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Association of Estrogen Receptor 1 PvuII and XbaI Polymorphisms and Peripheral Estrogen Receptor 1 mRNA Levels with Alzheimer's Disease in Turkish Patients

Östrojen Reseptörü 1 PvuII ve XbaI Polimorfizmleri ve Periferal ESR1 mRNA Düzeylerinin Türk Hastalarda Alzheimer Hastalığı ile İlişkisi

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Abstract

Objective: Estrogen receptor 1 (ESR1) polymorphisms are associated with Alzheimer's disease (AD) and polymorphisms in the first intronic region of the gene are known to affect ESR1 mRNA transcription. The first intronic region of the ESR1 contains two polymorphisms that have received the most attention: PvuII rs2234693 (NM 000125.3:c.453-397T>C) and XbaI rs9340799 (NM 000125.3:c.453-351A>G). Both polymorphisms have been shown to be associated with AD, but consistent findings across populations have not been established. This study aimed to determine whether ESR1 PvuII and XbaI polymorphisms are associated with the disease in a cohort of Turkish AD patients. Whether PvuII and XbaI polymorphisms affect disease susceptibility by influencing ESR1 mRNA expression was also examined.

Materials and Methods: Genotyping was performed in 424 patients with AD (mean age: 64.5 ± 11.1 yrs) and 302 controls (mean age: 56.4 ± 13.0 yrs). The polymerase chain reaction (PCR) and restriction enzyme digestion methods were used to determine the prevalence of the ESR1 PvuII and XbaI polymorphisms. The ESR1 mRNA expression was analyzed in the peripheral blood cells of 85 patients and 53 age-matched controls using quantitative real-time PCR.

Results: No significant difference in genotype and allele frequencies of ESR1 PvuII and XbaI polymorphisms between the patients and controls was found. However, the frequencies of the PvuII C and XbaI G alleles were significantly higher in the patients with the apolipoprotein-E (APOE) ε4 allele. The ESR1 mRNA levels were significantly lower in the patients with AD compared with the controls (P = 0.001). The XbaI A allele was significantly associated with lower ESR1 mRNA levels (P = 0.044) and this association remained significant even after adjusting for confounders such as age, gender and APOE ε4 carrier status (P = 0.035).

Conclusion: This study demonstrated that the distribution of PvuII and XbaI alleles is associated with the APOE ε4 allele. The XbaI polymorphism may be associated with a higher risk of AD by altering ESR1 mRNA levels.

Keywords: Alzheimer's disease, estrogen receptor 1, PvuII, XbaI, gene polymorphism, mRNA expression

Öz

Amaç: Östrojen reseptörü 1 (ESR1) polimorfizmleri, Alzheimer hastalığı (AH) ile ilişkilidir ve genin birinci intron bölgesindeki polimorfizmlerin ESR1 mRNA transkripsiyonunu etkilediği bilinmektedir. ESR1'in ilk intron bölgesi, iyi bilinen iki önemli polimorfizm içerir: PvuII rs2234693 (NM 000125.3:c.453-397T>C) ve Xbal rs9340799 (NM 000125.3:c.453-351A>G). Her iki polimorfizmin AH ile ilişkili olduğu gösterilmiştir, ancak popülasyonlar arasında tutarlı sonuçlar elde edilmemiştir. Çalışmamızda, Türk AH hastalarından oluşan kohortumuzda ESR1 PvuII ve XbaI polimorfizmlerinin hastalık ile ilişkili olup olmadığını belirlemeye çalıştık. Ayrıca PvuII ve XbaI polimorfizmlerinin ESR1 mRNA ekspresyonunu etkileyerek hastalığa katkı sağlayıp sağlamadığını da araştırdık.

Gereç ve Yöntem: Genotipleme 424 AH hastası (ortalama yaş: 64,5 ± 11,1 yıl) ve 302 kontrolde (ortalama yaş: 56,4 ± 13,0 yıl) gerçekleştirildi. ESR1 PvuII ve XbaI polimorfizmlerinin prevalansını belirlemek için polimeraz zincir reaksiyonu ve restriksiyon enzim kesimi yöntemi kullanıldı. ESR1 mRNA ekspresyonu, 85 hasta ve aynı yaştaki 53 kontrolün periferik kan hücrelerinde kantitatif gerçek zamanlı polimeraz zincir reaksiyonu ile analiz edildi.

