrogen. We determined the proportion of *ESR1 XbaI* and *PvuII* polymorphisms in Javanese woman in Yogyakarta, Indonesia and analyzed the correlation between genetic variations with mammogram density. *ESR1 XbaI* and *PvuII* polymorphisms of 50 cases and 58 controls were identified using PCR-RFLP. Breast density was assessed based on digitizer mammograms. Quantitative analysis was performed using an interactive program based on cumulus of two thresholds. Mean of density and frequencies of SNPs were compared between cases and controls to identify the association between SNPs and cancer susceptibility. Mammographic density was significantly higher in cases (52%) than controls (0.41%) ($p < 0.05$). Women with one or two copies of the *PvuII* T allele and *XbaI* A allele had higher mammographic density compared with women with C and G alleles, respectively. The proportion between PP and TT genotype was not statistically significant ($p > 0.05$), while the proportion between AA and GG was significantly different ($p < 0.05$). Haplotype 2 (CG/PX) was associated with lower sensitivity to estrogen and reflects a decrease of mammographic density. These findings were consistent with other studies that showed that *ESR1* polymorphisms may affect breast cancer risk through differences in breast density.

ABSTRAK

Estrogen memainkan peran penting dalam kanker payudara karena mengikat reseptor di jaringan payudara. Varian yang paling banyak dipelajari di reseptor estrogen α yang dikode oleh gen *ESR1* adalah polimorfisme *ESR1 PvuII* dan *XbaI*, yang terkait dengan sensitivitas yang lebih rendah untuk estrogen. Kami menentukan proporsi polimorfisme *ESR1 XbaI* dan *PvuII* pada wanita Jawa di Yogyakarta, Indonesia dan menganalisis korelasi antara variasi genetik dengan kepadatan mammogram. Polimorfisme *ESR1 XbaI* dan *PvuII* dari 50 kasus dan 58 kontrol diidentifikasi menggunakan PCR-RFLP. Kepadatan payudara dinilai berdasarkan *digitizer* mammogram. Analisis kuantitatif dilakukan dengan menggunakan program interaktif berbasis *cumulus* dari dua ambang batas. Rata-rata kepadatan dan frekuensi SNP dibandingkan antara kasus dan kontrol untuk mengidentifikasi hubungan antara SNP dan kerentanan terhadap kanker. Kepadatan mammografi secara signifikan lebih tinggi pada kasus (52%) dibandingkan kontrol (0,41%) ($p < 0,05$). Wanita dengan satu atau dua salinan alel *PvuII* T dan *XbaI* A memiliki kepadatan mammografi lebih tinggi

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dibandingkan dengan wanita dengan alel C dan G, secara berurutan. Proporsi antara genotipe PP dan TT tidak signifikan secara statistik ($p > 0,05$), sedangkan proporsi antara AA dan GG berbeda secara signifikan ($p < 0,05$). Haplotype 2 (CG / PX) berkaitan dengan sensitivitas yang lebih rendah terhadap estrogen dan mencerminkan penurunan kepadatan mamografi. Temuan ini konsisten dengan penelitian lain yang menunjukkan bahwa polimorfisme ESR1 dapat mempengaruhi risiko kanker payudara melalui perbedaan kepadatan payudara.

Keywords: breast cancer - DNA polymorphism - *ESR1 PvuII* - *ESR1 XbaI* - mammogram Digitizer

INTRODUCTION

Breast cancer (BC) is the highest ranked malignancy in women in the world and is particularly frequent in Yogyakarta, Java, Indonesia.^{1,2} Estrogen plays an important role in the occurrence of BC through inducing proliferation and genotoxic effects by mechanisms involving estrogen binding to its receptor in breast tissue. However, the detailed mechanism of the induction of BC carcinogenesis is not fully understood.³ There are two types of estrogen receptor, estrogen receptor alpha, which is the main estrogen receptor that is encoded by the *ESR1* gene, and estrogen receptor beta which is encoded by *ESR2* gene.⁴ *ESR1* is one of the most important mediators of hormonal response in estrogen-sensitive tissues such as the breast and plays a crucial role in breast growth and differentiation as well as in the development of cancer.⁵

