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A N I N T E R N A T I O N A L J O U R N A L

Prevalence of Estrogen Receptor Alpha *Pvu*II (c454-397T>C) and *Xba*I (c454A>G) Polymorphisms in a Population of Brazilian Women

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ABSTRACT

The aim of this work was to study the estrogen receptor alpha (ERS1) PvuII and XbaI gene polymorphisms prevalence in randomly selected women population from Rio de Janeiro and Espírito Santo states in Brazil by polymerase chain reaction restriction fragment lengths polymorphism (PCR_RFLP) methodology. It was shown that Rio de Janeiro women exhibited a significantly different prevalence of XbaI polymorphism comparing to Espirito Santo women. Nonetheless, similar prevalence of PvuII polymorphism was found in both women´s populations. Moreover, a strong linkage disequilibrium was observed between these SNPs reinforcing the hypothesis of differential pattern of inheritance observed on such populations.

Key words: Polymorphism, Estrogen Receptor, Brazilian Women, Prevalence, Linkage disequilibrium

INTRODUCTION

Estrogen is a steroidal hormone that influences many physiological processes, which include female reproduction, cardiovascular control, and bone integrity. Due to its lipophylic characteristic, estrogen diffuses through plasmatic membrane and binds to its receptor (ER), member of the nuclear receptor superfamily, located in the nucleus and cytoplasm, forming a estrogen/ER complex. This complex binds to estrogen response element sequences in the promoter region of estrogen-responsive genes, resulting in recruitment of co-regulatory proteins (co-activators or co-repressors) to the promoter and gene expression regulation (Jensen and Jacobsen, 1962; Reviewed in Nilsson et al., 2001). Two main isoforms of ER (called ERα and ERβ) are known, which are encoded by separate genes. ESR1 gene is located on chromosome 6q25.1 whereas ESR2 is located on chromosome 14q22-24 (Kuiper et al., 1997). ESR1 gene encopass 140kb of DNA composed by eight

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exons, encoding a 595 amino acids protein with a molecular weight of about 66 kDa. The first intron of a gene, like the promoter, usually contains a larger number of regulatory sequences than otherintrons. Several single nucleotide polymorphisms (SNPs) have been identified on ERS1 and some of them were associated with either an increased or a decreased risk of various diseases (Yoshidome et al., 2000; Gennari et al., 2005). The best characterized SNPs of ESR1 are the c454-397T>C and c454A>G site polymorphisms, both located in the first intron (Fig. 1). These polymorphisms are 397 and 351 bp upstream of exon 2 and have been described by the name of detecting restriction enzyme, PvuII or XbaI, or their reference ID numbers, rs2234693 and rs9340799, respectively (Hill et al., 1989; Yaich et al., 1992).

The PvuII and XbaI SNPs of the ESR1 gene were found to be associated with various pathological conditions, including cardiovascular disorders (Lawlor et al., 2006), venous thromboembolism (Straczek et al., 2005), miscarriage (Silva et al., 2010), and severe pre-eclampsia (Molvarec et al., 2007). Also, several estrogen-dependent characteristics such as the onset of menopause (Weel et al., 1999), lumbar spine bone mineral density (BMD), vertebral bone area and vertebral fracture risk in post-menopausal women (van Meurs et al., 2003), as well as waist circumference (Fox et al., 2005), blood pressure (Peter et al., 2005), coronary reactivity (Lehtimaki et al., 2008) and lipid profile (Molvarec et al., 2007) have been described.

The transcriptional regulation of ERα is poorly understood and only a small number of regulatory regions have been well characterized (Penolazzi et al., 2000). A possible functional mechanism attributed to PvuII and XbaI polymorphisms includes a change of ERα gene expression by altering the binding of transcription factors. This study aimed both to evaluate the populational distribution of ERS1 PvuII and XbaI genotypes of Brazilian adult women and to analyze de linkage between these SNPs in same women.

MATERIALS AND METHODS

Patients

Four hundred and nineteen adult Brazilian women ethnically mixed, aged 27-91 years (mean 62.5) were selected from the states of Rio de Janeiro and Espírito Santo in the Brazilian southeastern region between the years of 2007 and 2009. All of them consented to donate biological specimens for this study. Due to technical problems, DNA extraction of 23 women could not be achieved, decreasing the population to 396 patients. Genotyping was successfully done for 379 women for the PvuII polymorphism and 371 for the Xba polymorphism. The study was approved by the Ethics Committee of the Federal University of Espírito Santo and written consent was given by each participant.