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Bulgular: Hastalar ve kontroller arasında ESR1 PvuII ve XbaI polimorfizmlerinin genotip ve allel sıklığı açısından anlamlı bir farklılık bulunmadı. Bununla birlikte, PvuII C ve XbaI G allel frekansları, APOE ε4 alel taşıyıcısı hastalarda anlamlı olarak daha yüksek bulundu. ESR1 mRNA düzeyinin hastalarda kontrollere göre anlamlı derecede düşük olduğu belirlendi (P = 0,001). XbaI A allel taşıyıcılığı, hastalarda düşük ESR1 mRNA düzeyi ile ilişkili bulundu (P = 0,044) ve bu ilişkinin, yaş, cinsiyet ve APOE ε4 taşıyıcılık durumu gibi değişkenler için düzeltme yapıldıktan sonra bile anlamlı olduğu belirlendi (P = 0,035).

Sonuç: Çalışmamız, PvuII ve XbaI allel dağılımının APOE ε4 alleli ile ilişkili olduğunu gösterdi. XbaI polimorfizmi, ESR1 mRNA düzeyini etkileyerek AH riskini artırabilir.

Anahtar Kelimeler: Alzheimer hastalığı, östrojen reseptörü 1, PvuII, XbaI, gen polimorfizmi, mRNA ekspresyonu

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by memory problems and loss of cognitive functions. The disease is a serious public health issue that affects patients' quality of life and places a significant burden on the individual, family, and society. As a result, understanding the pathogenesis and risk factors of AD is critical for early diagnosis and treatment. Genetic, metabolic, and environmental factors all play a role in the disease's pathogenesis. Epidemiological studies show that the prevalence of AD varies by gender and is higher among women. The higher risk in women is mostly attributed to their longevity, but the findings of several studies suggest that there may be other factors at play. Reduced estrogen levels in postmenopausal women are a significant risk factor for the development of AD (1,2). Estrogen protects cognitive performance and verbal and visual memory, and delays cognitive decline in postmenopausal women. As a result, estrogen replacement therapy is recommended as a therapeutic approach to lowering the risk of AD and assisting patients with AD in maintaining their cognitive functions (3). The physiological effects of estrogen on the brain extend beyond the areas of the central nervous system that control reproduction. In addition to supporting neuronal cell survival, estrogen also lowers neuronal damage, guards against neurotoxins, promotes axonal sprouting and neuronal repair, and boosts synaptic transmission and neurogenesis (4).

Estrogen acts via two nuclear receptors known as estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which are encoded by estrogen receptor *(ESR1)* and *ESR2* genes, respectively. After binding to estrogen, these receptors function as transcription factors, regulating gene expression and function by interacting with the regulatory regions of target genes. Both receptors are located in the brain regions associated with cognitive function and emotion. The ERα receptor is predominantly expressed in the hypothalamus and amygdala, while ERβ is mainly expressed in the hippocampal formation and entorhinal cortex (5). It has been reported that polymorphisms in the *ESR1* gene are linked to several diseases, including neurodegenerative disorders. Polymorphisms in the first and second intronic regions of the *ESR1* gene are known to affect gene transcription (6,7). Among them, PvuII (rs2234693, NM_000125.4:c.453-397T>G) and XbaI (rs9340799, NM_000125.4:c.453-351A>G) are two common polymorphisms located in the first intronic region of the *ESR1* gene. It has been suggested that the PvuII polymorphism influences the splicing of ESR1 mRNA, resulting in a change in ESR1 protein synthesis (8). Previous research demonstrated that the PvuII and XbaI polymorphisms contributes to an increased risk of AD (9,10,11,12,13,14,15,16); however, there are also opposing findings (17,18,19,20,21). Additionally, numerous investigations have indicated that the presence of the apolipoprotein-E (APOE) ε4 allele influences the AD risk associated with the ESR1 polymorphisms (9,11,14).

Given the link between *ESR1* gene polymorphisms and AD, this paper examines the relationship between dementia and the most widely researched *ESR1* gene polymorphisms, PvuII (rs2234693) and XbaI (rs9340799). Additionally, the paper investigates whether these polymorphisms have an impact on ESR1 mRNA expression.

Materials and Methods

Patients and Controls

All patients were recruited from the Behavioural Neurology and Movement Disorders Unit Outpatient Clinic at the Istanbul University, Istanbul Faculty of Medicine and underwent detailed clinical and neuropsychological examinations. Clinical diagnoses were made in accordance with the appropriate clinical diagnostic criteria. The diagnosis of "probable AD" was based on the National Institute of Aging and Alzheimer's Disease Association Criteria (22). The exclusion criteria included the presence of non-Alzheimer's dementia [other than mild cognitive impairment (MCI)], active neurological, psychiatric, or inflammatory diseases, a history of severe head trauma or brain surgery, the use of immunosuppressive drugs and hormonal replacement treatments, and a history of active cancer or the use of cancer therapy, including anti-hormonal medications. The individuals in the control group had no past or ongoing history of any major neurologic, psychiatric, systemic disease and there was no use of hormonal replacement therapy. The mini-mental state examination test (MMSE) was used to evaluate the global cognitive status of the participants.

