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Potential genetic biomarkers in the early diagnosis of Alzheimer disease: *APOE* and *BIN1*

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Background/aim: Alzheimer disease (AD) is triggered by interactions of multiple genetic and environmental factors. The *APOE* gene E4 allele is the best-known risk factor for AD, yet it represents a small ratio of genetic factors. According to genome-wide association studies, the *BIN1* gene is the second important risk factor for AD, following the *APOE* gene. We aimed to identify a novel biomarker indicating susceptibility to AD by investigating *APOE* alleles and *BIN1* gene polymorphisms in a Turkish population.

Materials and methods: Fifty-three AD patients and 56 controls were included to examine polymorphism and allele frequency of the *APOE* and *BIN1* genes. Genomic DNAs were isolated from whole blood by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. RFLP was done for identification of polymorphisms in the *APOE* gene and allele-specific PCR was used for the *BIN1* gene.

Results: Frequency of the *APOE* E4 allele was higher in the AD patient group, while the frequency of the E2 allele was higher in controls. The E4/E4 genotype was detected in the AD patient group, while this genotype was not observed in the controls. The frequencies of *BIN1* alleles were similar in both groups.

Conclusion: There was a strong association between AD and the *APOE* E4 allele, while no such relation was observed with *BIN1* gene polymorphism.

Key words: Alzheimer disease, *APOE*, *BIN1*, polymorphism, biomarker, RFLP

1. Introduction

Alzheimer disease (AD) is an age-dependent, irreversible neurodegenerative disease, of which the most important risk factor is old age. Its prevalence is doubled with every 5 years after 65 years of age (1). Clinical diagnosis of AD is made by exclusion of other causes of dementia. Its accurate diagnosis is only possible with postmortem neuropathological examination (2). Therefore, there is a need for determination of specific biomarkers contributing to the diagnosis of AD. These biomarkers need to be able to determine susceptibility to AD as well as establishment of diagnosis and determination of prognosis; furthermore, they should also be useful for differential diagnosis from other causes of dementia (1).

The most important known risk factor for AD is the E4 allele of the apolipoprotein E (*APOE*) gene, although the prevalence of this allele in AD in Turkish society varies. Obviously, this single gene polymorphism is not able to be an explanatory factor for the pathophysiology of AD, and the influence of different genes on AD should be studied in detail. Another important candidate gene in the pathogenesis of AD identified in genome-wide association studies (GWASs) is the bridging integrator 1 (*BIN1*) gene. β-Amyloid peptide, tau proteins, and the isoform 4 type of cholesterol transporting APOE protein have been determined to be involved in the pathogenesis of AD (3,4).

Many candidate genes that are possible risk factors for AD have been studied and four genes from those (*APP*, *PSEN1*, *PSEN2*, and APOE) have been reported to be associated with the disease. Of these, APOE is the best-known gene to be associated with late-onset AD (LOAD) (5). According to a GWAS conducted by the Alzheimer's

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Disease Genetics Consortium, *APOE* and *BIN1* are among 10 genes responsible for susceptibility to LOAD (6).

APOE plays a central role in lipid metabolism and is involved in the repair of damaged neurons in the central nervous system (7,8). Therefore, in response to nerve damage, an apparent increase in levels of *APOE* is known to occur (9). *APOE* has three common isoforms, E2, E3, and E4 (10,11). First the involvement of *APOE* E4 in the pathophysiology of AD was reported (12). In the earliest works in this area, 62% of AD patients were reported to be carriers of the E4 allele among autopsied cases, and it was shown to be a major risk factor in a number of studies (3,13). In another study, dementia severity was positively linked to the number of neurofibrillary tangles in the brain, but not to the degree of senile plaque deposition (14).

A number of studies have determined functions of *BIN1*, its polymorphisms related to LOAD, and its possible physiological effects. Studies also revealed the involvement of the *BIN1* gene and tau protein in AD risk. The contribution and underlying mechanisms of the *BIN1* gene in the pathogenesis of AD is still not fully understood; however, many pathways have been discussed. Further investigations regarding detailed mechanisms and molecular pathways in AD pathogenesis are of high importance in terms of understanding LOAD biology and novel treatment protocols (3,4).

