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ORIGINAL RESEARCH

Genotyping Apolipoprotein E (APOE) Isoforms with Sequence-Specific-Primer (SSP)-PCR in Early-Onset Alzheimer's Disease Patients: A Rapid and Revised Methodology

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that causes progressive damage to brain cells, leading to impairment in cognitive functions. The Apolipoprotein E (APOE) variants play a significant role in the genetic basis of AD, especially late-onset AD (LOAD), and increase the disease risk at an earlier age. Although controversial, some studies reveal the association between *APOE* genotype and early-onset AD (EOAD) regardless of family history. Therefore, diagnostic laboratories widely perform routine tests to determine the *APOE* genotype. In the present study, we implemented a revised methodology for the Sequence-Specific-Primer-PCR (SSP-PCR) test for rapid *APOE* genotyping in 67 EOAD patients. Then, the findings were validated using automatic sequencing with newly designed primers for the related region of the *APOE*. We state clearly that the applicability of the SSP-PCR method was improved when the primer concentrations of control genes were increased 2-fold, as we reported. All data obtained from SSP-PCR were consistent with Sanger sequencing confirmations. Based on the genotyping results, the four different *APOE* genotypes were detected: E2/E4, E3/E3, E3/E4, and E4/E4. The frequencies were 1.5% (n=1) for E2/E4, 76.1% (n=51) for E3/E3, 16.4% (n=11) for E3/E4, and 6% (n=4) for E4/E4. In the study group, 23.9% (n=16) of the patients had homozygous or heterozygous APOE E4. However, we detected no significant association between the clinical features and the *APOE* genotype. As a result, this method is reliable, cost-effective, and rapid for performing genotyping analysis of the *APOE* for routine tests and research studies with larger EOAD cohorts.

Keywords: APOE. Early-onset Alzheimer's disease. SSP-PCR. Sanger sequencing.

Erken Başlangıçlı Alzheimer Hastalarında Sekans-Spesifik-Primer (SSP)-PCR ile Apolipoprotein E (APOE) İzoformlarının Genotiplendirilmesi: Hızlı ve Revize Bir Metodoloji

ÖZET

Alzheimer hastalığı (AD), beyin hücrelerinde ilerleyici hasara neden olan ve bilişsel işlevlerde bozulmaya yol açan nörodejeneratif bir hastalıktır. Apolipoprotein E (APOE) varyantları, özellikle geç başlangıçlı AD (LOAD) olmak üzere AD'nin genetik temelinde önemli bir rol oynar ve hastalık riskini daha erken yaşta artırır. Tartışmalı olmasına rağmen, bazı çalışmalar *APOE* genotipi ile erken başlangıçlı AD (EOAD) arasındaki ilişkiyi aile geçmişinden bağımsız olarak ortaya koymaktadır. Bu nedenle, tanı laboratuvarları *APOE* genotipini belirlemek için yaygın olarak rutin testler yapmaktadır. Mevcut çalışmada, 67 EOAD hastasında hızlı *APOE* genotiplemesi için Sekans-Spesifik-Primer-PCR (SSP-PCR) testi için revize edilmiş bir metodoloji uyguladık. Ardından, bulgular *APOE*'nin ilgili bölgesi için yeni tasarlanmış primerlerle otomatik sekanslama kullanılarak doğrulandı. Bildirdiğimiz gibi, kontrol genlerinin primer konsantrasyonları 2 kat artırıldığında SSP-PCR yönteminin uygulanabilirliğinin arttığını açıkça belirtiyoruz. SSP-PCR'den elde edilen tüm veriler Sanger dizileme doğrulamalarıyla tutarlıydı. Genotipleme sonuçlarına dayanarak dört farklı *APOE* genotipi tespit edildi: E2/E4, E3/E3, E3/E4 ve E4/E4. Sıklıklar E2/E4 için %1.5 (n=1), E3/E3 için %76.1 (n=51), E3/E4 için %16.4 (n=11) ve E4/E4 için %6 (n=4) idi. Çalışma grubunda hastaların %23.9'unda (n=16) homozigot veya heterozigot APOE E4 vardı. Ancak klinik özellikler ile *APOE* genotipi arasında anlamlı bir ilişki tespit etmedik. Sonuç olarak bu yöntem, rutin testler ve daha büyük EOAD kohortlarıyla yapılan araştırma çalışmaları için *APOE*'nin genotipleme analizini gerçekleştirmek için güvenilir, uygun maliyetli ve hızlıdır.

