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Screening for Mutations in the Coding Regions of *PSEN1* Gene, 16-17 Exons of *APP* Gene and *APOE* Genotyping in Patients with Alzheimer's Disease

Tugce KARADUMAN[1*], Erdal EROGLU[2], Emel SAGLAR OZER[1], Beril ERDEM TUNCDEMIR[1], Hatice MERGEN[1]

[1]Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey
[2]Faculty of Medicine, TOBB University of Economics and Technology, Ankara, Turkey
Tugce KARADUMAN ORCID No: 0000-0003-0728-0968
Erdal EROGLU ORCID No: ORCID-ID: 0000-0002-0412-8998
Emel SAGLAR OZER ORCID No: 0000-0002-8161-827X
Beril ERDEM TUNCDEMİR ORCID No: 0000-0002-9820-1147
Hatice MERGEN ORCID No: 0000-0002-7980-8197

**Corresponding author: tugcekaraduman@hacettepe.edu.tr*

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Alzheimer's disease, Polymorphism, Mutation, *PSEN1*, *APP*, *APOE*

Abstract: The aim of this study is to screen for mutations in the presenilin-1 (*PSEN1*) gene,16-17 exons of amyloid precursor protein (*APP*) gene and determining apolipoprotein-E (*APOE*) genotype in patients with Alzheimer's disease (AD). The coding regions of *PSEN1* gene, 16-17 exons of *APP* gene were screened by using DNA sequence analysis in 30 patients with late onset of Alzheimer's disease (LOAD) diagnosed based on Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria and 40 non-dementia controls. Additionally, genotype and allele frequencies of ε2, ε3 and ε4 polymorphisms of *APOE* gene were determined by using PCR-RFLP methods in both groups. No mutation was found in the coding regions of *PSEN1* gene and 16-17 exons of *APP* gene. On the other hand, rs165932 (G/T) polymorphism was found in intron 8 of *PSEN1* in 26 patients. There was no significant difference in genotype and allele frequencies of intronic polymorphism between control group and patients ($p>0.05$). The frequency of ε3/ε4 genotype was significantly higher in patient group ($p<0.05$) and frequencies of ε4 allele were also significantly higher among the patients with LOAD ($p<0.05$). When *PSEN1* genotype distribution and ε4 allele frequency were evaluated together in the patient group, no significant relation was found ($p>0.05$). We suggested that there was a potential association between LOAD and *APOE* ε4 allele; however, no result could found to link the between *PSEN1* gene polymorphism and disease pathogenesis.

Alzheimer Hastalarında *PSEN1* Geni Kodlayan Bölgelerinde ve *APP* Geni 16-17. Ekzonlarında Mutasyon Taraması ve *APOE* Genotiplendirmesi

Anahtar Kelimeler
Alzheimer hastalığı, Polimorfizm, Mutasyon, *PSEN1*, *APP*, *APOE*

Öz: Bu çalışmanın amacı, Alzheimer hastalarında (AD) presenilin-1 (*PSEN1)* geninin tüm ekzonları ve amiloid precursor protein (*APP*) geni 16-17. ekzonlarında mutasyon taraması gerçekleştirmek ve hastaların apolipoprotein-E (*APOE*) genotipini belirlemektir. *PSEN1* geni tüm ekzonları ve *APP* geni 16-17.ekzonları, DSM-IV kriterlerine göre teşhis edilen 30 geç başlangıçlı Alzheimer hastası bireyde (GBAH) ve 40 demans tanısı bulunmayan kontrol bireyde DNA dizi analizi ile taranmıştır. Ek olarak, *APOE* genine ait ε2, ε3 ve ε4 polimorfizmlerinin genotip ve allel frekansları her iki grupta PZR-RFLP methodu kullanılarak belirlenmiştir. *PSEN1* geni kodlayan bölgelerinde ve *APP* geni 16-17.ekzonlarında herhangi bir mutasyona rastlanılamamıştır. Ancak 26 hastada *PSEN1* geni intron 8 bölgesinde rs165932 (G/T) polimorfizmi tespit edilmiştir. Bununla birlikte intronik polimorfizmin, genotip ve allel frekansları açısından kontrol ve hasta grupları arasında anlamlı bir fark bulunamamıştır ($p>0.05$). *APOE* ε3/ε4 genotipi hasta grubunda önemli derecede yüksek oranda iken ($p<0.05$) ε4 alel frekansı GBAH olgularında anlamlı derecede yüksek bulunmuştur. Hasta grubunda *PSEN1* genotip dağılımı ve ε4 alel frekansı birlikte değerlendirildiğinde anlamlı bir ilişki bulunamamıştır ($p>0.05$). Çalışmamızda, GBAH ve ε4 alel