This study aimed to identify certain polymorphisms that may be effective biomarkers in the detection of susceptibility to AD in the Turkish population. Therefore, we aimed to determine the potential biomarkers indicating the risk of AD in elderly healthy individuals. For this purpose, healthy individuals and AD patients were examined for E2, E3, and E4 alleles of the *APOE* gene and *BIN1* gene polymorphisms.

2. Materials and methods

2.1. Patients

Of the patients admitted to the outpatient clinic of neurology of Turgut Özal University Hospital, a total of 53 AD patients (29 females, 24 males) ranging in age from 66 to 93 (mean ± SD: 78.28 ± 6.61) and 56 healthy controls older than 65 years old (36 females, 20 males) ranging in in age from 65 to 92 (mean ± SD: 72.55 ± 7.92) were included in the study. The control group members were chosen from elderly people who had no history or clinical signs of neurological or psychiatric or cognitive symptoms. Controls having had surgery or having suffered trauma in the past 20 days were excluded from the study. Controls unwilling or unable to fulfill the requirements of the study were also excluded from the study. Their Mini-Mental State Examination (MMSE) scores were well above the limit for the normal cognitive level and not satisfying DSM-IV criteria. The study was approved by the Local Ethics Committee of Turgut Özal University Medical School (29 January 2013, no. 16). After informed consent was obtained, neurological examination and the MMSE were performed for all participants (15). AD was diagnosed following the criteria for probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (16) and the DSM-IV. Disease severity was evaluated using MMSE scores; severe AD patients have a score of 16 or lower. The MMSE scores of the control group were well above the limit for the normal cognitive level and not satisfying DSM-IV and NINCDS-ADRDA criteria for diagnosis of AD.

2.2. DNA isolation

Blood samples from the antecubital vein were taken into EDTA-containing tubes and were stored at 4 °C for the short term and –70 °C for the long term until the DNA isolation procedure. Genomic DNAs were isolated from whole blood by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Briefly, 9 mL of lysis buffer (1X RBC lysis buffer) was mixed with 3 mL of peripheral blood. After obtaining a pellet by centrifuging at 2000 × *g* for 10 min, TE was added and mixed. After adding 10% SDS and proteinase K, samples were vortexed and incubated at 70 °C for 15 min. They were then incubated overnight at 37 °C. Following overnight incubation, complete dissolution of pellets was achieved and samples were centrifuged for 15 min at 5000 rpm. After obtaining supernatant, 20 µL of 5 M NaCl and 400 µL of phenol (Sigma P4557, USA) was added, followed by centrifugation at 15,000 rpm for 15 min at 4 °C. Two phases were detected and the upper transparent phase was transferred to a clean Eppendorf tube. Later, 400 µL of chloroform in isoamyl alcohol (CIA, 1/25 ratio; Sigma C0549-1PT, USA) was added and tubes were turned upside-down quickly. This was centrifuged at 15,000 rpm for 15 min at 4 °C. Two phases were detected and the upper transparent phase was transferred to a clean Eppendorf tube. This step was repeated twice. After centrifugation, the upper transparent phase was transferred to a clean Eppendorf tube. Consequently, 1 mL of 100% ethanol was added and the DNA pellet was obtained through centrifugation at 15,000 rpm for 15 min at 4 °C. The obtained DNA was rinsed with 1 mL of 75% ethanol and centrifuged at 15,000 rpm for 5 min at 4 °C. After centrifugation, the ethanol supernatant was removed and the DNA was left to dry for 10–15 min. Finally, dissolution of DNA was achieved by adding 100 µL of 1 mM TE buffer. The concentration of DNA was measured by a spectrophotometer and it was stored at 4 °C.

2.3. DNA amplification

To study polymorphisms rs429358 T>C and rs7412 C>T, a 303-bp length of the *APOE* gene was amplified. The primers of the *APOE* gene for PCR amplification were modified from a previous report as follows: 5' CGGGCACGGCTGTCCAAGGAG 3' (forward) and 5' CTGGTGGAACAGGGCCGCGTG 3' (reverse) (17). The polymorphism analysis of the *BIN1* gene at rs744373 T>C for a length of 766 bp was performed using allele specific oligonucleotide (ASO)-PCR and primers 5' CCATCTTCTTCTGCTCTCCCAG 3' (forward), 5' ATGCCTCCTCTCTCCGTCTT 3' (reverse), 5' AGGGACAGGCAGGTCTGAGGCC 3' (forward variant), and 5' TCTCAGAGGCTGCCCATG 3' (reverse wild) as previously reported (18). The stock solution (100 pmol/μL) was prepared from lyophilized primers using 1X TE. The working primers for PCR amplification were prepared as 20 pmol/μL from stock solution.

