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# Prevalence of estrogen receptor α *Pvu*II and *Xba*I polymorphism in population of Polish postmenopausal women

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**Abstract:** Numerous data indicate that polymorphism of estrogen receptor α (ERα) may predict lipid levels, lipid response to hormone replacement therapy (HRT), myocardial infarction risk, bone fracture risk, bone mineral density (BMD) and changes in BMD over time. In this study we aimed to evaluate distribution of ERα *Pvu*II and *Xba*I genotypes in population of Polish postmenopausal women qualified to different protocols of HRT. Subject of the study were 64 consecutive postmenopausal women aged from 45 to 65 years (mean 56.6) assigned to HRT. ERα *Pvu*II and *Xba*I polymorphism was determined by PCR-restriction fragment length polymorphism (RFLP). The absence of *Pvu*II and *Xba*I restriction sites were indicated by "P" and "X" and presence by "p" and "x", respectively. *Pvu*II genotype was distributed as follows: PP 17.2% (n=11), Pp 50% (n=32), pp 32.83% (n=21). Frequency of *Xba*I genotype was: XX 6.25% (n=4), Xx 34.4% (n=22), xx 59.4% (n=38). Four haplotypes with following frequencies were recognized: PX 17.3%, px 47.4%, Px 24.4% and pX 10.9%. Prevalence of estrogen receptor α *Pvu*II and *Xba*I polymorphisms in Polish women is similar to previously studied population.

**Key words:** Estrogen receptor - ER polymorphism - *Pvu*II - *Xba*I

## Introduction

In humans, estrogen influences many physiological processes, which include not only reproduction, cardiovascular health, bone integrity, but also cognition and behavior. Taking into consideration this widespread role in human health it is not surprising that estrogen is implicated in the development and progression of numerous diseases, among which there are many types of cancers (breast, ovarian, colorectal, prostate, endometrial), osteoporosis, neurodegenerative diseases, cardiovascular diseases, insulin resistance, lupus erythematosus, lupus nephritis, endometriosis, obesity and as it was shown in some recent studies- even occurrence of graft-versus-host disease [1-10].

There are several different mechanisms through which estrogens induce cellular changes. The most important estrogens action is binding to its receptor (ER). Estrogens diffuse into the cell and bind to ER located in the nucleus. This complex binds to estrogen response element sequences directly or indirectly through protein-protein interactions with activator protein 1 (AP1) or SP1 sites in the promoter region of estrogen-responsive genes. It results in recruitment of coregulatory proteins (coactivators or corepressors) to the promoter, increased or decreased mRNA levels and associated protein production, and a physiological response. There are evidence that estrogen can induce cellular response through ER situated in plasma membrane or through no-ER plasma membrane-associated

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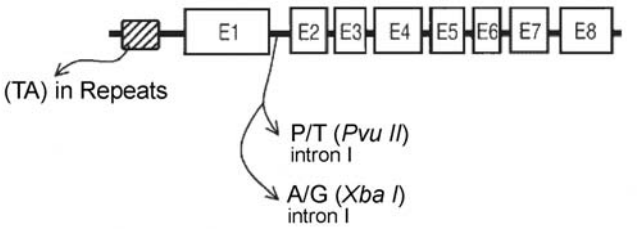


**Fig. 1.** Structure of polymorphisms *Pvu*II and *Xba*I in human estrogen receptor α gene. Coding exons (E) are in boxes [14, modified].

estrogen binding proteins, resulting in increasing levels of $Ca^{2+}$ or NO and kinase activation [11].