Anahtar Kelimeler: APOE. Erken başlangıçlı Alzheimer hastalığı. SSP-PCR. Sanger dizileme.

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Alzheimer's disease (AD), the most common neurodegenerative disorder in older adults, is characterized by severe memory loss, deterioration in learning, language, and comprehension, and a decline in cognitive functions such as reasoning and decision-making[1,2]. The primary risk factor for AD is age. The disease is classified as early- (EOAD) or late- (LOAD) onset based on the age 65 at which patients are diagnosed with AD[3]. Clinical symptom heterogeneity is frequently observed in both groups[4,5]. In addition to age, some environmental and genetic risk factors are the other significant contributors to the pathogenesis of AD. Among the genetic factors identified to date, the most reliable one is Apolipoprotein E (APOE)[6].

APOE, a lipid metabolism glycoprotein expressed by many tissues, including the brain, is encoded by the *APOE* gene (19q13.2), which is highly polymorphic at the two nucleotide residues, rs429358 (2059T/C) and rs7412 (2197C/T). The polymorphisms result in three *APOE* alleles as ε2, ε3, and ε4 compromised of six genotypes as ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4. The protein isoforms of these variants, which differ at the amino acid residues 112 and 158, are APOE 2 (cysteine 112 & cysteine 158), APOE 3 (cysteine 112 & arginine 158), and APOE 4 (arginine 112 & arginine 158)[7,8]. The frequencies of these variants vary population-based[9]. Individuals who carry homozygous APOE 4 have a substantial risk, up to 15-fold, for LOAD at an earlier age and also EOAD risk regardless of family history. In contrast, APOE 2 can potentially delay the age of onset[10-13]. Moreover, some studies have reported that the APOE genotype is associated with a faster or slower cognitive decline[14]. In an EOAD cohort, the lack of APOE 4 correlated with more rapid cognitive decline in non-memory domains[15]. Therefore, determining the APOE genotype is clinically important, considering that it contributes to disease progression and AD risk related to age of onset in both EOAD and LOAD.

There are currently many methods in the literature, some frequently used for routine *APOE* genotyping. However, among these methods, the most practical and cost-effective ones are fewer in number. The best-known techniques for *APOE* genotyping are Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), automatic DNA sequencing, Amplification Refractory Mutation System-PCR (ARMS-PCR), Real Time-PCR (RT-PCR), allele-specific RT-PCR with or without TaqMan probes, etc.[16-18]. All of them are one or two-step DNA-based tests with possible practical use in routine diagnostics. However, cost-effectiveness and accurate results with less equipment prevent the limitations that strain the laboratory's workforce and technical infrastructure. In this respect, one of the most advantageous tests is Sequence-Specific-Primer-PCR (SSP-PCR), introduced by Pantelidis et al.[19]. The steps of this method include amplification and agarose gel electrophoresis for the three APOE alleles in each patient's DNA sample, providing accurate results within a few hours at a lower cost and allowing to work with multiple patients simultaneously. In our study, we revised the allele-specific primer concentration and ensured a brief, detailed calculation, making it easier for the method to work in a highly optimized way. Then, to validate the results obtained, we performed automatic DNA sequence analysis by capillary electrophoresis using a single primer pair that we designed specifically for the relevant region, exon 4 of the *APOE* gene.

In the present study, we genotyped APOE isoforms using a revised and detailed Sequence-Specific-Primer (SSP)-PCR method confirmed by Sanger DNA sequence analysis. Thus, this study aims to test the usability of the modified method in our laboratory for the EOAD study group and to compare genotypic findings with clinicopathological features in a Turkish EOAD cohort.