2.4. Gradient PCR

Gradient PCR was performed to determine and modify the optimum temperature for annealing of primers for the *APOE* and *BIN1* genes. In the reaction Eppendorf tubes, the total volume used in gradient PCR was 25 μL comprising 10X buffer, $MgCl_2$ (25 mM), dNTP, primers, H_2O, Taq polymerase, and DMSO. The DNA samples were added at the end of all procedures. Total gradient PCR mix was constituted by 10X buffer (2.5 μL), $MgCl_2$ (2.5 μL), dNTP (0.5 μL), forward primer (0.5 μL), reverse primer (0.5 μL), Taq polymerase (0.2 μL, 1.0 U/25 μL reaction mixture), DMSO (1 μL), H_2O (15.3 μL), and DNA (2 μL) for the *APOE* and *BIN1* genes. Reaction conditions for *APOE* involved an initial denaturation of DNA at 94 °C for 5 min, followed by 38 cycles of amplification at 94 °C for 30 s (melting), 71 °C for 30 s (annealing), and 72 °C for 1 min, and final extension at 72 °C for 5 min. Reaction conditions for *BIN1* involved the same procedures for *APOE* except the annealing temperature (it was 64 °C).

2.5. Restriction fragment length polymorphism (RFLP)

PCR products provide the sequence of polymorphism sites for RFLP analysis. Detection of single nucleotide polymorphism alleles was done using restriction enzymes recognizing these regions. Enzymatic fragmentation was performed following the control of amplification of PCR products by agarose gel electrophoresis. After fragmentation, 15 μL of DNA was mixed with 1 μL of 6X loading dye and loaded into the well and run at 100 V for 45 min, followed by visualization in a UV transilluminator.

For enzymatic digestion of *APOE* gene rs429358 T>C and rs7412 C>T polymorphisms, a 303-bp PCR product was fragmented into fragments of 91, 83, 72, and 48 bp using CFO1 (Hhal) enzyme (New England Biosystems, USA). The enzyme sliced "C base" from the region of the "GCGC" sequence. The Hhal enzyme recognition sequence was 5'..GCG 'C…3', 3'…C'GCG…5'.

Risk determinations based on alleles are as follows: *APOE* E2 allele (rs429358 (T) + rs7412 (T)); *APOE* E3 allele (rs429358 (T) + rs7412 (C) = most common allele); *APOE* E4 allele (rs429358 (C) + rs7412 (C) = risk for AD); *APOE* E4/E4 allele (rs429358 (C;C) + rs7412 (C;C) = high risk for AD). Genotyping is as follows: E2/E2 (91 bp + 83 bp), E3/E3 (91 bp + 48 bp), E4/E4 (72 bp + 48 bp), E2/E3 (91 bp +83 bp +48 bp), E2/E4 (91 bp + 83 bp + 72 bp + 48 bp), E3/E4 (91 bp + 72 bp + 48 bp). Thus, polymorphic *APOE* includes three major isoforms: *APOE* E2 (cys112, cys158), *APOE* E3 (cys112, arg158), and *APOE* E4 (arg112, arg158).

2.6. ASO-PCR

After optimization trials, the total ASO-PCR reaction mixture was prepared to be 25 μL for each sample. Mixtures comprising 10X buffer, $MgCl_2$ (25 mM), dNTPs, primers, H_2O, DMSO, and Taq polymerase were distributed into each reaction tube, and finally DNA samples were added. Unlike normal PCR, two different specifically designed primers were used in ASO-PCR. The only difference in these primers was that one included the variant C allele primer, while the other included the wild-type T allele instead.

PCR products of *APOE* and *BIN1* were confirmed by 2% agarose gel electrophoresis. However, for RFLP fragment analysis of the *APOE* gene, 4% agarose gel was used.