Polymorphisms analysis

Genomic DNA was extracted from the specimens using a modified protocol of Vogelstein and colleagues (Goelz et al., 1985), briefly, biological specimens were incubated for two days in a solution of Proteinase K in 10% of Sodium-Dodecil-Sulfate (SDS) at 60ºC. A solution of formaldehyde and chloroform (pH 9.0) was added and after centrifugation (18,000g for 2 minutes), supernatant was collected and DNA was precipitated with a solution of ammonium acetate and ethanol. Genomic DNA samples were finally washed with 70% ethanol and resuspended in ultra-pure water. The PvuII and XbaI polymorphisms were analyzed by polymerase chain reaction restriction fragment lengths polymorphism (PCR-RFLP) (Bittencourt-Oliveira et al., 2009). A 109kb DNA fragment - that contained two polymorphic sites - was amplified using forward and reverse primers 5’ CTGTGTTGTCCATCACTTCATC 3’ and 5’ CCATTAGAGACCAATGCTCATC 3’. PCR was performed through 30 cycles by the following steps: desnaturation at 95°C for 60 s; annealing at 52°C for 30 s; and extension at 72°C for 30 s. PCR products were digested with the restriction endonucleases PvuII and XbaI (Invitrogen, Carlsbrad, CA, USA). Digested products were run onto 10% polyacrilamide gel stained with silver nitrate posteriorly. Heterozygous Pp genotype exhibited 119, 78 and 41 bp lengths and heterozygous Xx genotype exhibited fragments 119, 88 and 31 bp lengths (Fig. 2). Capital P or X represent the absence of restriction site while lower-case p or x indicate the presence of restriction site. Representative samples were confirmed by sequencing.

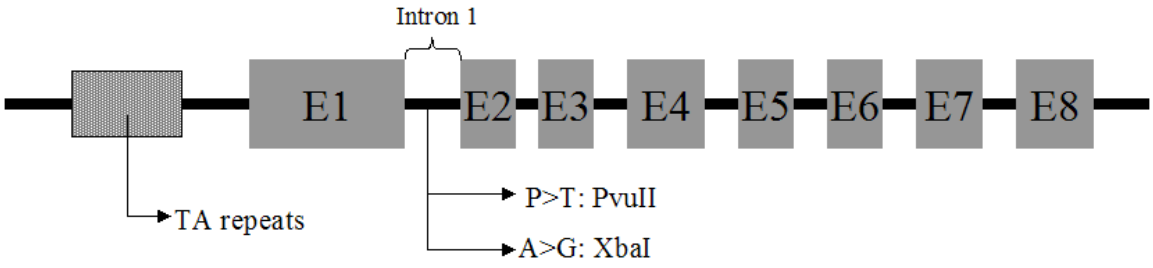

Figure 1 - Structure of polymorphisms TA, PvuII and XbaI in human estrogen receptor α gene. Exons are represented by the grey boxes and introns by the black line between the exons. TA tandem repeat polymorphism is represented in the grey box before Exon 1 (E1).

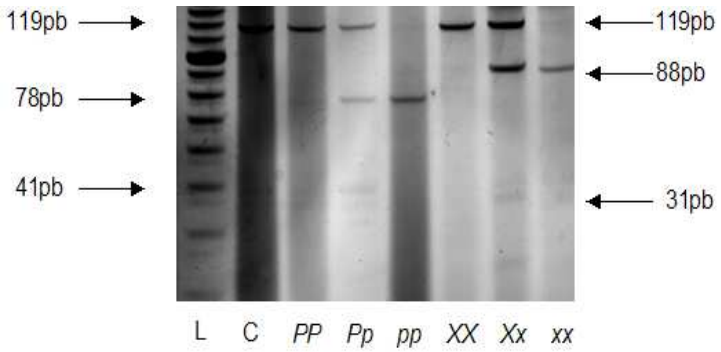

Figure2 - PCR-RFLP representative gel 10% polyacrylamide gel stained with silver nitrate representing the genotypes observed in this study. L: ladder 10 bp; C: not digested control.