Mammography density reflects the number of stromal and epithelial cells and is one of the strongest risk factors for BC. Some studies showed that women with a high density mammography pattern have a risk of BC 4–6 times higher than those with a low density pattern.⁶ An annual mammography examination is currently recommended for women over the age of 40 years,¹ but not for women younger than 40 years, due to pain from compression techniques during the examination and the risk of radiation hazard.⁷

Many studies have demonstrated evidence for the influence of estrogen on mammographic density.⁸⁻¹⁰ Women with hormone replacement therapy showed increased breast density.^{8,9} Conversely, tamoxifen, a selective ER modulator and anti-estrogen, has been shown to decrease breast density and cancer risk.¹⁰ This suggests that the association of mammographic density with BC risk may occur through an estrogenic mechanism.

Research on the relationship between *ESR1* polymorphism and mammographic density revealed different frequencies of *ESR1* polymorphisms in different ethnics.^{11,12} The most studied variants in the *ESR1* gene are the *PvuII* and *XbaI* polymorphisms, which have been associated with lower sensitivity to estrogen.¹³ Determining the proportions of *ESR1 XbaI* and *PvuII* polymorphisms in BC cases in Yogyakarta may provide information that can be used as a preventive measure, especially for younger women who cannot be examined with mammography and as a reference for some interventions to reduce BC risk.

MATERIALS AND METHODS

Subjects

This study included BC patients admitted to Dr. Sardjito General Hospital, Yogyakarta in 2009–2010 who underwent pre-surgery mammography examinations, had been

diagnosed with BC and undergone mastectomy. The controls included women with no benign lesion based on breast examination, either using ultrasonography or mammography. All subjects agreed to participate in the study and provided signed informed consent. One hundred and eight blood samples from cases (n=50) and controls (n=58) were transported to the Molecular Biology Laboratory Faculty of Medicine Universitas Gadjah Mada for DNA extraction. Clinical data were obtained from medical records and directly filling out the questionnaire.

Density analysis

Density analysis was carried out after the first mammogram scanning steps. The shape of a negative mammogram film is converted into digital form using a mammogram digitizer. Density analysis was performed using a special program. Quantitative analysis was performed using base system computer-assisted methods of measurement with an interactive program based on two thresholds. The first threshold distinguishes breast tissue from the background in the mammogram. The second threshold is used to distinguish tissue in the breast itself.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Genomic DNA was extracted with saturated NaCl method. PCR was conducted in a total reaction volume of 25 μ L, consisting of 100 ng genomic DNA, 2.5 μ L 10 X PCR buffer, 0.75 μ L MgCl 50 mm, 0.2 μ L 25-mm dNTPs, 0.1 μ L Platinum Taq (Invitrogen) and 10 pmol of each primer 1 μ L forward and reverse-coupled with DHPLC. The PCR reaction conditions were as follows: 95°C 5 min, followed by 35 cycles of 95°C 35 s, annealing temperatures of 15 s and 72°C for 25 s, followed by a final extension at 72°C

for 10 min. PCR products were digested with *Xba*I and *Pvu*II restriction enzymes (New England Biolabs) and visualized on 2.5–3% agarose gels containing ethidium bromide.

