ESTROGEN RECEPTOR ALPHA GENE POLYMORPHISMS IN PATIENTS WITH LATE ONSET ALZHEIMER’S DISEASE

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Evidences have been gathered from several studies suggest that a mechanism involving an estrogen-signaling pathway may contribute to modulate risk for Alzheimer’s disease. It was demonstrated that estrogen up-regulates the expression of apolipoprotein E gene, which has a role in the metabolism of β-amyloid that is related to the progress of Alzheimer’s disease. Case-control studies have found an increased frequency of PvuII and XbaI polymorphisms in affected subjects. In this study we explore the possible association of different polymorphic forms of human α-estrogen receptor (ER-α) with the risk to late onset Alzheimer’s disease in north-west Iranian population.

We conducted a case-control study in a dataset of 160 LOAD patients and 163 healthy controls that have been matched in gender and age. To evaluate the PvuII and XbaI polymorphisms in Alzheimer’s disease we used PCR/RFLP method and genotype frequencies were statistically determined. The PCR products prepared from 21 AD cases and 20 healthy controls were randomly purified by ethanol precipitation and bidirectionally sequenced.

The frequency of normal and mutated alleles for PvuII and XbaI locuses respectively, in the LOAD group were significantly higher than those in the control group (P<0.001, OR=0.51, 95 % CI 0.35-0.74 for XbaI locus; P<0.001, OR=0.41, 95 % CI 0.3-0.57 for PvuII locus). This result suggests that

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**ERα XbaI and PvuII polymorphism is an additional risk factor for late-onset Alzheimer’s disease.**

*Key words:* Alzheimer’s disease, α-estrogen receptor, polymorphism, North-west of Iran

**INTRODUCTION**

The most frequent neurodegenerative disease that affects 20-30 million individuals worldwide is Alzheimer (Selkoe 2005). It is an untreatable, degenerative and lethal disease and usually diagnosed in aged people (Brookmeyer et al. 2007). Alzheimer is clinically characterized by a progressive loss of memory and cognitive functions in later life (Culp et al. 2003). Inheritance of Alzheimer’s disease (AD) has a complex pattern and several genes and environmental factors play roles in its pathogenesis. Early-onset (<60 years) familial AD (EOFAD) almost always inherited as a Mendelian disorder and late-onset AD (LOAD) has less apparent or no familial aggregation and usually occur later in life (≥60 years) (Gharesouran et al. 2013a). There are many genes that because of their potential to alter the risk for late onset Alzheimer’s disease (LOAD) have been investigated, but until now only variations in the apolipoprotein E (APOE) gene have been always found to be associated with AD, where frequency of the 14 allele in AD cases is 2-3 times more compared to non-demented controls (Farrer et al. 1997). The E4 allele of the apolipoprotein E (APOE) gene located on chromosome 19 is the only recognized susceptibility gene for this late onset Alzheimer’s disease (Gharesouran et al. 2013b).

Evidences gathered from several studies suggest that a mechanism involving an estrogen-signalling pathway may contribute to modulate risk for AD. A sex difference has been recognized in the prevalence of AD in women which cannot be exclusively accounted for by long life. There are some reports that the risk of developing AD and cognitive decline in women with a history of hormone replacement therapy (HRT) use during the postmenopausal period is lower (Paganini-Hill and Henderson 1996; Tang et al. 1996).

The relationship between sex and APOE is also highlighted by the observation that women bearing at least one of 14 alleles present an increased risk of developing AD compared to men of the same APOE genotype (Farrer et al. 1997). Experimental evidences suggest that estrogen protects neuronal cells against oxidative-stress, improves survival of hippocampal neurons, increases dendritic spine density on CA1 pyramidal neurons, and enhances cholinergic and serotonergic neurotransmission (Mcewen 2001). In rodent tissues it has been shown that estrogen modulates the expression of Apolipoprotein E gene (APOE) (Srivastava et al. 1997) and activates synaptic sprouting in response to injury throughout an APOE-dependent mechanism (Stone et al. 1998). Neuroimaging data showed that ERT increases cerebral blood flow and modulates brain activity (Maki and Resnick 2001).