Statistical analysis

Genotype distribution of the polymorphism was tested for Hardy-Weiberg equilibrium by $\chi 2$. Haplotypes frequencies were estimated by using the software THESIAS (Tregouet et al., 2007) based on the maximum likelihood model and the SEM algorithm. Significance level of linkage disequilibrium resulting from the non-random association of the genotypes was assessed by $\chi 2$ using the software Arlequin 3.11 (Excoffier et al., 2005), which was also used to calculate the standardized disequilibrium values D' (Lewontin, 1998). The distribution of the genotypes in two age groups (less than 60 years old, and 60 years old or more) were also assessed by $\chi 2$. A p-value less than 0.05 was considered significant. Statistical analysis was performed with Graph Pad Prism version 5.0 software.

RESULTS

In the population genotyped for *Pvu*II, this polymorphism was distributed as follows: PP16.3% (n=62), Pp in 59.6% (n=226), pp 24.1% (n=91). The frequency of *Xba*I genotype in the population was XX 14.8% (n=55), Xx 79.2% (n=294), xx 6.0% (n=22). The distribution of genotypes was not in Hardy-Weinberg equilibrium for PvuII ($\chi 2$ p=0.025) and XbaI ($\chi 2$ p<0.0001). Table 1 shows the genotype frequencies of the ESR1 intronic polymorphisms *Pvu*II and *Xba*I in the studied population.

As expected, there was linkage disequilibrium between the *Pvu*II and *Xba*I polymorphisms ($\chi 2$=172.18, df=2, p< 0.000001, D'=0.5901 R^2=0.2446). After combining the two polymorphisms, four haplotypes with following

frequencies were recognized: PX 37.1%, px 38.2%, Px 7.8% and pX 16.7%. Table 2 shows the haplotype frequencies of the ESR1 intronic polymorphisms *Pvu*II and *Xba*I in the studied population. The population was segregated by the geographic region and the following allele and genotype frequency in Espírito Santo state population were observed: X: 56.7%; x: 43.3% XX: 15.6%; Xx: 82.0% and xx: 2.4%. Woman from Rio de Janeiro showed a significantly different prevalence of each allele and genotype as X: 46.1%; x: 53.9%; XX: 11.6% Xx: 68.8% and xx: 19.4% ($\chi 2$ $p<0.0001$ data not shown). Performing the same analysis for the PvuII polymorphism, the following allele and genotype for PvuII prevalence in Espírito Santo population were observed: P: 46.6%%; p: 53.4% PP: 17.4%; Pp: 58.4% and pp: 24.2%; for Rio de Janeiro population, these were as follows: P:44.6%; p: 55.4% PP: 12.2%; Pp: 64.8% and pp: 23.0%. No significant differences in genotype prevalence was observed between the groups ($\chi 2$ p=0.48 data not shown).

Analysis was also made to find out if genotypes distribution in the two age groups was distinct. $\chi 2$ analysis showed no significant difference in the distribution of both PvuII ($\chi 2$ p=0,26) and XbaI ($\chi 2$ p=0,90) SNPs when compared to a younger group (less than 60 years old) and an older group (more than 59 years old).

Table 1: Frequency distribution of estrogen receptor polymorphisms PvuII and XbaI on the population.

Genotype	Number of Subjects	%
PP	62	16,3
Pp	226	59,6
pp	91	24,1
XX	55	14,8

Table 2 - Distribution of the nine possible genotypes for PvuII and XbaI polymorphisms on the studied population. Haplotypes derived from each genotype and their frequency as calculated by THESIAS software.

Genotype	Number of Subjects	Derived Haplotypes	Haplotypes	%
PPXX	27	PX PX	PX	37.1
PPXx	27	PX Px		
PPxx	2	Px Px	Px	7.8
PpXX	17	PX pX		
PpXx	182	PX px or Px pX	pX	16.7
Ppxx	8	Px px		
ppXX	7	pX pX	px	38.2
ppXx	70	pX px		
ppxx	11	px px		

DISCUSSION

In this study, the prevalence of P, p, X and x alleles in a Brazilian ethnic mixed population of adult women was evaluated, similar to what was already done for other gene polymorphisms (Lopes et al., 2007). Results for P vuII allele frequencies were similar to most of other studies around the world, although concerning the XbaI allele frequencies, the present data differed from the previous other studies (Jakimiuk et al., 2007; Gennari et al., 2005, Becherini et al., 2000; Long et al., 2005; Shearman et al., 2003; Demissie et al 2006 and Huang et al., 2006). The main difference of the women inserted in this one was the prevalence of the allele x which was very low for the experimental population.