The study was approved by the Ethics Committee of the Istanbul University, Istanbul Faculty of Medicine, while the procedures used adhered to the tenets of the Declaration of Helsinki (date: 10/09/21/no: 16). Informed consent was obtained from the participants or from legal guardians for those unable to consent.

Estrogen Receptor 1 Genotyping

Genomic DNA was extracted from ethylenediaminetetraacetic acid blood samples using the salting-out method. Genotyping of PvuII (rs2234693, NM_000125.4:c.453-397T>G) and XbaI (rs9340799, NM_000125.4:c.453-351A>G) was carried out via polymerase chain reaction (PCR)-based restriction fragment length polymorphism analysis. A fragment of 255 base pairs (bp) that contains the two polymorphic sites was amplified by using forward 5'-CAGGGTTATGTGGCAATGAC-3' and reverse 5′-TACCTATAAAAATGACAAAATGAAAT-3') primers. The PCR amplification was carried out in a 50-µl reaction mixture

using the following conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and final extension at 72 °C for 5 min followed by a hold at 4 °C. The PCR products were digested overnight with PvuII and XbaI restriction enzymes (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C, producing fragments of 255 bp (C allele) or 97 + 158 bp (T allele) and of 255 bp (G allele) or 142 + 113 bp (A allele), respectively. The cleavage products were electrophoresed on 4% agarose gel and stained with ethidium bromide.

Apolipoprotein-E Genotyping

The presence of at least one APOE ε4 allele was included as a potential confounder due to its strong association with AD risk in previous studies. The presence of the APOE ε4 allele was determined via the real-time (RT)-PCR method using hydrolysis probes on the Lightcycler 480® Instrument RT-PCR system (Roche Diagnostics, Mannheim, Germany).

Estrogen Receptor 1 mRNA Expression Analysis

The total RNA was extracted from peripheral blood leukocytes using the TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Here, 1 µg of total RNA was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT-PCR (RT-qPCR) analysis was performed using AMPIGENE qPCR Green Mix Hi-ROX with SYBR® Green dye (Enzo Life Sciences, Farmingdale, NY, USA) on the Lightcycler 480 system. The RT-qPCR analysis was carried out under the following conditions: 5 min at 95 °C; 45 cycles of 10 s at 95 °C, 20 s at 62 °C, and 10 s at 72 °C, with the qPCR reactions run in duplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene for the normalization of the data and the relative expression levels of ESR1 mRNA were determined using the $2^{-\Delta\Delta Ct}$ method. Primers targeting the ESR1 exon 3 and 4 boundary were used as previously described (23). The specificity of the primers and amplicons was confirmed in silico using the BLASTN tool, and the size of the PCR products was confirmed via gel electrophoresis. To confirm the existence of one gene-specific peak and the lack of primer dimmers, melting curve analysis was performed. The primer sequences were as follows: ESR1 forward primer: 5′-GCTACGAAGTGGGAATGATGAAAG-3′, ESR1 reverse primer: 5′-TCTGGCGCTTGTGTTTCAAC-3′; GAPDH forward primer: 5′-ATCTTCCAGGAGCGAGATC-3′, GAPDH reverse primer: 5′- CAGGAGGCATTGCTGATGA-3′.

Statistical Analysis

Genotype and allelic distributions were compared using the Pearson χ^2 test. Hardy–Weinberg equilibrium (HWE) was computed to the expected genotype distribution. A t-test was used for comparison of means and Fisher's exact test for percentages. Maximum likelihood estimates of odds ratios (OR) and associated 95% confidence intervals (CIs) were calculated via logistic regression analysis. Due to the skewed distribution of mRNA expression, the data were log transformed to achieve normal distribution. An independent t-test was used to compare the ESR1 mRNA levels between groups and to assess the differences in ESR1 mRNA levels and genotypes. The differences in mRNA levels were tested via analysis of covariance (ANCOVA) with age, gender, and APOE ε4 as the covariates. Correlations between ESR1 mRNA levels and demographic characteristics were tested using the Pearson correlation test. All the statistical analysis was performed using SPSS version 24.0 software (IBM Corp., USA). The criterion for significance was set at $P < 0.05$.