2.7. Sequencing

In order to confirm the RFLP and ASO-PCR results, the sequencing of some samples was conducted as follows. First 2 μL of ExoSAP IT (GML A.G., Switzerland) was placed into PCR tubes, and then 5 μL of PCR product was added and tubes were placed in a thermal cycler. Reaction conditions for the presequence procedure involved an initial denaturation of DNA at 37 °C for 30 min, followed by final extension at 80 °C for 15 min and storage at 4 °C.

A mixture prepared for this purpose (8 μL) was placed into each tube and 2 μL of DNA-treated ExoSAP was added. As a control, pGEM was used. Reaction conditions for the sequence involved an initial denaturation of DNA at 96 °C for 1 min, followed by 25 cycles of amplification at 96 °C for 15 s (melting), 50 °C for 15 s (annealing), and 60 °C for 4 min, with no more final extension. PCR products were purified using Sephadex column purification after sequencing PCR amplification. Samples were analyzed with the Applied Biosystems 3130 Genetic Analyzer.

2.8. Statistical evaluation

SPSS 16.0 was used for assessment of the relationship between genotypes and clinical findings. The chi-square test and Fisher's exact test were used as needed for comparisons between allele and genotype frequencies of patients and controls. $P < 0.05$ was considered significant.

3. Results

3.1. *APOE* PCR electrophoresis and Hhal enzyme digestion

For evaluation of PCR products of the searched region for *APOE*, samples were run on 2% agarose gel (fragments of 303 bp in length) and visualized in a UV transilluminator (Figure 1).

In order to assess RFLP results of the investigated region, enzyme digestion fragments were run on 4% agarose gel, and fragments of 91 bp, 83 bp, 72 bp, and 48 bp were visualized in a UV transilluminator (Figure 2).

3.2. *BIN1* PCR electrophoresis results

To evaluate PCR products of the investigated region, ASO-PCR products were run on 2% agarose gel, and fragments of 766 bp, 498 bp, and 308 bp were obtained and visualized in a UV transilluminator (Figure 3).

3.3. Sequence confirmation

After detecting the presence of PCR products, certain reference samples underwent DNA sequencing, and the *BIN1* and *APOE* genes were confirmed. PCR products were purified using Sephadex column purification after PCR amplification. Samples were analyzed with the 3130 Genetic Analyzer. *APOE* gene polymorphism results were as follows: E2/E3 polymorphism (Figure 4), E3/E4 polymorphism (Figure 5), and E3/E3 polymorphism (Figure 6). PCR polymorphism results of *BIN1* were defined as TT and CT polymorphisms (Figure 7).

3.4. Allele distributions of *APOE* and *BIN1* genes

APOE and *BIN1* allele distributions of patient and control groups are shown in Table 1. *APOE* allele distribution percentages in AD patients were determined as follows: 58.5% for E3/E3, 5.7% for E4/E4, 7.5% for E2/E3, 0% for E2/E4, and 28.3% for E3/E4. *APOE* allele distribution percentages in the control group were determined as follows: 67.9% for E3/E3, 0% for E4/E4, 17.9% for E2/E3, 1.8% for E2/E4, and 12.5% for E3/E4. The difference between the prevalence of the E4 allele in AD patients and the control group was statistically significant ($P < 0.05$) (Table 2). Regarding *BIN1* allele distribution, there was no statistically significant difference for CC genotype prevalence between patients and controls ($P > 0.05$). E2 allele prevalence was significantly higher in the control group compared to AD patients ($P = 0.067$). Sex distribution ratios and its relationship with *APOE* and *BIN1* were similar between the patient and control groups (Table 3) ($P > 0.05$).

Although a statistically significant difference was observed between patient and control groups in terms of ischemic heart disease prevalence, there was no significant difference in terms of prevalence of hypertension, diabetes, stroke history, cholesterol and triglyceride levels, and family history of AD (Table 4). Furthermore, no statistically significant difference was observed between patient and control groups in terms of the relationship of *APOE* genotype with cholesterol, stroke history, ischemic heart disease, triglyceride levels, and family history of AD ($P > 0.05$) (Table 5). There was significant difference between patient and control groups in terms of relationship of *APOE* E4 allele with hypertension, while no significant difference was observed in terms of relationship of *APOE* E4 allele with stroke history, ischemic heart disease, diabetes, and family history of AD ($P > 0.05$) (Table 5). There was a statistically significant difference between patient and control groups in terms of relationship of *BIN1* gene CC allele with hypertension prevalence ($P < 0.001$) (Table 6); however, no significant difference was observed in terms of diabetes, family history of AD, cholesterol and triglyceride levels, stroke history, or ischemic heart disease ($P > 0.05$). The distribution of *APOE* gene E4 allele and *BIN1* C allele frequencies and their relationship with MMSE scores in AD patients is shown in Table 7.