Material and Method

Patient group

A total of 67 EOAD patients were enrolled in the study. All patients were diagnosed with EOAD by the neurologist MB, as previously reported[20]. The same neurologist carried out clinical examinations and neuropsychological assessments of the patients. All patients were screened with the Mini-Mental State Examination as a neuropsychological test (NPT). Then, the results were grouped as normal (25-30), mild (20-24), moderate (13-20), and severe (12 and below) based on the total test score of 30[21]. There was no association between the age of onset and sex in the study group. Family history was considered positive if a patient had at least one relative previously diagnosed with dementia. A signed voluntary consent form was obtained from all patients. This study was also approved by the Medical Ethics Committee (2023-19/37) and complied with the ethical standards of the Helsinki Declaration.

DNA isolation and sequence-specific-primer SSP-PCR analysis

DNA isolation was obtained from the peripheral blood samples of the patients according to the E.Z.N.A. Blood DNA Mini Kit (Omega Bio-Tek, Norcross, GA) instructions. Then, the SSP-PCR protocol was performed as the following. For each patient, the three PCR tubes were prepared, comprising primer mix 1 for *APOE* 2 (P1-P3), primer mix 2 for *APOE 3* (P1-P2), and primer mix for 3 *APOE 4* (P2-P4). The primers P1, P2, P3, and P4 were synthesized with the same sequence reported in the previous study[19].

However, we revised and briefly reported the primer stock concentrations and serial dilutions for each primer (Table I). All details, including revisions regarding the primers, are indicated in bold in Table I. Then, the touchdown-PCR method was applied to all PCR mixes with these primer pairs. The touchdown-PCR components used, including 5 U/µl Hot-Start AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) DNA Polymerase, were mixed for each *APOE* allele, as shown in Table II. The PCR conditions were as follows: 96°C for 1 min to denature DNA samples initially; subsequently by 5 cycles of 96°C for 20 s, 70°C for 45 s, and 72°C for 25 s; 21 cycles of 96°C for 25 s, 65°C for 50 s, and 72°C for 30 s; 4 cycles of 96°C for 30 s, 55°C for 60 s, and 72°C for 2 min. After PCR, we carried out a 2% Tris-boric acid-EDTA agarose gel electrophoresis with 2 µl ethidium bromide for the three alleles in each patient and a simultaneous control amplification for the *HLA-DRB1* gene. Based on the band profile, the patients were defined as homozygous or heterozygous carriers of APOE 2, APOE 3, or APOE 4. Suppose the band was visualized and obtained from primer 1 mix alone; in that case, the patient was defined as homozygous E2/E2, or if the bands were detected obtained from primer 1 and primer 3, the patient was categorized as heterozygous E2/E4, etc. This analysis was repeated for samples for which no band was obtained from the control amplification of the *HLA-DRB1* gene.

Table I. The primers used in the SSP-PCR and Sanger sequence validation analysis for *APOE* genotyping

Primers	Region	Analysis	Sequence (5'→3')	Orientation	Stok concentration	First dilution w/dH_2O	Second dilution w/dH_2O	Final volume for PCR	Final concentration for PCR
DSYM1 (F1)	***APOE*, exon 4 region**	**Sanger sequencing**	**CGGCTGTCCAAGGAGCTG**	**Forward**	**100 µM, 125 µg**	**1:10**	**None**	**1.25 µl in 25 µl reaction mix**	**0.625 µg**
DSYM2 (R1)	***APOE*, exon 4 region**	**Sanger sequencing**	**CCTGTTCCACCAGGGGC**	**Reverse**	**100 µM, 117 µg**	**1:10**	**None**	**1.25 µl in 25 µl reaction mix**	**0.625 µg**
DSYM3 (F2)	Internal control (*HLA-DRB1*)	SSP-PCR	TGCCAAGTGGAGCACCCAA	Forward	100 µM, 133 µg	**1:10**	**1:10**	**0.98 µl in 13 µl reaction mix**	**13 ng**
DSYM4 (R2)	Internal control (*HLA-DRB1*)	SSP-PCR	GCATCTTGCTCTGTGCAGAT	Reverse	100 µM, 180 µg	**1:10**	**1:10**	**0.72 µl in 13 µl reaction mix**	**13 ng**
DSYM5 (P1)	APOE_112 cysteine site	SSP-PCR	CGGACATGGAGGACGTGT	Forward	100 µM, 90 µg	1:10	None	1.1 µl in 13 µl reaction mix	50 ng
DSYM6 (P2)	APOE_158 arginine site	SSP-PCR	CTGGTACACTGCCAGGCG	Reverse	100 µM, 143 µg	1:10	None	0.70 µl in 13 µl reaction mix	50 ng
DSYM7 (P4)	APOE_158 cysteine site	SSP-PCR	CTGGTACACTGCCAGGCA	Forward	100 µM, 88 µg	1:10	None	1.14 µl in 13 µl reaction mix	50 ng
DSYM8 (P3)	APOE_112 arginine site	SSP-PCR	CGGACATGGAGGACGTGC	Reverse	100 µM, 131 µg	1:10	None	0.76 µl in 13 µl reaction mix	50 ng