Results

The study enrolled 726 participants, including 424 patients with AD and 302 controls. The patients included 238 women and 186 men, with a mean age of 64.5 ± 11.1 years. The control group included 156 women and 146 men, with a mean age of 56.4 ± 13.0 years. Of these patients, 242 (57.1%) had a positive family history of dementia among first-degree relatives. Parental consanguinity was reported in 14.9% of the patients. Table 1 presents the demographic information of the study participants. The patient's performance in MMSE was significantly lower in the patient group ($P < 0.001$), as was expected. Furthermore, individuals carrying the ε4 allele were significantly more common among the patients than among the controls, whereas individuals carrying the ε2 allele were significantly more frequent among the controls than the patients.

The distribution of PvuII and XbaI genotypes did not significantly deviate from HWE among the patients and controls (Table 2). The PvuII minor C allele frequencies were 0.471 and 0.483 and the XbaI minor G allele frequencies 0.433 and 0.442 among the patients and controls, respectively. The distribution of PvuII and XbaI alleles and genotypes did not differ significantly between the two groups (Table 2). The linkage disequilibrium between PvuII and XbaI polymorphisms was examined in earlier studies due to the close proximity of these two polymorphisms, and the analysis showed that they were in strong linkage disequilibrium (9,10,24,25,26). After combining two polymorphisms, nine genotype combinations were recognized. Table 3 shows nine possible genotypes for the PvuII and XbaI polymorphisms in the studied population. The frequencies of genotype combinations among the patients and controls were similar and no significant difference was observed.

Whether the APOE ε4 allele affects the distribution of PvuII and XbaI genotypes was also examined (Table 4). Due to the low number of individuals who were homozygous for the rare alleles of PvuII and XbaI, homozygotes were grouped with heterozygotes as rare allele carriers for the statistical analysis. When the study group was divided based on APOE ε4 allele status, the frequency of PvuII C allele was lower (66.8%) among the patients who did not have the APOE ε4 allele compared with the controls (74.4%). Furthermore, in the APOE ε4 non-carriers, the PvuII C allele had an almost significant protective effect on AD, with an OR of 0.692 (95% CI: 0.474–1.009, $P = 0.055$). However, the frequency of the PvuII C allele was significantly higher (77.3%) among the patients with the APOE ε4 allele than among the controls (62.5%). Furthermore, the individuals carrying the PvuII C allele together with the APOE ε4 allele had a 2.047-fold increased risk for AD (OR: 2.047, 95% CI: 1.018–4.115, $P = 0.042$). Similar to the PvuII C allele, the XbaI G allele was less prevalent among the patients (62.4%) without the APOE ε4 allele than among the controls (68.9%); however, in terms of APOE ε4 allele carriers, it was significantly more common among the patients (70%) than the controls (52.1%), and the individuals with both the XbaI G allele and the APOE ε4 allele had a 2.147-fold increased risk for AD (OR: 2.147, 95% CI: 1.104–4.175, $P = 0.023$).

To investigate whether leukocyte ESR1 expression is associated with AD, the ESR1 mRNA levels among the patients and the controls were compared, with the levels analyzed in 85 patients and 53 age-matched controls. The analysis indicated significantly reduced mRNA levels among the patients compared with the controls (P = 0.001) (Figure 1). Furthermore, the analysis was repeated after stratifying the subjects based on PvuII and XbaI alleles to investigate whether this relationship varies accoriding to ESR1 polymorphisms. In the patient group, it was found that the XbaI A allele carriers (AA + AG) had significantly lower ESR1 mRNA levels than the non-carriers (P = 0.044) (Figure 2a), but no significant difference was found between the PvuII T allele

Table 1. General characteristics of the patients and controls

	Patients with AD (n = 424)	Controls (n = 302)	*P* value
Age, years, mean ± SD	64.5 ± 11.1	56.4 ± 13.0	**<0.001**
Age of onset, years, mean ± SD	60.8 ± 10.7	-	
Gender			
Female, n (%)	238 (56.1)	156 (51.7)	0.233
Male, n (%)	186 (43.9)	146 (48.3)	
MMSE score, mean ± SD (n)	17.8 ± 7.7 (261)	28.9 ± 2.1 (59)	**<0.001**
APOE ε4 carrier status			
ε4 carrier	150 (35.4)	48 (15.9)	**<0.001**
ε4 non-carrier	274 (64.6)	254 (84.1)	
APOE ε2 carrier status			
ε2 carrier	36 (6.8)	29 (11.9)	**0.018**
ε2 non-carrier	266 (93.2)	395 (88.1)	

Continuous variables are presented as mean ± SD and dichotomous variables as a percentage. A t-test was used for comparison of means and an χ^2 test for percentages. AD: Alzheimer's disease; MMSE: Mini mental state examination, n: Number of individuals, SD: Standard deviation, APOE: Apolipoprotein-E

Table 2. Allele and genotype frequencies of PvuII and XbaI polymorphisms among the patients and controls