ER exists in two main forms, ERα and ERβ, encoded by separate genes, ESR1 and ERS2 respectively, found in different chromosomes. The ESR1 is located on chromosome 6q25.1 [47,72]. The ESR1 gene is large, encompassing 140kb of DNA, includes 8 exons, encodes a protein of 595 amino acids with a molecular weight of about 66 kDa [14]. The first intron of a gene, like the promoter, usually contains a larger number of regulatory sequences than other introns. Several sequence variations or single-nucleotide polymorphisms (SNPs) in the ERα gene have been identified and found to be associated with either an increased or a decreased risk of various diseases. The best characterized SNPs of ERS1 are the *Pvu*II and *Xba*I restriction site polymorphisms, both located in the first intron (Figure 1). The polymorphisms, c454-397T→C and c454A→G, are 397 and 351 bp upstream of exon 2 and have been described by the name of detecting restriction enzyme, *Pvu*II or *Xba*I, or their reference ID numbers, rs2234693 and rs9340799, respectively [15,16]. Possible functional mechanisms attributed to these polymorphisms include a change of ERα gene expression by altering the binding of transcription factors and influence on alternative splicing of ERα gene.

In different studies, these polymorphisms have been associated with several pathologic conditions such as breast and prostate cancer, osteoporosis, Alzheimer's disease and cardiovascular diseases [1,2,5,9,17,18,19]. Results are still conflicting and molecular mechanisms by which these polymorphisms influence receptor activity are as yet unclear. *Pvu*II and *Xba*I RFLPs lie in an intronic and apparently nonfunctional area of the gene and, as would be expected by two polymorphisms separated by 50 base pairs, are in strong linkage disequilibrium.

In this study we aimed to evaluate distribution of ERα *Pvu*II and *Xba*I genotypes in population of Polish postmenopausal women qualified to different protocols of HRT.

**Table 1.** Frequency distribution of estrogen receptor α *Pvu*II and *Xba*I polymorphisms in studied female population

| Genotypes | Number of subjects | % |
|---|---|---|
| PP | 11 | 17.2 |
| Pp | 32 | 50 |
| pp | 21 | 32.8 |
| XX | 4 | 6.25 |
| Xx | 22 | 34.4 |
| xx | 38 | 59.4 |

## Materials and methods

**Patients.** The study population comprised 64 white postmenopausal women aged 45-65 years (mean 56.6) from the Lublin region attending outpatient clinic. Women were considered postmenopausal if they had no menstruation for at least 12 months with no other obvious physiological or pathological cause or bilateral oophorectomy. Body mass index of the participant ranged between 20.3 and 46.9 (mean 29.5). Eleven (17.2%) of women included into the study were smokers. Exclusion criteria included cardiovascular, endocrinological, kidney, liver and any other serious disorders. Women did not receive any hormonal treatment during the last 1 year prior to study.

**Examination of polymorphisms.** Genomic DNA was extracted from the peripheral leukocytes using Genomic Prep Blood DNA isolation kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacture's instruction. *Pvu*II and *Xba*I polymorphisms were analyzed by polymerase chain reaction restriction fragment lengths polymorphism (PCR-RFLP). A 1.372 kb DNA fragment that contains the 2 polymorphic site was amplified using forward and reverse primers 5'CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3' and 5' TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA 3'. PCR was performed through 30 cycles by the following steps: denaturation at 64°C for 60 s; annealing at 50°C for 60 s; and extension at 72°C for 90 s. PCR products were digested with the restriction endonucleases *Pvu*II and *Xba*I (Promega, Madison, WI, USA). Digested products analyzed on 2% agarose gel stained with ethidium bromide. Heterozygous Pp genotype exhibited fragments 1372, 936 and 436 bp lengths and heterozygous Xx genotype exhibited fragments 1372, 982 and 390 bp lengths. Capital P or X represent the absence of restriction site while lower-case p or x indicate the presence of restriction site.

**Ethical issues.** The study was approved by the Ethics Committee of the Medical University of Lublin and written consent was given by each participant.

**Statistics.** Genotype distribution of the polymorphism was tested for Hardy-Weiberg equilibrium by $\chi^2$. Linkage disequilibrium resulting from the non-random association of the genotypes was also assessed by $\chi^2$. A p-value less than 0.05 was considered significant. Statistical analysis was performed with Statistica Statsoft version 6.0 software.