A recent study comprising a population of 64 postmenopausal Polish woman showed similar results for the prevalence of the PP Pp and pp genotypes: 17.2%; 50.0% and 32.8%, respectively. The same study, however, showed significant differences in the prevalence for the XX, Xx and xx genotypes: 6,2%; 34,3% and 59,4%, respectively (Jakimiuk et al., 2007). For the Polish women, xx genotype was very frequent, while this genotype was observed only in 6% for Brazilian women.

Aléssio et al., 2007 reported the association between the ESR1 polymorphisms and deep vein

thrombosis in a Brazilian population with both genders (Aléssio et al., 2007). Simlar data were observed for the PvuII polymorphism prevalence in Brazilian population comparing to their results (PP: 17,5%, Pp: 49,6% and pp: 32,9%) while for XbaI polymorphism. Contrasting date were observed to these in the prersetn study (XX: 9,5%, Xx: 41,3% and xx: 49,2%). Another study conducted in Brazil, but with a post-menopausal women population, showed a higher prevalence of the x allele (X: 35%, x: 65%) differing X: 54,4%, x:45,6%) also, PvuII polymorphism prevalence did not show significant differences between their population (P: 42% p: 58%) and the present population (P: 46,3%, p: 53,7%) (Almeida et al., 2008). It might be worth to note that these studies were conducted in different regions of Brazil. The first one was in Rio Grande do Sul state, where there was a caucasian majority and the second one was in São Paulo state using afro-descendant and caucasian subjects. Taken together, one could conclude that the present observations for XbaI polymorphism differed from the previously published data in Brazil, while for PvuII polymorphism, the present results were in consonance to what has been already described in literature.

Segregation of the population by geographic region showed that the low prevalence of the allele x in the studied population was due to the influence of the woman from Espírito Santo state, where the following allele and genotype frequency were found: X: 56,7%; x: 43,3% XX: 15,6%; Xx: 82,0% and xx: 2,4%. These frequencies were significantly different from the observed in the population of from Rio de Janeiro: X: 46,1%; x: 53,9%; XX: 11,6% Xx: 68,8% and xx: 19,4%. Although a higher prevalence of xx genotype was observed in the women from Rio de Janeiro, this value was still under the commonly found in Brazilian women.

The THESIAS software was used to estimate the prevalence of the haplotypes based on the maximum likelihood model and the SEM algorithm (Tregouet et al., 2007). Interestingly, the less prevalent haplotype in the population was Px that appeared only in 7.8% of the subjects. Studies using Japanese (Yamada et al., 2002), Korean [Han et al., 1999] and Polish (Jakimiuk et al., 2007) women reported a prevalence for this haplotypehigher than 20%, while in UK (Albagha et al., 2001), Italy (Becherini et al., 2000) and Canada (Patel et al., 2000), this value was lower than 10%, as observed in the present study. Another interesting fact was that the usually rare pX haplotype was observed in 16.7% of the population, while in other studies, this value was rarely more than 2%.

Arlequin 3.11 (Excoffier et al., 2001) software was used to calculate the significance level of linkage disequilibrium between these two polymorphic sites. Results showed a strong linkage disequilibrium in this population, similar to commonly described in the literature (Becherini et al., 2000, Almeida et al., 2008, Yamada et al., 2002, Okura et al., 2003). This was obviously due to the short distance (46pb) between these polymorphisms, which minimized the probability of genetic rearrangements during the crossing-over phase of meiosis.

PvuII and XbaI polymorphisms are possible markers for several human diseases. These genotype variations have been implicated in the development and progression of numerous diseases, among which there are many types of cancers, osteoporosis, neurodegenerative diseases, cardiovascular diseases, insulin resistance, lupus erythematosus, lupus nephritis, endometriosis and obesity (Brandi et al., 1999; Dunning et al., 1999; Kitawaki et al., 2001; Liu et al., 2002; Shearman et al., 2006 and Tanaka et al., 2003). It is not clear how the intronic polymorphism of the ERα gene influences the receptor function but its positions in an intron, near the gene promoter suggests a possible role in either transcription regulation or mRNA processing and stability. It was recently shown that transition to P allele resulted in asite for myb binding, hence, the presence of this allele possible augments ERα transcription (Herrington et al., 2002). Another explanation to the observations of associations between these SNPs and human illness is a possible linkage disequilibrium between the PvuI and XbaII polymorphisms with other polymorphisms in the ERα gene,such as TA tandem polymorphism (Fig. 1) in the promoter region of ESR1 gene.

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