		Patients with AD (n = 424) n (%)	Controls (n = 302) n (%)	χ^2	*P* value	Odds ratio (95% CI)	*P* value
PvuII	**Genotype**						
	TT	125 (29.5)	83 (27.5)			**Reference**	
	TC	199 (46.9)	146 (48.3)	0.345	0.842	0.91 (0.64–1.28)	0.576
	CC	100 (23.6)	73 (24.2)			0.91 (0.60–1.37)	0.650
	HWE-test	χ^2: 1.428, P = 0.489	χ^2: 0.306, ***P = 0.858***				
	Allele						
	T	449 (52.9)	312 (51.7)	0.236		**Reference**	0.627
	C	399 (47.1)	292 (48.3)			0.95 (0.77–1.17)	
XbaI	**Genotype**						
	AA	148 (34.9)	102 (33.8)			**Reference**	
	AG	185 (43.6)	133 (44)	0.115	0.944	0.96 (0.68–1.34)	0.806
	GG	91 (21.5)	67 (22.2)			0.94 (0.62–1.40)	0.422
	HWE-test	χ^2: 5.250, P = 0.072	χ^2: 3.473, P = 0.176				
	Allele						
	A	481 (56.7)	337 (55.8)	0.123		**Reference**	0.726
	G	367 (43.3)	267 (44.2)			0.96 (0.78–1.19)	

Genotype and allele frequencies were compared using the chi-square test. AD: Alzheimer's disease, HWE: Hardy–Weinberg equilibrium, n: Number of individuals, CI: Confidence interval

carriers (TT + TC) and the non-carriers (P = 0.070) (Figure 2b). In addition, in the control group, no significant effect of these polymorphisms on ESR1 mRNA levels was found (Figure 2a, b). The significant interaction between ESR1 mRNA and the XbaI A allele was further analyzed via ANCOVA. The significant difference in mRNA levels between the XbaI A allele carriers and non-carriers persisted following the analysis using age, gender, and APOE ε4 status as covariates (P = 0.035). The correlation between ESR1 mRNA levels and age, age at onset, and MMSE scores was also investigated. The Pearson correlation analysis revealed a significantly positive correlation between ESR1 mRNA levels and MMSE scores among the patients (r = 0.281, P = 0.041), but not between age or age at onset. In addition, no significant correlations were found among any of the parameters in the control group.

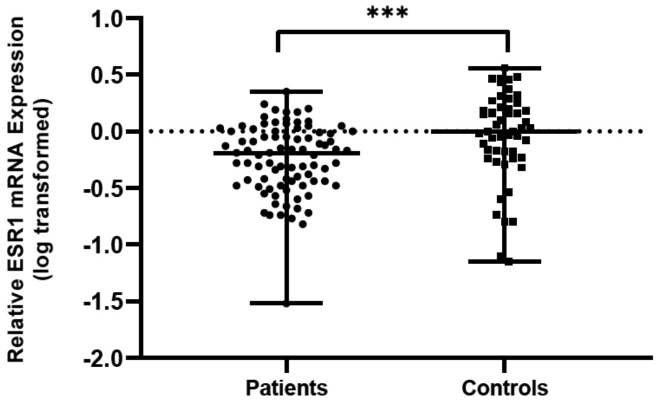

Figure 1. Estrogen receptor 1 mRNA levels in the patients with AD and the controls

The mean is indicated together with the standard deviation

****P < 0.001, AD: Alzheimer's disease, ESR: Estrogen receptor*

Discussion

Many human diseases, varying from breast cancer to AD, have been linked to common polymorphisms in the first intronic region of the *ESR1* gene. These findings raise the possibility that variations in this region are linked to disease susceptibility. The first intron contains two common polymorphisms, which have been described in terms of the name of the detecting restriction enzyme, PvuII or XbaI (27). The PvuII polymorphism occurs due to a T–C transition located 397-bp upstream of exon 2, while XbaI is characterised by an A–G transition 351-nucleotides upstream of exon 2. For each endonuclease, the restriction site has traditionally been denoted by a lowercase letter (p or x for PvuII and XbaI endonucleases, respectively), with an uppercase letter (P or X) denoting the lack of the restriction site. Specifically, P and p are the C and T alleles of the PvuII polymorphism, while X and x are the G and A alleles of the XbaI polymorphism, respectively.