4. Discussion

In this study we investigated rs429358 and rs7412 polymorphisms for E2, E3, and E4 alleles of the *APOE*

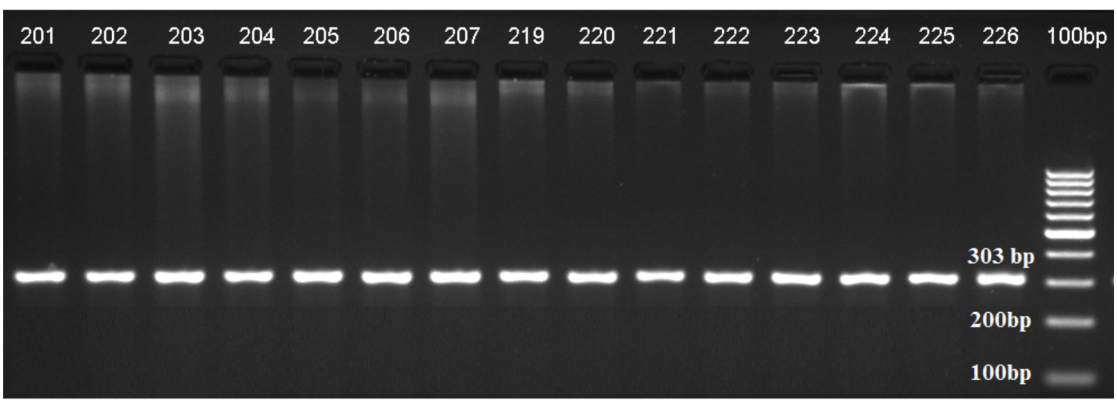

Figure 1. Agarose gel image of *APOE* PCR amplification products.