Table II. The PCR components used for determining *APOE* alleles

Components	E2	E3	E4	
Control primer F		0.98 µl	0.98 µl	0.98 µl
Control primer R		0.72 µl	0.72 µl	0.72 µl
P1 (Forward)		1.1 µl	1.1 µl	
P2 (Reverse)			0.70 µl	0.70 µl
P3 (Reverse)		0.76 µl		
P4 (Forward)				1.14 µl
Primer total			~ 3.5 µl	
1X PCR Buffer (10X)			1.3 µl	
2 mM $MgCl_2$ (25mM)			1.5 µl	
150 µM dNTPs			1.25 µl	
0.5 U Taq polymerase (5 U/µl)			0.1 µl	
0.15 µg DNA			3 µl	
dH_2O			2.35 µl	
Total PCR reaction			**13 µl**	

Capillary electrophoresis

After obtaining SSP-PCR results, genotyping data were confirmed by Sanger sequencing. The region covering both rs429358 and rs7412 in the exon 4 of the *APOE* gene was amplified by the specific primer pair that was first designed by the tool at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The primer pairs (5'→3') were synthesized as F: CGGCTGTCCAAGGAGCTG and R: CCTGTTCCACCAGGGGC and used to amplify the 289 base pairs *APOE* exon 4 products at the annealing temperature of 66°C with a classical PCR condition of 35 cycles. Then, conventional Sanger sequencing was performed with the purified samples using a PCR-

product sequencing kit in CEQ-8000 Automated DNA Sequencing System (Beckman Coulter Inc., Fullerton, CA), as previously reported[20]. All genomic data was compared to detect sequence variations of the transcript, APOE-001 ENST00000252486. The results were compared to the findings from SSP-PCR analysis.

Statistics

For statistics, the IBM SPSS Statistics 25.0 (SPSS Inc., USA) program was used. While the frequency was reported for categorical variables, the mean ± SD was reported for continuous ones. The $\chi 2$ test was used to compare all categorical variables. One-way ANOVA and *t*-test were used to compare the age of onset and genotyping results. A *p*-value less than <0.05 was considered statistically significant.

Results

A total of 67 unrelated EOAD patients were enrolled in the study. While 58.2% (n=39) of the patients were female, 41.8% (n=28) were male. Family history was positive for 43.3% (n=29) of the patients. The age of onset of EOAD was 52.10 ± 8.10 in the study group. The NPT results were grouped as normal, mild, moderate, and severe based on the total test score. Thus, 9% (n=6) of the patients had normal, 23.9% (n=16) had mild, 47.8% had moderate, and 19.4% (n=13) had severe dementia findings. Among the patients, the folic acid levels were low in 19.4% (n=13), the TSH levels were low in 7.5% (n=5), and the B12 levels were low in 13.4% (n=9). Atrophy was detected on MRI in 70.1% (n=47) of the patients.