frekansı arasında önemli bir ilişki olduğu belirlenirken, *PSEN1* geni rs165932 (G/T) polimorfizmi ve hastalık patogenezi arasında herhangi bir ilişki bulunamamıştır.

1. INTRODUCTION

AD has a genetic structure that complex. Firstly, a genetic defect causing autosomal-dominant AD on chromosome 21 was identified and subsequently it was displayed to be mutations in the *APP* gene [1].

Up to now more than 30 pathogenic mutations have been identified in the *APP* gene [2], various mutations or polymorphisms in this gene lead to excessive of Aβ proteins or affect its separating into Aβ40 and Aβ42 peptides. Aβ42 is a major peptide playing a role in the pathogenesis of AD. Additionally, most of the genetic alterations are located in exons 16 and 17 of *APP* gene, these exons code the Aβ region of APP protein. Changes of sequences in these regions affect the cleavage recognition sites in APP protein structure and result in altered proteolytic processing of APP [3].

EOAD is associated with mutations in the *PSEN1* gene located on 14q24.2. The *PSEN1* gene has ten coding (exons 3-12) and three non-coding regions (1A, 1B and 2). This gene encodes a protein of 467 amino acid residues and this protein contains 9 transmembrane domains and a one large hydrophilic loop region [4]. To date, more than 230 different mutations, have been identified in the *PSEN1* gene [5]. The presence of missense mutations in *PSEN1* gene is responsible for the aggressive progression of EOAD. The role of *PSEN1* gene is not clear for the case of LOAD except the contributions to familial EOAD. Recent studies show that the biological pathway involving *PSEN1* gene, is quite important and defects in this pathway could contribute to LOAD pathogenesis.

APOE is one of the lipid transport proteins in the plasma and central nervous system. In humans, the *APOE* gene is located in the 19q13.2 region and ε2, ε3 and ε4 are three common isoforms of APOE. According to previous studies, heterozygous individuals with *APOE* ε4 allele have 2-3 fold risk of developing LOAD, whereas homozygous individuals with *APOE* ε4 allele have more than 8 fold risk of this disease [6]. Additionally, it is suggested that the ε2 allele has a protective effect on the developing of AD, whereas ε3 allele has no effect [7].

In this study, it was aimed to screen the *PSEN1* gene, 16-17 exons of *APP* gene and to evaluate the genetic association of *APOE* gene polymorphisms and LOAD in a Turkish patient group. The possible effects of these genes were investigated on disease severity.

2. MATERIAL AND METHODS

2.1. Patients and Control Group

30 patients with LOAD and 40 non-affected, age-matched controls in Gulhane Military Medical Academy, Department of Neurology, Ankara, Turkey participated in this study. The demographic variables of the study groups were shown in Table 1. The diagnosis of LOAD was based on the criteria of the DSM-IV. All clinical and neurological examination were performed for each participant. Given detailed family history, 6 patients had a family history of dementia.