Estrogen has been shown to act as a neuroprotectant and neurotrophic agent. In vitro circumstance estrogen can increase APP secretion and prevents Ab1-42 aggregation. It is one family of sex hormones and exerts many of its effects during the activation of nuclear receptors (estrogen receptors, ERα and β) (Enmark and Gustafsson, 1999). The detection of ERα in the regions of the brain affected by AD pathology demonstrates the local distribution of these receptors in the brain overlaps that of the neuro(Mcewen and Alves 1999; Mitra et al. 2003).

The most important genetic risk factor for late-onset AD is the ε4 allele of the APOE (Farrer et al. 1997). Previous studies showed a relation between ERα PvuII and XbaI
polymorphisms and the APOE ε4 allele in increasing the risk of AD (BRANDI et al. 1999; MATTILA et al. 2000). It is established that estrogen up-regulates the expression of apolipoprotein E (ApoE) gene (SRIVASTAVA et al. 1997) pathological lesions of AD, which has a role in the metabolism of b-amyloid related to the progress of Alzheimer’s disease. There are two polymorphic loci with only 50 bp apart in intron 1 of ER-α gene, Pvu II locus and Xba I locus (CASTAGNOLI et al. 1987). These polymorphic loci may influence the expression of ER-α gene. Several studies have examined the association between AD and ERα gene PvuII and XbaI polymorphisms. Some case-control studies have found an increased frequency of the PvuII and XbaI polymorphisms in AD subjects compared to controls (BRANDI et al. 1999; ISOE et al. 1997; JI et al. 2000). These findings suggest a role for ERα in the pathogenesis of AD. Studies in HeLa cells indicated that xp haplotype of ERα has higher expression than the XP one, but there was no significant difference between them (MARUYAMA et al. 2000).

Based on the above findings implying the potential importance of ER-α in the pathogenesis of AD and different frequency of related polymorphisms of the genes in various populations, we studied the distribution of ERα PvuII and XbaI polymorphisms in 160 AD patients as well as in 163 age and gender matched controls, in order to determine whether they influence the susceptibility on development of the disease in eastern Azerbaijan of Iran.

MATERIALS AND METHODS

Subjects sample Preparation

The study included 160 AD patients (women & men, the mean age 76.06±7.75 year, ranging from 65 to 99 and healthy control group included 163 healthy individuals with the same ethnicity to subject group (women & men, the mean age 75.29±6.75 year, ranging from 65 to 89 years) which were randomly selected from a diagnostic pathobiology lab. All Alzheimer subjects were diagnosed by expert clinicians according to the MMSE criteria (DOODY et al. 2001). The age of onset was above 65 years, and the sporadic form of the disease was ensured where no affected individuals were present in first degree relatives of subjects. All subjects included in the study were Azeri Turk originating from a limited population area in northwest of Iran. Samples of this study were used to evaluate several SNPs in other susceptible genes (GHARESOURAN et al. 2013a; GHARESOURAN et al. 2013b; GHARESOURAN et al. 2014).

DNA preparation and genotyping

Blood specimens were collected in sterile tubes containing EDTA and the DNA was extracted using the salting out method. The ERα gene polymorphism identification was determined by PCR/RFLP. Products of 1.3 kb long were obtained with a pair of 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'). The primer designing was carried out using online primer 3 software and Ensembl Genome Browser used for blasting. The PCR reaction was prepared in a total volume of 25 µl, containing 0.1 μg of genomic DNA, 0.01 μg each of primers, 2.5 μg of 10xPCR buffer (670 mM Tris-HCl pH 8.8, 160 mM (NH4)2SO4, 0.1%Tween-20), dNTP mix (10mM each), 50 mM MgCl2, Taq DNA polymerase (5000u/ml). After denaturation of template DNA at 94°C for 2 minutes, 30 cycles of PCR reactions were optimized and performed by denaturation at 94°C for 30 seconds, annealing at 68.5°C for 30 seconds and extension at 72°C for 50 seconds. The PCR products were digested with restriction endonucleases Pvu II and Xba I to identify the Pvu II and Xba I polymorphism, respectively. The
PCR products were digested using 2unit/µl of Pvu II and Xba I restriction enzyme in a total volume of 25µl in separate reactions, containing 5µl PCR product in supplied buffer. The mixture was incubated at 37°C for 24h. The digested PCR product was fractionated on 8% polyacrylamide gel and visualized after staining by AgN03. Capital letters (P or X) denoted the absence of the restriction site, and small letters (p or x) denoted its presence. The restriction endonucleases were purchased from Roche applied science. The purified PCR products from 21 AD cases and 20 healthy controls were randomly sequenced bidirectionally.