We first analyzed the band profile of each patient after the SSP-PCR step. We showed that the bands associated with amplifying the control *HLA-DRB1* gene were visibly obtained from all reactions with the primer concentration of 13 ng. Among the study group, four different band profiles were detected from primer mix 1 and primer mix 3 (heterozygous E2/E4), primer mix 2 (homozygous E3/E3), primer mix 2 and primer mix 3 (heterozygous E3/E4), and primer mix 3 (homozygous E4/E4), as indicated in Figure 1A. All results were confirmed by Sanger sequencing with automatic capillary electrophoresis (Figure 1B). No mismatch was detected in any of the findings, and we obtained 100% concordance between the SSP-PCR method and Sanger sequencing analysis for *APOE* genotyping.

Based on the genotyping results, the four different *APOE* genotypes were detected in the study group: E2/E4, E3/E3, E3/E4, and E4/E4. The frequencies were 1.5% (n=1) for E2/E4, 76.1% (n=51) for E3/E3, 16.4% (n=11) for E3/E4, and 6% (n=4) for E4/E4. In the study group, 23.9% (n=16) of the patients had homozygous or heterozygous APOE 4 allele. However, when the clinical parameters and the genotyping results were compared, we did not find any statistically significant association for any group, specifically regarding family history and age of onset. Moreover, since the APOE 4 allele is considered a risk factor for AD, a comparison was made between APOE 4 carriers and other patients; no statistically significant result was detected for E4 carriers, as shown in Table III.

Table III. Comparison of the clinical features and *APOE* genotyping results in the study group

Feature	*APOE* genotype (%, n)				*p*-value	*APOE* alleles (%, n)		*p*-value
	E2/E4	E3/E3	E3/E4	E4/E4		E3 (+)	E4 (+)	
Sex								
Female	1 (2.6%)	31 (79.5%)	5 (12.7%)	2 (5.2%)	0.636	31 (79.5%)	8 (20.5%)	0.637
Male	-	20 (71.4%)	6 (21.5%)	2 (7.1%)		20 (71.4%)	8 (28.6%)	
Age of onset (mean ± SD)	50.2 ± 9.1	52.5 ± 8.06	50.25 ± 6.94	58	0.633	52.54 ± 8.06	50.68 ± 8.39	0.622
Family history								
Positive	1 (3.4%)	22 (75.8%)	3 (10.4%)	3 (10.4%)	0.251	22 (75.8%)	7 (24.2%)	1.00
Negative	-	29 (76.3%)	8 (21.1%)	1 (2.6%)		29 (76.3%)	9 (23.7%)	
NPT Results								
Normal	-	6 (100%)	-	-	0.415	6 (100%)	-	0.127
Mild	-	12 (75.0%)	4 (25.0%)	-		12 (75.0%)	4 (25.0%)	
Moderate	1 (3.2%)	21 (65.6%)	6 (18.7%)	4 (12.5%)		21 (65.6%)	11 (34.4%)	
Severe	-	12 (92.3%)	1 (7.7%)	-		12 (92.3%)	1 (7.7%)	
Folic acid								
Normal	1 (1.9%)	42 (77.8%)	7 (12.9%)	4 (7.4%)	0.344	42 (77.8%)	12 (22.2%)	0.493
Low	-	9 (69.2%)	4 (30.8%)	-		9 (69.2%)	4 (30.8%)	
TSH								
Normal	1 (1.6%)	48 (77.4%)	9 (14.5%)	4 (6.5%)	0.49	48 (77.4%)	14 (22.6%)	0.586
Low	-	3 (60.0%)	2 (40.0%)	-		3 (60.0%)	2 (40.0%)	
B12								
Normal	1 (1.8%)	44 (75.8%)	10 (17.2%)	3 (5.2%)	0.85	44 (75.8%)	14 (24.2%)	1.00
Low	-	7 (77.8%)	1 (11.1%)	1 (11.1%)		7 (77.8%)	2 (22.2%)	
Atrophy on MRI								
Positive	1 (2.1%)	35 (74.5%)	9 (19.1%)	2 (4.3%)	0.578	35 (74.5%)	12 (25.5%)	0.76
Negative	-	16 (80.0%)	2 (10.0%)	2 (10.0%)		16 (80.0%)	4 (20.0%)	