Table 1. Demographic characteristics of control and patient group

	Control group (n=40)	**Patient group** (n= 30)
Age	60.68	69.3
Gender	32M/8W	13M/17W
FHD*	0	6

*FHD: Family history of dementia

Blood samples were collected according to ethical rules determined by the Hacettepe University, Non-Invasive Clinical Research Ethics Committee Blood samples were collected according to ethical rules determined by the Hacettepe University, Non-Invasive Clinical Research Ethics Committee (Decision number: TBK12/10-15). Genomic DNA was isolated from peripheral blood leucocytes via the phenol/chloroform protocol. The amount and purity of DNA samples were quantified and were stored at -20°C.

2.2. *PSEN1* and *APP* Sequence Analysis

All patients were subjected to sequencing for all exons of *PSEN1* gene and 16-17 exons of *APP* gene. Primers were used to amplify exons of *PSEN1* and *APP* amplification sizes were shown in Table 2. Amplification condition was 94°C for 2 min for denaturation, followed by 30 cycles a 94°C for 30 s, 64°C for 30 s, and 72°C for 30 s, and a final extension phase at 72°C for 2 min. PCR products were visualized on 1,5% agarose gel by electrophoresis. PCR products were purified with enzymatic purification (Exo-SAP) method before sequencing.

Table 2. For amplification of *PSEN1* and *APP* gene primer pairs

Primers	5'-3' sequence	Amplification size
PSENI-3F PSENI-3R	5'-TCTGCTTAATGTAATCTATGAAAGTG-3' 5'-AGATGATAAGTGAATCCAGTCTGG-3'	277 bp
PSENI-4F PSENI-4R	5'-CCAGGTCTAACCGTTACCTTGAT-3' 5'-GCCTTCAAGGTGATGATGACATG-3'	399 bp
PSENI-5F PSENI-5R	5'-TCTGTGTTGGAGGTGGTAATGTG-3' 5'-CTAGATCAGTTAAGTTACTGTGAC-3'	303 bp
PSENI-6F PSENI-6R	5'-AAGTGAGACCCTGTCTCAAAAAAG-3' 5'-CTTCAGAGTAATTCATCAACAAAGTA-3'	262 bp
PSENI-7F PSENI-7R	5'-GAGCCATCACATTATTCTAAATAATG-3' 5'-AAAGAAAACACTCCAGTGGGGCA-3'	359 bp
PSENI-8F PSENI-8R	5'-TACCACCCATTTACAAGTTTAGCC-3' 5'-TTACATGTGCTTCAGTTCCGATAAA-3'	233 bp
PSENI-9F PSENI-9R	5'-AAGACTGGCGATTTGTGTGGAGA-3' 5'-AGTCTATGACCAAAGAAAGACGATA-3'	282 bp
PSENI-10F PSENI-10R	5'-CTGTTTCCATGTAATTTTCTTAAAGG-3' 5'-AATTAGACTGTAACACTCTCAATTCA-3'	352 bp
PSENI-11F PSENI-11R	5'-ATTCATTGTGGGGTTGAGTAGGG-3' 5'-ACAGAACTGCCTTAAAGGGACTG-3'	297 bp
PSENI-12F PSENI-12R	5'-ATGTTAAAAACCAAGACTTGTGATTG-3' 5'-CCGGGAATCTTGACTTTGTTAGAT-3'	356 bp
APP-16F APP-16R	5'CTAACTTCAGGCCTAGAAAGAAGT-3' 5'CCTTAATTTGATTTCTAGCACAGGA-3'	364 bp
APP-17F APP-17R	5'ATCCAAATGTCCCCTGCATT+-TAAG-3' 5'GAGATACTTAGCTAGTTCTTAGCAA-3'	348 bp

Sequencing of purified PCR products was performed by using the Big Dye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction condition was at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, followed by 25 cycles. Then the products were purified using ethanol/sodium acetate precipitation method and electrophoresed on an ABI PRISM 310 Genetic Analyzer.