**Table 2**. Comparison of frequency of *Pvu*II-*Xba*I haplotypes of the human estrogen receptor α gene in different ethnic groups

| Ethnic group | Place of study | No.of subjects | *Pvu*II and *Xba*I haplotypes | | | | Study |
|---|---|---|---|---|---|---|---|
| | | | px | PX | Px | pX | |
| Asians | Japan | 238 | 54.5 | 18.7 | 26.5 | 0.3 | Kobayashi *et al.*[22] |
| Asians | Japan | 2238 | 59.4 | 18.3 | 22.3 | 0 | Yamada *et al.* [68] |
| Asians | Korea | 598 | 57.7 | 18.5 | 22.3 | 2.3 | Han *et al.* [21] |
| Caucasians | Denmark | 454 | 53.0 | 33.7 | 13.3 | 0 | Bagger *et al.* [53] |
| Caucasians | Netherlands | 1100 | 53.0 | 36.1 | 10.9 | 0 | van Meurs *et al.* [27] |
| Caucasians | United Kingdom | 206 | 56.1 | 33.5 | 9.2 | 1.2 | Albagha *et al.* [52] |
| Caucasians | Italy | 610 | 52.1 | 40.9 | 5.7 | 1.3 | Becherini *et al.* [28] |
| Caucasians | Canada | 662 | 54.9 | 35.6 | 9.5 | 0 | Patel *et al.* [12] |
| African Americans | United States | 19 | 36.8 | 50.0 | 13.6 | 0 | van Meurs *et al.* [27] |
| Caucasians | Poland | 64 | 47.4 | 17.3 | 24.4 | 10.9 | Jakimiuk *et al.* [this study] |

## Results

In our studied population *Pvu*II genotype was distributed as follows: PP 17.2% (n=11), Pp in 50% (n=32), pp 32.8% (n=21). Frequency of *Xba*I genotype was: XX 6.25% (n=4), Xx 34.4% (n=22), xx 59.4% (n=38). The distribution of genotypes was in Hardy-Weinberg equilibrium. As expected, linkage disequilibrium between *Pvu*II and *Xba*I polymorphisms was observed ($\chi^2$=32.7, df=2, p< 0.000001). After combining the two polymorphisms, four haplotypes with following frequencies were recognized: PX 17.3%, px 47.4%, Px 24.4% and pX 10.9%.

Table 1 shows the allele frequencies and heterozygosity index for intron *Pvu*II and *Xba*I polymorphisms in our population.

## Discussion

As it is presented in Table 2, the distribution of *Pvu*II and *Xba*I polymorphisms was very similar to what was previously reported in Caucasian populations of European ancestry and differed significantly from what was observed in populations of Asiatic ancestry [20-26]. Asian populations showed an increased frequency of the Px haplotype and a reduced frequency of the PX haplotype with respect to Caucasian populations of European ancestry, while in an African population haplotype px was present at a lower frequency [27]. In fact, a differential degree of linkage disequilibrium among different ethnic populations may partly explain previous discrepancies among ERα polymorphism studies [28].

Haplotype pX was not observed in the majority of studies, whereas haplotype Px was detected, albeit in a low frequency. This frequency of haplotypes may result from the disequilibrium which is not complete and may be due to recombination or multiple mutations which have occurred between or at these two polymorphic sites.

There are a lot of evidences that ERα gene polymorphism influence many physiological processes in humans, women in particular, as well as may be the etiopathological factor of various diseases.

The PX haplotype may be important in regulating not only the onset, but also the end of high tissue estrogen exposure during the lifetime of an individual. One group of investigators has suggested that ERα gene polymorphisms, in particular *Pvu*II, may affect the age of menopause [29,30]. In that study, the P allele showed a dose-effect relationship with a 0.5 year earlier onset of natural menopause per each copy of the P allele and the risk of surgical menopause was higher, by 2.4-fold, in women carrying the PP genotype as

compared with pp homozygotes. Two other studies, carried out in a Japanese and Dutch population, did not replicate this finding [31,32].