Table 3. Distributions of PvuII and XbaI genotype combinations in the patients and controls

ESR1 genotypes	Patients wih AD n (%)	Controls n (%)	χ^2	*P* value
TT/AA	120 (28.3)	79 (26.2)		
TT/AG	3 (0.7)	4 (1.3)		
TT/GG	2 (0.5)	0 (0)		
TC/AA	27 (6.4)	19 (6.3)	11.896	0.156
TC/AG	161 (38.0)	123 (40.7)		
TC/GG	11 (2.6)	4 (1.3)		
CC/AA	1 (0.2)	4 (1.3)		
CC/AG	21 (5.0)	6 (2.0)		
CC/GG	78 (18.4)	63 (20.9)		

AD: Alzheimer's disease; n: Number of individuals, ESR: Estrogen receptor

Table 4. Distribution of PvuII and XbaI genotypes according to APOE ε4 allele status

Polymorphism	APOE ε4 status	Genotype	Patients with AD n (%)	Controls n (%)	*P* value	Odds ratio (95% CI)
PvuII	APOE ε4-	TT	91 (33.2)	65 (25.6)	0.055	**Reference**
		TC + CC	183 (66.8)	189 (74.4)		0.692 (0.474–1.009)
	APOE ε4+	TT	34 (22.7)	18 (37.5)	**0.042**	**Reference**
		TC + CC	116 (77.3)	30 (62.5)		**2.047 (1.018–4.115)**
XbaI	APOE ε4-	AA	103 (37.6)	79 (31.1)	0.117	**Reference**
		AG + GG	171 (62.4)	175 (68.9)		0.749 (0.522–1.075)
	APOE ε4+	AA	45 (30.0)	23 (47.9)	**0.023**	**Reference**
		AG + GG	105 (70)	25 (52.1)		**2.147 (1.104–4.175)**

+ denotes carrying at least one copy of APOE ε4 allele, - denotes without carrying any copies of the APOE ε4 allele. Significant *P* values and odds ratios are indicated in bold. AD: Alzheimer's disease, APOE: Apolipoprotein-E; n: Number of individuals, CI: Confidence interval

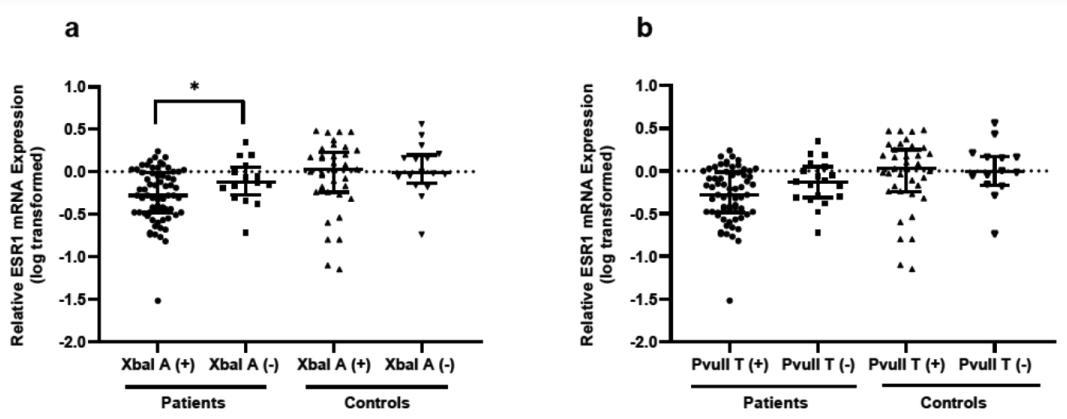

Figure 2. Estrogen receptor 1 mRNA levels in the patients with AD and the controls based on PvuII and XbaI genotypes. (a) XbaI A allele carriers (AA + AG) vs. non-carriers (GG); (b) PvuII T allele carriers (TT + TC) vs. non-carriers (CC). The mean is indicated together with the standard deviation

**P < 0.05, AD: Alzheimer's disease, ESR: Estrogen receptor*

The role of these polymorphisms in late-onset AD has been widely explored among European and Asian populations in small-scale case-control studies. In terms of Europeans, five studies reported no association with AD in Spanish (17), British (20,28), Swedish (29), and Dutch (30) populations, whereas eight studies showed positive associations in the Italian (9,10,15,16), Spanish (24), Swedish (14), and Finnish populations (14). In terms of Asians, two separate investigations among the Japanese population (20,21) revealed no association, while three studies in the Japanese (11) and Chinese (12,13) populations did. Given these contrasting findings, the role of ESR1 in AD remains debatable. Several meta-analyses have been conducted to overcome the constraints of individual research, such as a limited sample size or insufficient power. One meta-analysis revealed that ethnicity has a considerable effect on the *P* and *X* allele frequencies of PvuII and XbaI polymorphisms, particularly among Caucasians and Asians. While not present in the European population, the *P* (OR: 1.48, 95% CI: 1.1–2.0, *P* = 0.006) and *X* (OR: 1.51, 95% CI: 1.1–2.0, *P* = 0.004) alleles were significantly linked to an increased risk of AD in the Asian population (31). In contrast, Cheng et al. (32) found that only the PvuII polymorphism was associated with the risk of AD under the dominant (CC +CT vs. TT, OR: 1.14, 95% CI: 1.02–1.28, *P* = 0.03) and heterozygous (CT vs. TT, OR: 1.16, 95% CI: 1.02–1.31, *P* = 0.02) models among Caucasians, but not Asians. Furthermore, Wang (33) demonstrated that the relationship between the PvuII polymorphism and AD risk differs across different European regions. The PvuII *p* allele was shown to be strongly associated with a lower risk of AD among South Europeans, but not among North Europeans (33). The authors claimed that the significance of geographic regions in the association between ESR1 variants and AD indicates that gene-environment interactions influence AD development and should be investigated further (33).