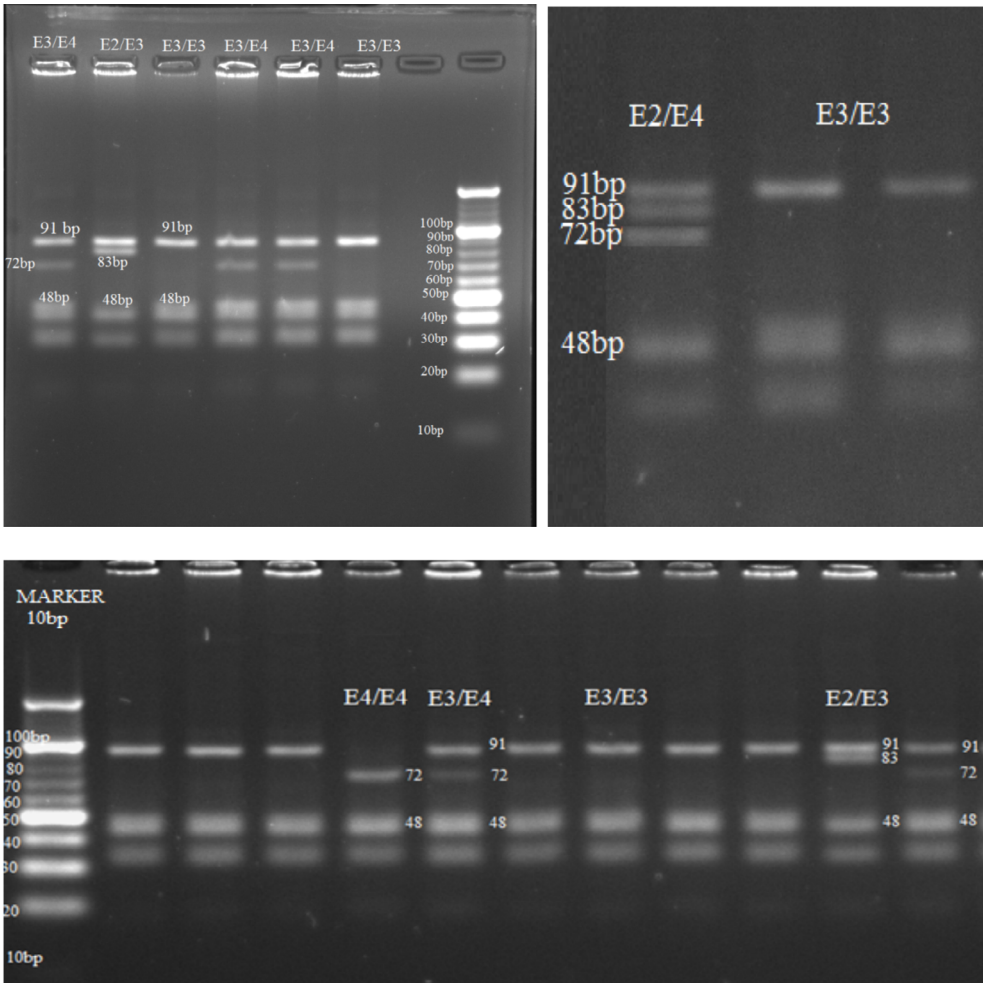

Figure 2. Agarose gel image of *APOE* RFLP products.

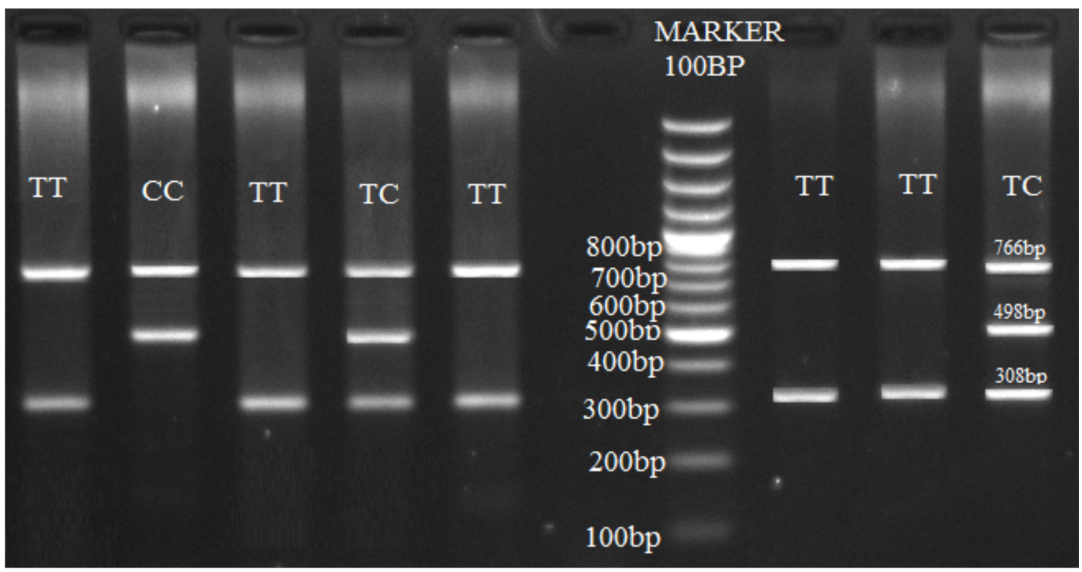

Figure 3. Agarose gel image of *BIN1* ASO-PCR amplification products.