2.3. *APOE* Genotyping

APOE gene that consisted of polymorphic regions (codons 112 and 158) was amplified using PCR and a 295-bp fragment was obtained. The forward and reverse primers were 5'-GAA CAA CTG ACC CCG GTG GCG-3' and 5'-GGA TGG CGC TGA GGC CGC GCTC-3', respectively. PCR was carried out in a total volume of 25 µl containing 2 µl of genomic DNA, 2,5 µl of 10X PCR buffer (without Mg^{2+}), 1,5 µl 25 mM of $MgCI_2$, 1 µl of 2.5 mM dNTP mix, 1 µl of 10 µM for each primer, 10% DMSO, 1U Taq DNA Polymerase Sigma ® (5 U μl^{-1}) and 13,5 µl sterile distilled water. PCR conditions were as follows: initial denaturation at 94°C for 4 min, denaturation at 94°C for 45 s, annealing at 62°C for 45 s, extension at 72°C for 45 s and further elongation at 72°C for 5 min, for 30 cycles. PCR products were subjected to digestion with the restriction enzyme, *HhaI*, Thermo Scientific ® (10 U μl^{-1}) at 37°C overnight and visualized on 10% polyacrylamide gel stained with ethidium bromide. According to restriction enzyme digestion results, fragment sizes of ε2/ε2 were 91 and 83 bp, ε3/ε3 were 91 bp and 48 bp, ε4/ε4 were 72 and 48 bp and these were homozygous genotypes. For heterozygous genotypes; ε2/ε3 were 91, 83 and 48 bp, ε3/ε4 were 91, 72 and 48 bp, ε2/ε4 were 91, 83, 72 and 48 bp [8]. In our study, five different *APOE* genotypes were observed (ε3/ε3, ε2/ε3, ε3/ε4, ε2/ε4 and ε4/ε4).

2.4. Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) Version 21 (Chicago, IL, USA). Genotype and allele frequencies were presented as percentages. The comparison of allele and genotype frequencies between patients and controls was performed by chi-square ($\chi 2$) test. In patient group, the relationship between *APOE* gene ε4 allele and *PSEN1* gene rs165932 genotypes were compared using $\chi 2$ test. $\chi 2$ test was used to compare the presence of ε4 allele between LOAD individuals with or without familial dementia history.

3. RESULTS

3.1. Sequencing Analysis of *PSEN1* and *APP* Genes

As a result, we did not find any mutations in the coding regions of *PSEN1* gene and 16-17 exons of *APP* gene. On the other hand, rs165932 (G/T) polymorphism was found in intron 8 of *PSEN1* in 26 patients. To investigate the relationship between this polymorphism and LOAD, this region was screened by DNA sequence analysis in the control group. The distribution of genotype and allele

frequencies of rs165932 (G/T) polymorphism were shown in Appendix A.

However, there was no significant difference between control group and patients in terms of genotype and allele frequencies of rs165932 (G/T) polymorphism ($p>0.05$).

3.2. Genotyping *APOE* Alleles

The assessments were performed on 30 patients diagnosed with LOAD and 40 controls without a history of dementia. The *APOE* genotype was identified by the digested fragments obtained from *APOE* gene PCR products. The results of *APOE* genotype were shown in Figure 1.

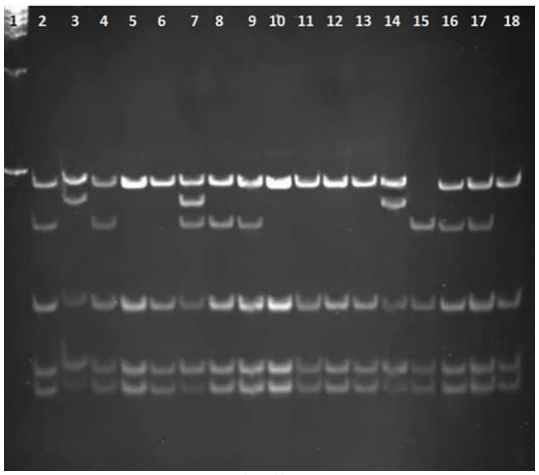

Figure 1. PAGE results of different APOE genotypes. Sample 1 is a DNA ladder (100 bp). Sample numbers of 2, 4, 8, 9, 16 and 17 are type ε3/ε4. Sample numbers of 5, 6, 10, 11, 12, 13 and 18 are type ε3/ε3. Sample numbers of 3 and 14 are type ε2/ε3. Sample 7 is type ε2/ε4. Sample 15 is type ε4/ε4

Appendix B shows the distribution of each genotype and the allele frequencies of *APOE* gene.