To the best of our knowledge, to date, no study has investigated the relationship between PvuII and XbaI polymorphisms and AD risk in the Turkish population. Therefore, the current study is the first to examine the PvuII and XbaI polymorphisms among Turkish patients with AD. The observed minor PvuII allele frequency (MAF: 0.474) in this study was comparable to the data from the GenomAD database pertaining to Europeans, but the present study reported higher values for the XbaI (MAF: 0.431). Nevertheless, this study failed to demonstrate the existence of an association between these polymorphisms and AD. This result agrees with many previous studies that reported that the PvuII and XbaI polymorphism did not influence the risk of AD (18,19,20,21,29,30), while also contradicting many of the previous studies' findings (9,10,11,12,13,14,15,16,34). Several factors could explain the contrasting results among the studies. First, the racial or ethnic differences among populations may result in differences in AD susceptibility. Second, it is widely recognized that gene-environment interactions and a variety of genetic and environmental factors contribute to AD, which means that differences in environmental risk factors may alter the affects of genes. Finally, the sample size varied depending on the study design, and the lack of association between ESR1 genotypes could be due to insufficient statistical power.

Since AD is a multifactorial disease, and since the APOE ε4 allele is one of the major genetic risk factors of the disease, whether the presence of APOE ε4 affects the distribution of ESR1 polymorphisms was also investigated. Numerous studies have reported functional interactions between estrogen and APOE. *In vivo* and *in vitro* analyses demonstrated that estradiol increases the APOE mRNA expression in astrocytes and microglia (35). Furthermore, the effect of estrogen on APOE expression differs depending on APOE allele status (36). Additionaly, APOE expression is regulated by ERs and is isoform specific. The activation of ERa increased and ERb decreased APOE expression in the hippocampus both *in vitro* and *in vivo* (37). According to several studies, the APOE ε4 allele interacts with the PvuII and XbaI polymorphisms to influence AD risk (9,10,11,14,16,19). In an Italian study, individuals with homozygous APOE ε4 and the *PPXX* genotype had a 7.6-fold increased risk of developing AD compared with ε4-negative individuals (9). Another Italian study reported that the risk of AD was two times higher in individuals carrying *PP* and/or *XX* genotypes in addition to the APOE ε4 allele compared with individuals carrying only the APOE ε4 allele (10). In the same study, it was shown that ESR1 polymorphisms have a significant effect on APOE plasma levels,

with the *PP* and *XX* genotypes being associated with the reduced APOE concentrations. In a study conducted among Swedish familial patients with AD, the APOE ε4 allele, in combination with both the *xx* and *pp* genotypes, was found to increase the risk of familial AD among Swedish women (14). In a Brazilian study, neither the alleles and genotypes of the PvuII and XbaI polymorphisms were independently associated with the risk of MCIa or AD, while the combination of *X* and *P* alleles with the APOE ε4 allele did increase the risk (19). All such pieces of evidence indicate that ESR1 and APOE polymorphisms may interact to influence the risk of AD. However, the interaction between *ESR1* and *APOE* genes on AD also appears to be erratic, much like the relationships between *ESR1* gene polymorphisms and the incidence of AD. Several studies did not report significant interaction between *APOE* and *ESR1* genotypes (15,38,39). Our results revealed that the distribution of PvuII and XbaI alleles was associated with the *APOE* gene. The PvuII C (or *P* allele) allele had an almost significant protective effect against AD among the APOE ε4 non-carriers, but co-occurring with the APOE ε4 allele increased the risk of AD. Based on our findings, it can be concluded that the PvuII C (or *P* allele) allele may have a protective effect on AD in the absence of the APOE ε4 allele, but the presence of the APOE ε4 allele diminishes the protective effect of the PvuII C allele.