Statistical analysis

Statistical analysis was performed using the Sigma Stat 2.0 software. Allelic and genotypic frequencies were obtained by direct counting. Fisher’s exact test was used for differences in genotypes and haplotypes between the groups. Statistical significance was set at P< 0.05 and the odds ratio (OR) was calculated at 95% CI.

RESULTS

A total of 323 individuals were examined in the present study to evaluate the association of ERα PvuII and XbaI polymorphisms in ERα gene with AD using PCR/RFLP procedure. The patient and control groups were matched by age, gender status.

The statistical analysis of patients, genotypes and allele frequencies (Table 1) indicated a significant difference in ERα genotype and allele frequencies between the AD patients and healthy subjects. When PvuII and XbaI RFLPs were analyzed separately we observed an increased prevalence of both PP (P< 0.001) and XX (P= 0.007) alleles in individuals affected with LOAD. When we grouped AD patients according to different ERα genotypes it revealed that there were no significant differences in age at onset.

Table 1. Pvu II and Xba I polymorphism of ERα gene in the investigated groups.

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>AD patients(N)</th>
<th>Healthy controls (N)</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvuII Allele</td>
<td></td>
<td></td>
<td>Reference group</td>
<td></td>
<td>0.30-0.57</td>
<td>89%</td>
</tr>
<tr>
<td>P</td>
<td>166(51%)</td>
<td>101(31%)</td>
<td>&lt;0.001</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>154(49%)</td>
<td>225(69%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PvuII Genotype</td>
<td></td>
<td></td>
<td>Reference group</td>
<td></td>
<td>Undefined</td>
<td>Undefined</td>
</tr>
<tr>
<td>PP</td>
<td>36(22.5%)</td>
<td>169(9.8%)</td>
<td>&lt;0.001</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp</td>
<td>94(58.8%)</td>
<td>69(42.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp</td>
<td>30(18.8%)</td>
<td>78(47.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XbaI Allele</td>
<td></td>
<td></td>
<td>Reference group</td>
<td></td>
<td>0.35-0.74</td>
<td>91%</td>
</tr>
<tr>
<td>X</td>
<td>95(29.7%)</td>
<td>58(17.8%)</td>
<td>&lt;0.001</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>225(70.3%)</td>
<td>268(82.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XbaI Genotype</td>
<td></td>
<td></td>
<td>Reference group</td>
<td></td>
<td>Undefined</td>
<td>Undefined</td>
</tr>
<tr>
<td>XX</td>
<td>27(16.9%)</td>
<td>11(6.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xx</td>
<td>41(25.6%)</td>
<td>36(22.1%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xx</td>
<td>92(57.5%)</td>
<td>116(71.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Using the PP or Pp genotype as a reference, the odds ratio for AD in subjects with pp genotype was 0.25 (95% CI = 0.15 – 0.41). Using the XX or Xx genotype as a reference, the presence of the odds ratio for AD in subjects with xx genotype was 0.54 (95% CI = 0.34 – 0.87) (Table 2).

After analysis of the combined genotypes of the two loci, 9 genotypes were obtained due to the linkage disequilibrium between the PvuII and XbaI loci several kinds of genotypes were infrequent in the genotyped individuals. PPXx has a significantly more frequency in the LOAD patients than that in the control group.

### Table 2: The sum of ins or del allele frequencies in patients and healthy controls

<table>
<thead>
<tr>
<th>Allelic frequencies</th>
<th>AD patients(N)</th>
<th>Healthy controls (N)</th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvuII Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pp</td>
<td>30(18.8%)</td>
<td>78(47.9%)</td>
<td>&lt;0.001</td>
<td>0.25</td>
<td>0.15-0.41</td>
<td>93%</td>
</tr>
<tr>
<td>Pp + PP</td>
<td>130(81.2%)</td>
<td>85(52.1%)</td>
<td></td>
<td>0.54</td>
<td>0.34-0.87</td>
<td>88%</td>
</tr>
<tr>
<td>XbaI Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xx</td>
<td>92(57.5%)</td>
<td>116(71.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xX + XX</td>
<td>68(42.5%)</td>
<td>47(28.8%)</td>
<td></td>
<td>0.01</td>
<td>0.34-0.87</td>
<td></td>
</tr>
</tbody>
</table>

### DISCUSSION

XbaI and PvuII polymorphic loci were found in the intron 1 of ER-α gene. It is demonstrated that there are key regulation sequences such as enhancer and promoter sequences are existed in this region, so it proposed that mutations in this intron may influence the expression and biological function of ER-α (Hill et al. 1989).