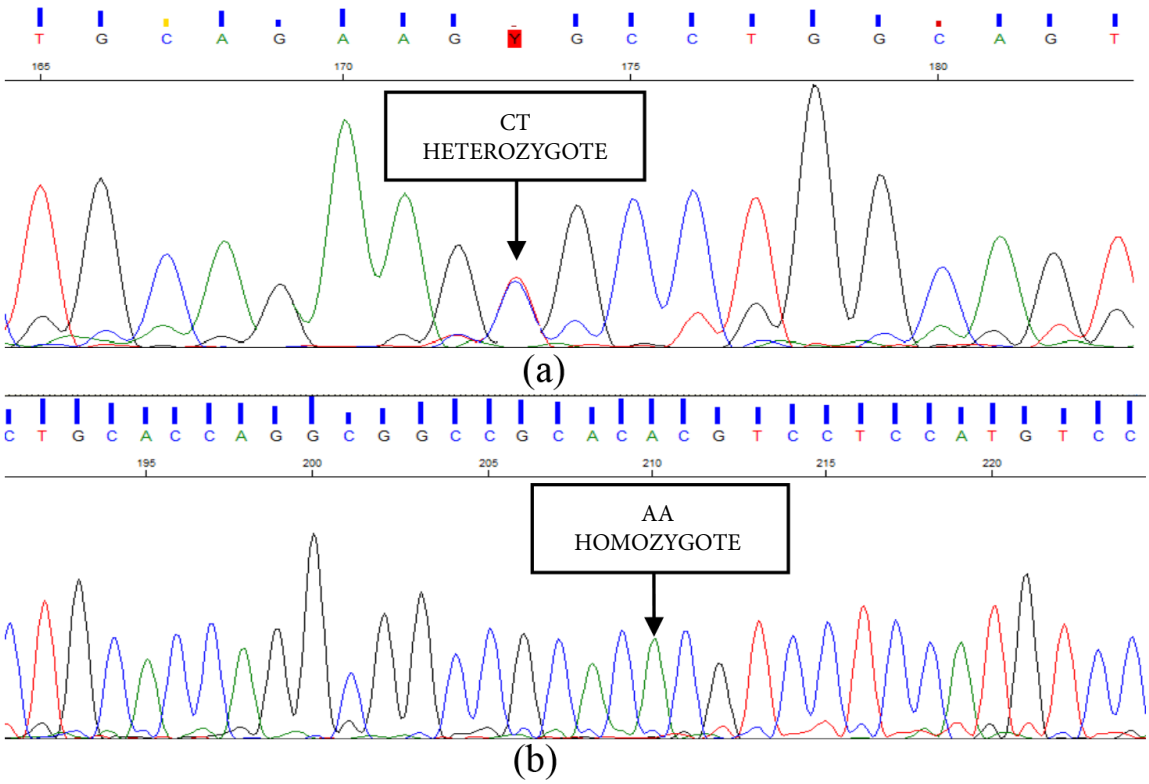

Figure 4. *APOE* Rs429358 and Rs7412 polymorphisms.