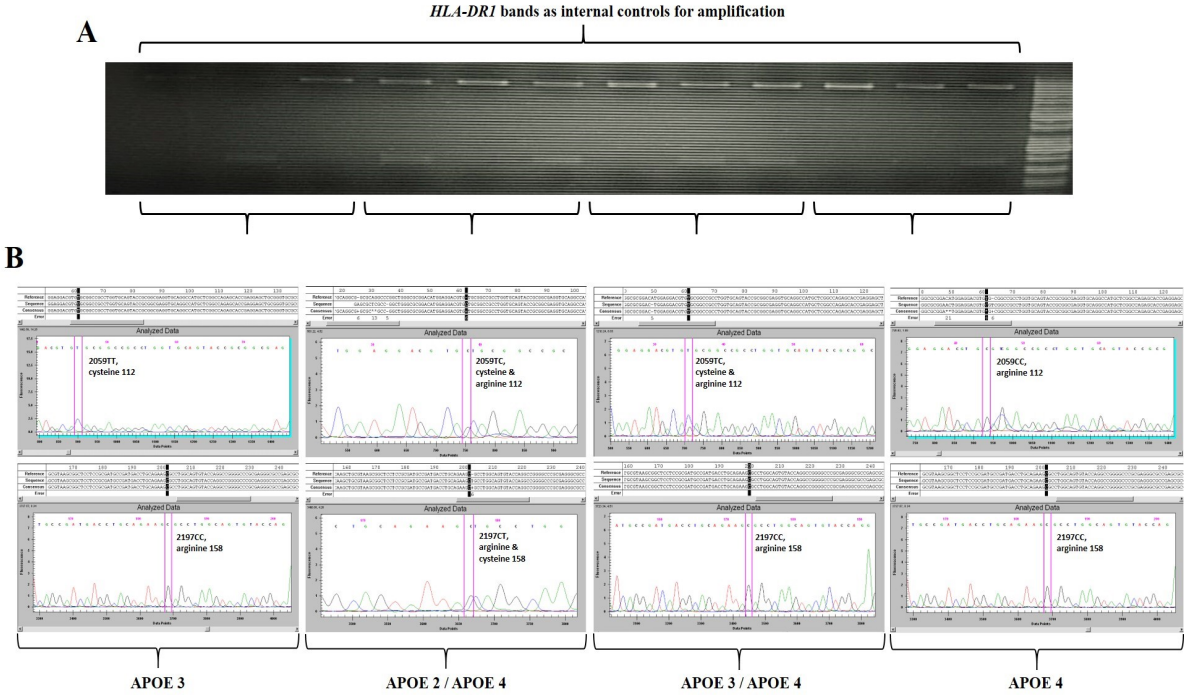

Figure 1.

(A) The SSP-PCR analysis results for the different APOE genotypes detected in the study cohort. The bands of 785 bp indicate HLA-DR1 control gene amplification in each reaction tube. The 173-bp bands are obtained from the amplification positive tubes based on the patients' APOE alleles. ***(B)*** *Sanger sequencing validates the detected genotypes for APOE 3, APOE 2 / APOE 4, APOE 3 / APOE 4, and APOE 4.*

Discussion and Conclusion

In the present study, we aimed to optimize the laboratory implementation of SSP-PCR analysis and evaluate the effectiveness of the test for routine *APOE* genotyping. We also compared the genotyping results with the clinical parameters in Turkish EOAD patients.