Different ER-α genotypes may rather determine individual’s differences in ER-α expression than influence the final effect of ER-α. In present study we found that subjects who had AD represented “p or x” allele and in AD patients, PPXX genotype frequency was not higher than that in the healthy patients significantly. According to the statistical analysis the ppxx genotype is clearly associated with AD as a “protective” factor. A study that recently accomplished in UK proposed that px allele of ER-α might be a “protective” haplotype to AD (Lambert et al. 2001). The sample size was not large enough to provide an accurate interpretation after stratification for gender and genotype.

We did find an association between the ERα PvuII and AD either a cumulative effect of XbaI and PvuII in contributing to increase the risk of AD.

Among previous reports, several case-control studies reported a significant association between PvuII and XbaI polymorphisms and AD (Brandi et al. 1999; Isoe et al. 1997; Ji et al. 2000). Two Japanese case-control studies found higher frequencies of both PvuII P allele and PP
genotype or XbaI X allele and XX genotype in AD subjects compared to controls (ISOE et al. 1997; JI et al. 2000). In an Italian sample Brandi et al., combining the alleles of the two ERα gene polymorphisms, demonstrated that the PPXX genotype was significantly more frequent in AD than in controls (BRANDI et al. 1999).

Our data confirm those reported by Brandi et al. in a dataset of Italian patients with AD. They found an increased frequency of both the X and P alleles of the ERα XbaI and PvuII polymorphisms in AD (BRANDI et al. 1999). Our results and several previous studies on the association between ERα and AD confirm this hypothesis (ISOE et al. 1997; JI et al. 2000; MATTILA et al. 2000). Differences between different studies may have several reasons like sample size and mode, different ethnic and geographical origins. Lastly, it is also possible that the increased risk of AD associated with ERα polymorphisms is due to linkage disequilibrium with linked genes that may cause and increased risk for developing AD.

There are no functional studies that report functional effect of the ERα PvuII and XbaI polymorphisms in AD. ERα are to be found throughout the brain, especially in structures implicated in learning and memory such as the CA1–CA3 region of the hippocampus (MITRA et al. 2003), which is early affected in AD (JELLINGER 1998). ERα has an intronic localization; they bind to the estrogen response element in the promoter region of target genes, with subsequent stimulation of the expression of specific genes involved in neuroprotective processes. Therefore, neurons expressing low ERα may be more vulnerable to excitotoxins and free radicals (MCEWEN 2001). In addition, it is demonstrated that estrogens have a cognitive enhancing effect and appear to be able to protect the brain from neurodegeneration (MCEWEN 2001).

CONCLUSION
This study confirms the hypothesis that ERα XbaI and PvuII polymorphism are associated with increased risk of LOAD. For investigation of functional consequences of this polymorphism and mechanisms to which the ERα polymorphisms could lead to AD there must be fundamental studies at biochemical level to be established.

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POLIMORFIZAM ESTROGEN RECEPTORA ALFA GENA KOD PACIJENATA SA KASNOM POJAVOM ALZHAJMER-OVE BOLESTI

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Izvod
Vrsena su istraživanja 160 bolesnih (LOAD) i 163 zdrava, kontrolna pacijenta istog pola i starosti. Za utvrđivanje polimorfizma PvuII i XbaI kod Alzhajmerove bolesti primenjene su PCR/RFLP metode a učestalost genotipova je utvrđivana statističkim metodama. Produkti PCR reakcije pripremljeni od 21 slučaja bolesnih i 20 zdravih kontrolnih pacijenata su prečkreni u oba pravca. Utvrđena je veća učestalost mutiranih alela u PvuII i XbaI lokusima kod LOAD (bolesne) grupe u odnosu na kontrolnu grupu (P<0.001, OR=0.51, 95 % CI 0.35-0.74 za XbaI lokus; P<0.001, OR=0.41, 95 % CI 0.3-0.57 za PvuII lokus). Rezultati sugerišu da su Era XbaI i PVUII polimorfizam dodatni faktori rizika za kasnu pojavu Alzhajmerove bolesti.

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