When we compared the different *APOE* genotype frequencies among the patient and control group, ε3/ε3 genotype frequency was significantly higher in the control group while ε3/ε4 genotype frequency was higher in the patient group ($p<0.05$). In our study, ε2/ε2 homozygous genotype was not detected in both groups (Figure 2).

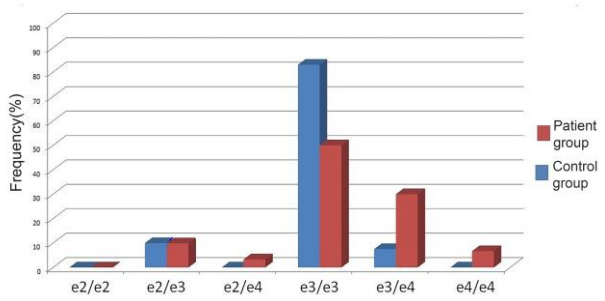

Figure 2. Distrubution of *APOE* genotype frequencies in control and patient groups

The frequency of the ε3 allele in the control group was significantly higher than in patient group ($p<0.05$). In addition, the frequency of ε4 allele in the patient group was significantly higher than in the control group ($p<0.05$). There was a significant difference between control and the patient groups in terms of ε4 allele carrier ($p<0.05$). When we compared the presence of ε4 allele in LOAD individuals with familial dementia history and without familial dementia history, we found that ε4 allele frequency was significantly higher at LOAD individuals with familial dementia history ($p<0.05$).

When we consider the distribution of *PSEN1* genotype and ε4 allele carrier in our patient group, we did not find any significant difference between them ($p<0.05$).

4. CONCLUSION

AD has two main subtypes, EOAD and LOAD. EOAD starts before the age of 65 and accounts for 1-5% of all cases. Both subtypes of AD may have familial and sporadic conditions [9].

The presence of missense mutations in *PSEN1* gene is responsible for the aggressive progression of EOAD. The role of *PSEN1* gene is not clear in the pathologic pathway of LOAD except the contributions to familial EOAD [10]. Recent studies show that the biological pathway including *PSEN1* gene is quite important and defects in this pathway could contribute to LOAD [11-15].

A study on RT-PCR analysis of *PSEN1* gene in individuals that historically confirmed sporadic cases and in individuals that had normal brain tissues who were at the same age with sporadic cases was conducted and no change was observed in the coding region of *PSEN1* gene. Therefore, it was claimed that the mutation frequency of *PSEN1* coding region is low in sporadic cases [16]. Similar to that study, in our study, we did not find any *PSEN1* coding region mutation in our patient group. As well as some results showed that variations of *PSEN1* have no effect on LOAD pathogenesis [17], there is also an evidence that *PSEN1* rare variants have a contribution to this pathogenesis [13,15,16]. However, many of these studies investigating the relationship between *PSEN1* and LOAD on molecular basis could not find a strong relationship as in our study. Investigation of the promoter region of *PSEN1* gene or the interactions of other variables with the regulatory regions of *PSEN1* gene, and also the determination of the relationship of disease variables are really important. Thus, these studies may highlight the function of this polymorphism. In our study, we detected polymorphism in intron 8 of the *PSEN1* gene which is associated with LOAD. Our results do not support the relationship between the 1/1 and 2/2 genotypes and the increase of the AD risk [18-20]. It is important to determine how a polymorphism affects an alternative splice process.

In addition, LOAD patients' samples were examined for mutations in exon 16 and exon 17 of *APP* gene by DNA sequence analysis. Similarly, mutation screening efforts in *APP*, which have been restricted to exons 16 and 17,

have included on LOAD families [3,13,21-24]. Some studies found variations of *APP* gene on LOAD patients [3,13,23] but the others did not find mutations or polymorphism on LOAD patients [21,22,24].