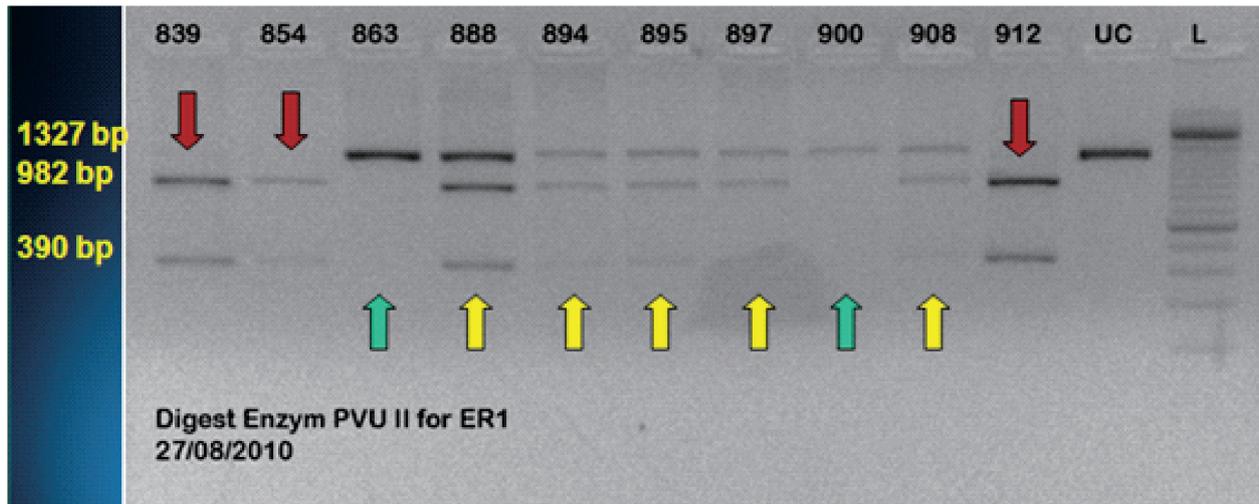
The homozygous mutant in *ESR1 Xba*I AA shows two bands sized 936 and 436 bp, the heterozygous AG produces three bands (1327, 936 and 436 bp), and the homozygous wildtype GG shows a single band size of 1327 bp.⁹ For *ESR1 Pvu*II, the TT homozygous mutant shows two bands sized 982 and 390 bp, the heterozygous CT produces three bands (1372, 982 and 390 bp), and the homozygous CC wildtype shows a single band of 1327 bp.¹⁴

Statistical analysis

Data were analyzed with student t-test statistical analyses. *P*-values less than 0.05 were considered significant.

RESULTS

This study included 108 BC research subjects in Dr. Sardjito General Hospital, Yogyakarta between 2009 and 2010. The average age of subjects was 50.6 years and average age of controls was 48.72 years. The youngest subject among them was 33 years old and the oldest was 68 years old. There was no significant difference of age between cases and controls ($p > 0.05$). The percent mammogram density of cases was 52% and controls was 41%. Compare means with independent sample t-test between cases and controls was significantly different ($p < 0.05$). We next performed PCR-RFLP of all cases. The *ESR1 Pvu*II TT homozygous mutant shows two bands of 982 and 390 bp, the heterozygous CT produces three bands of 1372, 982 and 390 bp, and the homozygous CC wildtype shows one band of 1327 bp (FIGURE 1).¹⁴ Furthermore the frequency of *ESR1 Pvu*II polymorphism is presented in TABLE 1.