Estrogen is a neuroprotective hormone that exerts its effects by decreasing the toxicities of glutamate and Ab, improving synaptic plasticity, controlling neurotrophic factors, enabling transcription factor activation, lowering brain inflammation, and reducing tau hyperphosphorylation (40). Both ERα and ERb play a major role in mediating the estrogen response. Estrogen receptors are ligand-activated transcription factors composed of several domains important for hormone binding, DNA binding, and activation of transcription. These receptors act as transcription factors upon estrogen binding, regulating gene expression and function by interacting with the regulatory regions of the target genes involved in proliferation, differentiation, and survival (41). Both ERα and ERb are widely distributed in the brain, with ERα mediating the stimulatory effect of estrogens and ERb mediating the inhibitory effect of estrogens (40). Meanwhile, ESR1 polymorphisms have been shown to affect ESR1 mRNA expression, as well as ERα and estrogen function (28,42,43). Although the PvuII and XbaI polymorphisms are both found in the intronic region of ESR1 and do not result in any amino acid change, it is possible that they could have phenotypic impacts through a number of different mechanisms. Intronic polymorphisms might increase or decrease gene transcription and might have an impact on RNA splicing and mRNA processing and stability (44). Even if they do not have a functional consequence, they may be in linkage disequilibrium with other functional polymorphisms. While the precise mechanism by which the PvuII and XbaI polymorphisms influence receptor function is unknown, evidence suggests that these polymorphisms effect estrogen activity by modifying the transcription of the *ESR1* gene through altered transcription factor binding. A study by Herrington et al. (42) showed that the C allele but not the T allele of the PvuII polymorphism produced a functional binding site for the transcription factor, B-Myb. When co-transfected with B-Myb, the presence of the C allele was associated with a four-times-higher expression of a downstream reporter construct when compared with the T allele, suggesting that the C allele may contribute to the upregulation of ESR1 expression (42). The T allele of PvuII lacks a functional binding site for the transcription factor B-Myb, which may cause a decrease in ESR1 transcription or the production of an ESR1 isoform with distinct functional properties (42,45). The actions of estrogen that are mediated by ESR1 may therefore be diminished, resulting in a relative estrogen deficit (8). The present study demonstrated a decrease in ESR1 mRNA levels in the presence of the XbaI A allele in patients with AD. The functional consequences of XbaI polymorphism are not yet known. However, similar to PvuII, the A–G transition of XbaI may have an effect on ESR1 expression and receptor function, which may explain the lower ESR1 mRNA levels among the XbaI A allele carriers in this study. The findings suggest that the XbaI polymorphism could change ESR1 gene expression, influencing downstream pathways and circulating estradiol levels. In support of this suggestion, a study by Schuit et al. (43) reported lower plasma estrodiol levels among postmenopausal women who carried the ESR1 PvuII-XbaI TA haplotype. However, it cannot be ruled out that the XbaI polymorphism co-segregates with another truly functional polymorphism in the *ESR1* gene.

Study Limitations

Several limitations should be considered when interpreting the results. The present study was the first to examine PvuII and XbaI polymorphisms among Turkish individuals with AD. Therefore, the replicability of our results cannot be tested. Second, AD is a complex multifactorial disease involving interactions between genes or between genes and the environment, but the necessary data for assessing such interactions to investgate the independent role of ESR1 polymorphisms in AD risk are lacking. Third, due to the limited data, subgroup analysis was not performed in terms of gender, age, or clinical stage of AD. Finally, the serum levels of estradiol were not measured.

Conclusion

This study demonstrated that the distribution of PvuII and XbaI alleles are associated with the APOE ε4 allele. The XbaI A allele is associated with lower ESR1 mRNA levels and this association is not explained or influenced by risk factors of AD such as age, gender, or APOE ε4 carriage. Taken together, the current findings suggest that the XbaI polymorphism may affect ESR1 expression. Future studies will be needed to better understand the mechanisms behind AD. Larger cohorts of patients from a variety of ethnic groups are still required to expand our understanding of the *ESR1* gene.

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Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of the Istanbul University, Istanbul Faculty of Medicine, while the procedures used adhered to the tenets of the Declaration of Helsinki (date: 10/09/21/no: 16).

Informed Consent: Informed consent was obtained from the participants or from legal guardians for those unable to consent.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.L., B.S., H.G., H.H., B.B., Concept: G.G., E.L., B.S., H.G., H.H., B.B., Design: G.G., F.G., Data Collection or Processing: G.G., P.K-B., M.K., D.U., B.Ç., F.G-G., Analysis or Interpretation: G.G., P.K-B., M.K., D.U., B.Ç., E.L., B.S., H.G., H.H., B.B., Literature Search: G.G., P.K-B., Writing: G.G.

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