The age of menarche was associated with the *Xba*I and possibly the *Pvu*II polymorphism of the ERα gene in healthy adolescent Greek girls. In particular, menarche occurred 6 months later in girls with the AA genotype of the 351 A→C polymorphism than in girls with AC or CC genotypes and tended to occur later in TT homozygotes of the 397T→C polymorphism than in TC and CC genotype carriers. *Xba*I XX homozygotes or, in more general terms, subjects homozygous for the PX haplotype seem to have a modest delay in the age of menarche. The effects of *Pvu*II are not as clear on their own as the effects of *Xba*I. It may reflect the strong linkage disequilibrium with *Xba*I and the PX haplotype shows the strongest association with the age of menarche. The biological pathway for *Xba*I and *Pvu*II that may affect the age of menarche is unknown. Restriction sites of both polymorphisms are located in the intron 1 of the ERα gene. Some introns contain regulatory sequences such as enhancers, which means binding sites for elements that regulate the level of gene expression and thus also affect protein synthesis [14,33,34]. The observed association may reflect linkage disequilibrium with some other functional polymorphisms in the *Xba*I vicinity. Regardless of the exact mechanism, if ERα gene polymorphisms can alter the estrogenic biological activity at the cellular level, this may influence the maturation of the hypothalamic-pituitary-gonadal axis, which determines the onset of menarche.

Salmén suggested that the PP genotype of the *Pvu*II polymorphism is a relatively estrogen-insensitive genotype and that women with the p allele may benefit more from the protective effect of the hormone replacement therapy on fracture risk and atherosclerotic severity than women with the pp genotype [25,35]. These results stress the importance of estrogen receptor genotype and suggest that estrogen receptor has potential to explain recent conflicting data on estrogen replacement therapy and cardiovascular disease susceptibility in women.

Data regarding the influence of genetic variations in ERα gene are contradictory. Numerous studies also report an association between estrogen therapy and a lower risk of cardiovascular disease. Polymorphisms in ERα that link it to severity and risk of coronary artery disease have been identified. In the Framingham Heart Study, the ESR1 *Pvu*II polymorphism is associated with an increased risk of myocardial infarction in men and with blood pressure variation in men [7]. In addition, the haplotype encompassing both ESR1 *Pvu*II and *Xba*I polymorphisms is associated with an increased risk of myocardial infarction and ischemic heart disease in postmenopausal women in the Rotterdam Study [36]. Nordström demonstrated that the presence of the p allele was associated with the increased risk of aortic valve sclerosis [37]. ESR1 polymorphisms (pp genotype) are associated with increased HDL cholesterol levels in postmenopausal women with coronary disease in response to estrogen therapy or HRT [38]. Moreover, *Xba*I polymorphism (XX) was associated with an increasing risk of coronary heart disease in postmenopausal women with familial hypercholesterolemia [39]. ERα is expressed in vascular endothelial cells, vascular smooth muscle cells and cardiomyocytes [11]. ERα might be involved in some elements of atherosclerotic plaques formation which comes from observation that estradiol reduces the histological complexity of plaques in the compound gene-targeted animals. On the other hand, several studies showed lack of association between ERα gene polymorphism and increased risk of cardiovascular diseases in women after the menopause [7,40,41].

The *Pvu*II polymorphism is associated with increased breast cancer risk, as well as risk for other disease in which estrogen is implicated. XX homozygotes are protected from breast cancer and endometrial cancer [42,43]. A non-significant trend for protection against endometrial cancer has also been seen for PP homozygotes [43]. It is unknown to what extent these findings reflect differential cumulative exposure to estrogens due to different estrogen levels, different expression of ERs, different time duration of the estrogen exposure, or a combination of these factors. Part of this protective effect may be mediated by a delayed menarche and this may also be the case for *Xba*I XX homozygotes [44]. A delayed menarche is a strong protective factor against breast and endometrial cancer and is related to reduced lifetime estrogen exposure of the target tissues, as has been shown in several studies.

Studies in ERα knockout (αERKO) mice demonstrated that ERα is required for normal mammary gland development, because adult αERKO females exhibit mammary glands lacking ductal development end terminal end buds, resembling prepubertal stage [45]. Loss of ERα in Neu/ErbB2 knock-in transgenic mice resulted in lack of tumor development, while these mice develop mammary glands tumors with long latency period [46]. Another data suggesting function ERs in tumor development and progression is that mammary tumor incidence is minimized with loss of ERα expression in the C3/T (AG) mouse model of estrogen-promoted mammary tumorigenesis [47].