Currently, many methods are frequently used in the literature for routine *APOE* genotyping, such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), automatic DNA sequencing, Amplification Refractory Mutation System-PCR (ARMS-PCR), Real Time-PCR (RT-PCR), allele-specific RT-PCR with or without TaqMan probes, etc.[16-19]. All of these methods have their advantages and disadvantages, which increase or limit the usability rate of tests. However, the reliability of the genotyping test, its speed, affordability, and the requirement for minimal laboratory equipment and workforce are among the features that can transform the test into a gold standard. One of the cost-effective ones, strip assay, has been routinely used in laboratories for *APOE* genotyping based on PCR and reverse-hybridization. Despite being commercialized, this test requires many solutions and specific materials for the occurrence of enzymatic reactions, rendering the test unusable with not resistant solutions to long-term storage conditions[22,23]. Fluorescence-based methods, on the other hand, can be faster but may be more expensive, hindering the accessibility of the test for laboratories[17]. An anti-apoE4 monoclonal antibody-based method has been introduced by Calero et al. as an alternative technique to *APOE* genotyping for patient screening. However, they reported that their method cannot discriminate homozygous APOE E4/E4 from heterozygous E3/E4 and E2/E4[24]. Therefore, fast, cost-effective, long-term usability, and reliable tests are preferred.

One of the most practical and cost-effective tests is SSP-PCR[19], introduced by Pantelidis et al. We have made this test available with some modifications in our laboratory for *APOE* genotyping in the present study. Then, the results were compared using the Sanger method, the gold standard for DNA sequencing. First, we could not achieve successful results in the SSP-PCR with 6 ng control gene primers, as previously reported[19]. Thus, we increased the control gene primer concentration in each PCR mix to 13 ng rather than 6 ng for *APOE* alleles, enhancing the detection of control bands; it ensured that the test was more reliable and functioning correctly. Second, in this study, we experienced that Hot-Start Taq polymerase provided more efficient bands than those without a non-hot start feature. Moreover, with this Taq polymerase, we did not use

mineral oil for reactions, as recommended[19]. Finally, we demonstrated that a newly synthesized one primer pair could target the related region of the *APOE* gene exon 4, containing both residues of rs429358 and rs7412, indicating that the region can be analyzed by Sanger sequencing even after a single conventional PCR. However, when we compared the cost and labor per sample between Sanger sequencing and SSP-PCR, it is clear that SSP-PCR has a distinct advantage over Sanger sequencing for routine *APOE* genotyping. Furthermore, no additional findings were encountered when the results obtained from the SSP-PCR for each patient were confirmed by Sanger sequencing. Thus, *APOE* genotyping with the SSP-PCR test is reliable, practical, and cost-effective for routine applications in diagnostic laboratories.

APOE 4 allele is a strong and reliable risk factor for LOAD pathogenesis. Moreover, in recent years, some studies have reported the *APOE* genotype associated with a faster or slower cognitive decline. Moreover, in an EOAD cohort, the lack of APOE 4 correlated with more rapid cognitive decline in non-memory domains[14,15]. In our study, the genotype frequencies were detected as 1.5% (n=1) for E2/E4, 76.1% (n=51) for E3/E3, 16.4% (n=11) for E3/E4, and 6% (n=4) for E4/E4. The APOE genotype frequencies show variation based on ethnicity, as previously indicated. The genotype frequencies were reported as 1% for E2/E4, 58.3% for E3/E3, 25.8% for E3/E4, and 5.6% for E4/E4 in a study from the Turkish population with AD patients between 45-90 years[24]. We did not detect APOE E2/E2 or E2/E3 genotypes in our study group with EOAD patients alone. Despite this, E3 and E4 allele frequencies were consistent with the previous report[24]. However, since we did not show any association with the clinical disease parameters of the EOAD patients in our study, we propose the need for broader comparative studies with the larger study groups of Turkish EOAD patients to determine the contribution of the *APOE* gene to the disease symptoms.

Consequently, our study suggests that SSP-PCR is reliable, cost-effective, and rapid for performing genotyping analysis of the *APOE* in AD patients. Genotyping analysis can also be performed with the specific primer pair in a Sanger sequencing reaction. However, the SSP-PCR technique is faster and has fewer steps than the Sanger sequencing method, and it can be easily optimized and readily usable in many diagnostic laboratories.

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Idea and design: I.E.E., Ü.E.; Data collection and processing: I.E.E, M.B.; Analysis and interpretation of data: I.E.E., Ü.E., G.Ç.; Writing of significant parts of the article: I.E.E., M.B.

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