In addition, we have investigated the genetic association of *APOE* gene polymorphisms with LOAD in a group of Turkish patients and we found a significant association between *APOE* and LOAD. The frequency of ε4 allele was significantly higher in patients with LOAD ($p<0.05$). There were 6 individuals with familial dementia history in our patient group. In the patients with family history of dementia, ε4 allele frequency was significantly higher than the patients without dementia history ($p<0.05$). The studies have shown that the *APOE* ε4 allele is related to LOAD in several populations. It is suggested that the carrying of ε4 allele increases the risk of developing AD, whereas the carrying of ε2 allele decreases the risk of developing AD [25]. Taken together, in this study, there was no significant difference in both groups in terms of ε2 allele carrying ($p>0.05$) (Appendix A).

We have investigated the combined effect of *PSEN1* and *APOE* polymorphisms on the risk of LOAD, which have also been not observed in other studies [1,6,26]. Also, when we have considered together *PSEN1* genotype distribution and ε4 allele carrying in our patient group, it was not found any significant differences between them ($p>0.05$).

However, we can explain this observation with the small size of *APOE* ε4 allele carriers. In substance, previously performed studies on the genetics of AD showed that AD is a complex disorder in which both genetic and environmental factors are effective [25]. A family history of dementia has shown that the presence of dementia in pedigree increases the risk of AD. It has been reported that this risk is a relative increase and varies according to situations such as having first degree relatives, evaluation based on the whole family history, maternal and paternal transition [27,28,29].

There are many different results about *APOE* and *PSEN1* gene polymorphisms related to AD in the literature [3,4,6,7,18,19,20,25,26,30,31,32].

These different results may be explained by ethnic or regional differences and different sample sizes. In our study, while there was a strong association between AD and APOE ε4 allele, there was no relationship between *PSEN1* gene rs165932 (G/T) polymorphism and disease pathogenesis. As a conclusion, *APOE* and *PSEN1* polymorphisms or also other related gene should be studied in comprehensive trials.

Declaration of interest

This study is not subject to any conflicts of interest.

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Appendix

Appendix A. Distribution of the *PSEN1* genotype and allele frequencies in control and patient group (*p* values were obtained from $\chi 2$ test).

Appendix B. Distribution of the *APOE* genotype and allele frequencies in control and patient group (*p* values were obtained from $\chi 2$ test).

Appendix A. Distribution of the *PSEN1* genotype and allele frequencies in control and patient group (*p* values were obtained from $\chi 2$ test).

Genotype	Control group (N=40)(%)	Patient group (N=30)(%)	*p*
1/1(TT)	13(32.25)	11(36.7)	0.455
1/2(TG)	21(52.5)	15(50.0)	0.514
2/2(GG)	6(15.0)	4(13.3)	0.563
Allel			
1(T)	47(58.75)(n=80)	37(61.7)(n=60)	0.432
2(G)	33(41.25)(n=80)	23(38.3)(n=60)	0.432

Appendix B. Distribution of the *APOE* genotype and allele frequencies in control and patient group (*p* values were obtained from $\chi 2$ test).

Genotype	Control group (N=40)(%)	Patient group (N=30)(%)	*p*
ε2/ε2	0(0.0)	0(0.0)	-
ε2/ε3	4(10.0)	3(7.5)	0.660
ε2/ε4	0(0.0)	1(3.3)	0.429
ε3/ε3	33(82.5)	15(50.0)	0.004
ε3/ε4	3(7.5)	9(30.0)	0.016
ε4/ε4	0(0.0)	2(6.7)	0.180
Allel			
ε2	4(5.0)(n=80)	4(6.7)(n=60)	0.437
ε3	73(91.3)(n=80)	42(70.0)(n=60)	0.001
ε4	3(3.7)(n=80)	14(23.3)(n=60)	0.001