Green arrow: C/C, yellow arrow: C/T, red arrow: T/T

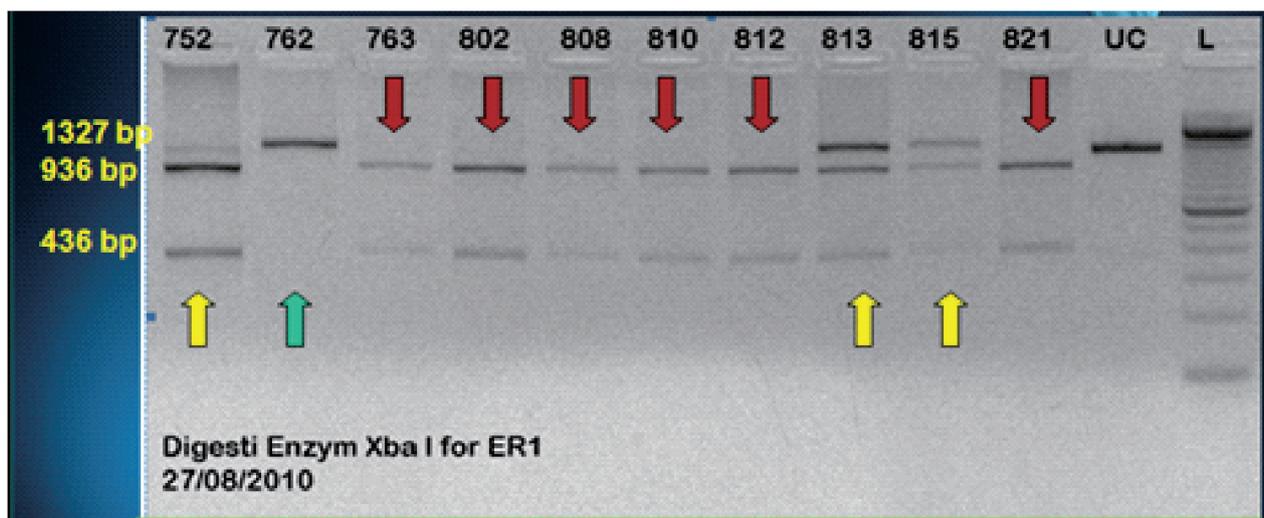
FIGURE 1. ESR1 PvuII polymorphism

TABLE 1. Frequency of ESR1 PvuII polymorphism

Allele	Case		Control	
	Frequency	%	Frequency	%
TT	15	30	6	10.3
TC	28	56	33	56.9
CC	7	14	19	32.8

The homozygous mutant in the *ESR1* *XbaI* AA shows two bands sized 936 and 436 bp, the heterozygous AG produces three bands (1327, 936 and 436 bp), and the homozygous

wildtype GG shows a band size of 1327 bp (FIGURE 2).¹⁴ Furthermore the frequency of *ESR1 XbaI* polymorphism is presented in TABLE 2.



Green arrow: G/G, yellow arrow: G/A, red arrow: A/A

FIGURE 2. Polymorphism ESR1 XbaI

TABLE 2. Frequency of *ESR1 XbaI* polymorphism

Allele	Case		Control	
	Frequency	%	Frequency	%
GG	2	4	10	17.2
AG	20	40	25	43.1
AA	28	56	23	39.7

We also analyzed and compared the mammography density in all groups. The mean percentage density in women with one or two copies of the *PvuII* p allele (CT/Pp and TT/pp, 49% and 48%, respectively) was higher than in those with the CC/PP genotype (39%). However, this difference did not show statistical significance (TT vs. CC, $p > 0.05$). Women with one or two copies of the *XbaI* x allele had a higher mean percentage density (AG/Xx and AA/xx, 49% and 47%, respectively) than those with the GG/XX genotype (32%), and we detected a significant difference between AA and GG genotypes ($p < 0.05$). The percentages of heterozygous (CT) and homozygous (TT) *PvuII* in BC cases were higher (86%) than those in control (66%). The percentages of heterozygous (AG) and homozygous (AA) *XbaI* were also higher in BC cases (96%) compared with controls (82%).

DISCUSSION

The average age of the subjects in this study was 50.6 years. Age less than 50 years as many as 25 (50%), equal to or more than 50 years 25 subjects (50%). A study in the United Kingdom in 2007 reported that most GC patients are over 50 years of age (81%).¹⁵ These data reflect the high number of young GC survivors in Indonesia, especially in Java, which is consistent with the pattern of GC in Asian females.¹⁶ In general, the frequency of cancer incidence will increase in line with age.

This increased incidence can be explained by the accumulation of somatic mutations in the human body. Another factor that plays a role is the decline in immune competence that accompanies the aging process. Although very few cases of BC occur in women in their teens or early 20s, BC is the second most common cancer diagnosed in women under 35 years. Approximately 1,400 BC cases are diagnosed each year among women aged 35–39 years. BC incidence rates generally increase with age, with the largest rate of increase before menopause.¹ The differences in rates between patients after BC in Indonesia and in developed countries is probably because of the lower life expectancy in Indonesia and may also be due to specific genetic factors.