The ER is present in approximately two-thirds of human ovarian tumors, not only of epithelial but also stromal origin [11]. ERα plays role in the estrogenic actions in the prostate which is supported by the observation that adult αERKO mice treated with DES do not develop the prostatic squamous metaplasia observed in DES-treated normal mice [8]. As it was

proved, in about 70-80% endometrial carcinomas ER is expressed [11]. The *Xba*I RFLP genotype XX has been associated with a decreased risk of endometrial cancer in a Northern European study group [43].

In one study it had been shown that the allelic variants of the ERα gene are associated with endometriosis, adenomyosis and leiomyomata [3]. The frequency of PP genotype was low in the patients with endometriosis, adenomyosis and leiomyomata, whereas it was high in the disease-free patients, suggesting that the P allele is protective against endometriosis, adenomyosis and leiomyomata. Similar results were reported in Greek patients with endometriosis, who had significantly lower frequency of PP genotype in the ERα gene compared with that in the control group [48].

Although numerous studies have investigated a possible association between ER, including ESR1 *Pvu*II and *Xba*I polymorphisms and osteoporotic risk, it remains controversial and requires further investigation. A role for the ER in the development of osteopenia and in skeletal growth and maintenance is exemplified by the case of one estrogen-resistant male patient carrying a loss-of-function mutation in gene encoding ERα [49].

Estrogens are known to be important for preservation of bone mass in females during menopause. Several studies have shown that PP genotype has higher bone mineral density than the Pp and pp genotypes and Caucasian women have observed no association between ERα gene and bone mineral density [22,24,26,50]. These findings suggest that the local estrogenic action is more potent in women carrying the P allele than those carrying the p allele. This is also supported by the finding that the PP genotype has a higher risk of premenopausal hysterectomy and earlier onset of natural menopause due to menorrhagia and fibroids than the Pp and pp genotypes [30]. These findings however contradict those of the studies that showed that women carrying the PP genotype have a lower risk for estrogen-dependent uterine disease. Indeed, endometriotic implants and adenomyotic tissues express a reduced amount of ER protein [25,51].

Haplotype px showed a significant association with decreased BMD at the lumbar spine in women, while haplotype PX was associated with increased LS-BMD and haplotype Px did not show any association [27]. In contrast, two other studies found the *Pvu*II-*Xba*I haplotype Px to be associated with decreased BMD, whereas others showed no association [21,52,53]. In Japanese women, the ERα *Pvu*II PP genotype is associated with low BMD, whereas in studies on a largely Caucasian population from the USA or from Finland, the *Pvu*II pp genotype is associated with low BMD [22,26,54].

There is a hypothesis that ESR1 polymophisms leads to a difference in bone growth, which might be explained by a genotype-dependent estrogen sensivity locally at the site of bone growth. In support of this hypothesis there is an association of ESR1 polymorphism with stature [36].

ERα is an isoform more highly expressed than ERβ in mature human adipocytes and the only one expressed in preadipocytes. ERα implication in adipocyte growth and proliferation and - consequently- obesity development, is supported by observation that αERKO mice possess 50-100% more adipose tissue than wild-type controls, which is result of increase in adipocyte number and size [11].

The *Xba*I polymorphism has been found to be significantly associated with upper-body obesity in middle-aged persons [55]. Other observations indicated an association between ERα intron I RFLPs and height or body mass index [56,57]. In one study the association of the A→G polymorphism or the combination of the T→C and A→G polymorphisms with not only a greater BMI, but also larger % fat mass, FM, waist circumference and WHR in middle-aged women had been shown [55].