Mammogram density

High breast density on mammography is one of the strongest risk factors for BC. Breast density seen on a mammogram reflects differences in the number of stromal and epithelial cells and fatty tissue in the breast. Stroma and epithelium are radiologically will provide the density, while fat provides a radioluscent.¹⁷ The average mammographic density of BC cases is 52% with an average age 50.6%, but in control group 41% with an average age 48.7 % ($p < 0.05$). A previous study reported an average mammographic density in normal pre-menopausal women with an average age of 44.75 years of 28.7% and 23.64% in post-menopausal with an

average age of 55.95 years.¹⁸ Another study reported an average density in BC patients with an average age of 57.4 years of 36.7%, while the control group showed 30.6% with an average age of 56.8 years.¹⁹ These data showed that our study subjects had a higher average mammogram density than previous studies. Martin and Boyd proposed a hypothesis that the biological occurrence of mammogram density is determined by cell proliferation (mitogenesis) and cell damage caused by mutagens (mutagenesis). Both mitogenesis and mutagenesis are affected by many factors, including age, reproductive status, endogenous hormones and growth factors.²⁰ The high-density mammogram data in this study support the hypothesis that mammogram density is one of the risks for BC.

DNA polymorphisms *XbaI* and *PvuII* in *ESR1*

Estrogen is a mitogen that affects physiological processes including cell growth. Estrogen is found in breast tissue and affects the regulation of cell growth. High estrogen levels are also reportedly associated with an increased risk of BC. Estrogen mostly exerts its cellular action through binding its receptor. Between the two forms of estrogen receptor α and β , estrogen receptor α plays an important role in BC because of its high prevalence in breast tissue.²¹ The *ESR1* gene, also known as *ESR α* , is located on human chromosome 6q25. *ESR1* is a core receptor that mediates the action of estrogen or other steroid hormones that regulate gene transcription, especially in estrogen-sensitive tissues such as breast, and plays a crucial role in the growth and differentiation of BC. Most research has focused on the *ESR1 PvuII* (C/T) and *XbaI* (G/A) polymorphisms in intron 1.²¹ Both variants have implications in

affecting transcription and gene expression. Some studies also indicate an increased risk of BC with polymorphisms at allele A and T (homozygous mutant) of *XbaI* and *PvuII*.²²

In this study, percentage of heterozygous (CT) and homozygous mutant (TT) of *PvuII* in breast cancer cases (86%) higher than controls (66%). The percentage of heterozygous (AG) and homozygous mutant (AA) of *XbaI* in breast cancer cases (96%) also higher than controls (82%). The average percentage density was higher in women with one or two copies of the *PvuII* p allele (CT/Pp and TT/pp, 49% and 48%, respectively) than in those with the CC/PP genotype (39%). However, this difference did not show statistical significance (TT vs. CC). Women with one or two copies of the *XbaI* x allele had higher mean percentage density (AG/Xx and AA/xx, 49% and 47%, respectively) than those with the GG/XX genotype (32%), and we detected a significant difference between AA and GG genotypes.

The results of our study are consistent with those published by van Duijnhoven.²³ Mammographic density was significantly higher in women with one or two copies of p (T) allele in *ESR1 PvuII*. Haplotype *ESR1* gene 1 (px/TA) was also associated with increased mammographic density while haplotype 2 (PX/CG) was associated with a decrease in density. On the whole subject is a breast cancer contained a high percentage and high mammographic density of heterozygous and homozygous mutant compared with homozygous wild type at *ESR1 XbaI* and *ESR1 PvuII*. The difference between the two groups which show high estrogen that effect mammographic density that is strong risk of breast cancer. Crandal *et al.* showed that genetic variation in sex steroids or estrogen receptors are in production in breast showed different degrees of proliferation that affect radiological picture as a mammogram density

differences.²² The existence of sex steroid metabolic enzymes and ESRs in breast tissue caused an activation of local estrogen that could potentially lead to reactive metabolites in breast tissue and may have a role in the initiation and promotion of carcinogenesis.

CONCLUSION

Our study showed that haplotype 1 (px/TA) of *ESR1* gene is associated with high sensitivity to estrogen and reflects an increase of mammographic density. Haplotype 2 (CG/PX) is associated with lower sensitivity to estrogen and reflects a decrease of mammographic density. Our findings support the view that *ESR1* polymorphisms may affect BC risk through differences in breast density.

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