The ESR1 *Pvu*II polymorphism is associated with a higher risk of stroke in men [58]. Both ERα and ERβ are found in various regions of the human brain, including the hypothalamus, hippocampus, cerebral cortex, midbrain, brainstem and forebrain, and ER-mediated effects are thought to provide neuroprotection, with suggestion that ERα is more important in this process [59]. The expression of ERα appears to dominate in the hypothalamus and amygdale, suggesting that this subtype is an important mediator of effects of estrogen on emotional and reproductive behavior [59,60]. There are suggestions based on some clinical studies that ESR1 may influence various aspects of personality in women and an association between ERα polymorphism and personality traits (measured by means of temperament and character inventory) as well as with conduct disorder had been proved [61]. It had been found that in women ERα haplotypes created by the *Pvu*II and *Xba*I polymorphisms increased the likehood of having an anxiety but not of having a depressive disorder [19]. The RFLPs for *Pvu*II and *Xba*I in intron 1 have been associated with likelihood of developing Alzheimer's disease, again with differences in the specific allelic associations being seen between Japanese and European subject groups [17,18].

From clinical observations that the onset of symptoms of rheumatoid arthritis is often associated with menopausal transition came the idea of role of ERα polymorphism [62]. Indeed, there was a significant difference between six genotype groups in female rheumatoid arthritis age at onset [63].

ERα gene polymorphism is probably one from the genetic factors contributing the development of clini-

cal heterogeneity and sexually dimorphic manifestations of Lupus Nephritis, which is an autoimmune disease with high prevalence among childbearing women and its manifestation differs in women and men. The PpXx genotype may be associated with the susceptibility of SLE in male [35].

Polymorphism of ERα (presence of PX haplotype) in the patients genotype associates with occurrence of acute graft-versus-host disease and lower overall survival, following the correction for known clinical and genotypic risk features [6].

Recently, a possible relation of ERα polymorphisms with male infertility has been reported in Greek and Japanese populations [64,65]. The *Xba*I RFLP showed a significant association with infertility, whereas this association was not found for the *Pvu*II polymorphism.

Polymorphisms in the intron 1 region of the ERα gene are being linked to an increasing number of human pathologies. It is unclear how the anonymous intronic polymorphism of the ERα gene influences its protein function. Their position in an intron near the gene promoter might suggest a possible role in either transcription regulation (ERα gene transcription can occur from alternative promoters) or mRNA processing and stability (numerous alternate splice variants of the primary ERα transcript are reported in human cells).

Recently, Herrrington noted that T→C transition associated with loss of *Pvu*II site (P allele) results in a potential binding site for myb transcription factors that, in the presence of B-myb, is capable of augmenting in vitro transcription of a downstream reporter construct tenfold [66]. Thus, in some settings, the presence of the P allele might amplify ERα transcription. An alternative explanation is that the to polymorphisms in intron 1 may be in linkage disequilibrium with causal polymorphisms elsewhere in the ERα gene or, less likely, in an adjacent gene [14]. In this regard, it has been well established that intron 1 polymorphisms are in linkage disequilibrium with the upstream TA repeat polymorphism in the promoter region of the ERα gene (Fig. 1) [28].

A promising and interesting field in studies of ER gene polymorphisms comes from their possible pharmacogenomic implications in determining the response to hormone replacement therapy. The positive influence of postmenopausal HRT on bone mass is well established and its antifracture effect is widely accepted. However, it seems that there are some women who do not respond to RT, and a possible explanation for their less favorable responsiveness to estrogen may be related to ER genotypes. This issue has been investigated in a few studies performed in different ethnic populations, with conflicting results. There is also very interesting evidence that *Pvu*II polymorphism in the ERα gene influences individual response to HRT with regard to cholesterol levels and other cardiovascular markers [67]. If, in the future, we could determine women with a greater or lesser response to HT, the treatment could be directed to women most able to benefit from it.

In the future, studies will have to further examine the spectrum of variation across the ESR1 gene and to establish whether any significant association results can be replicated both within and between ethnic groups. The number of studied groups should be large enough in order to test gene-gene and gene-environment interactions that may potentially be involved at the interface of ESR1 variation and human diseases. It would be worthwhile to conduct studies in other heterogeneous populations in order to assess the replication validity of present findings.

We hope that future studies will further clarify the role of ERα in the development and progression of different diseases and may help to identify diagnostic or therapeutic markers. It would allow tailoring the type and duration of hormone therapy based on genetic profile and provide a means to optimize treatment for each patient.

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