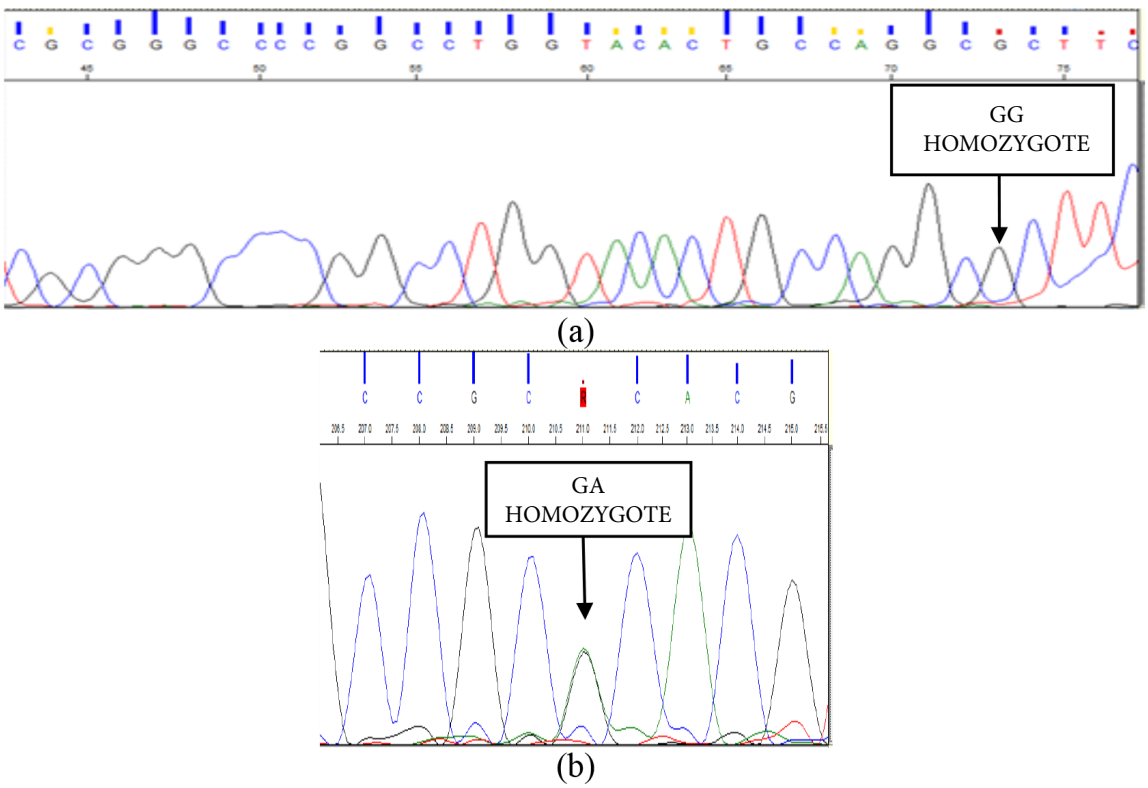

Figure 5. a) *APOE* rs429358 and **b)** *APOE* rs7412 polymorphisms.

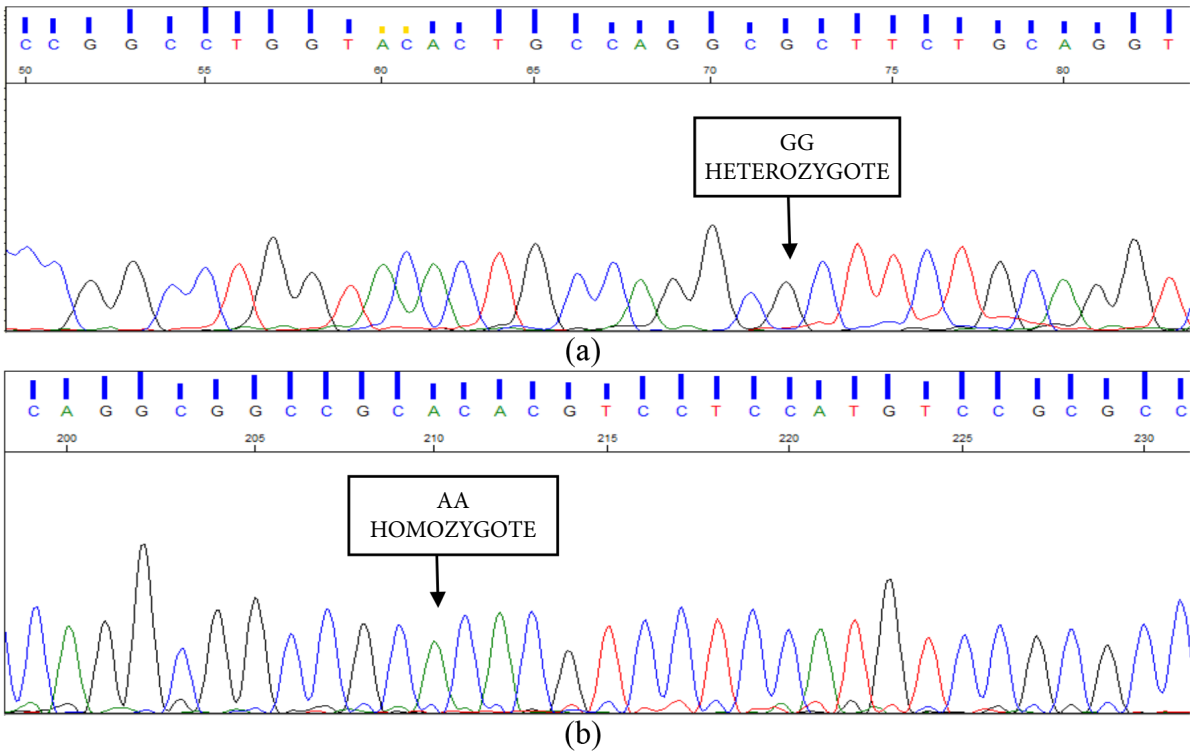

Figure 6. a) *APOE* rs429358 and **b)** *APOE* rs7412 polymorphisms.

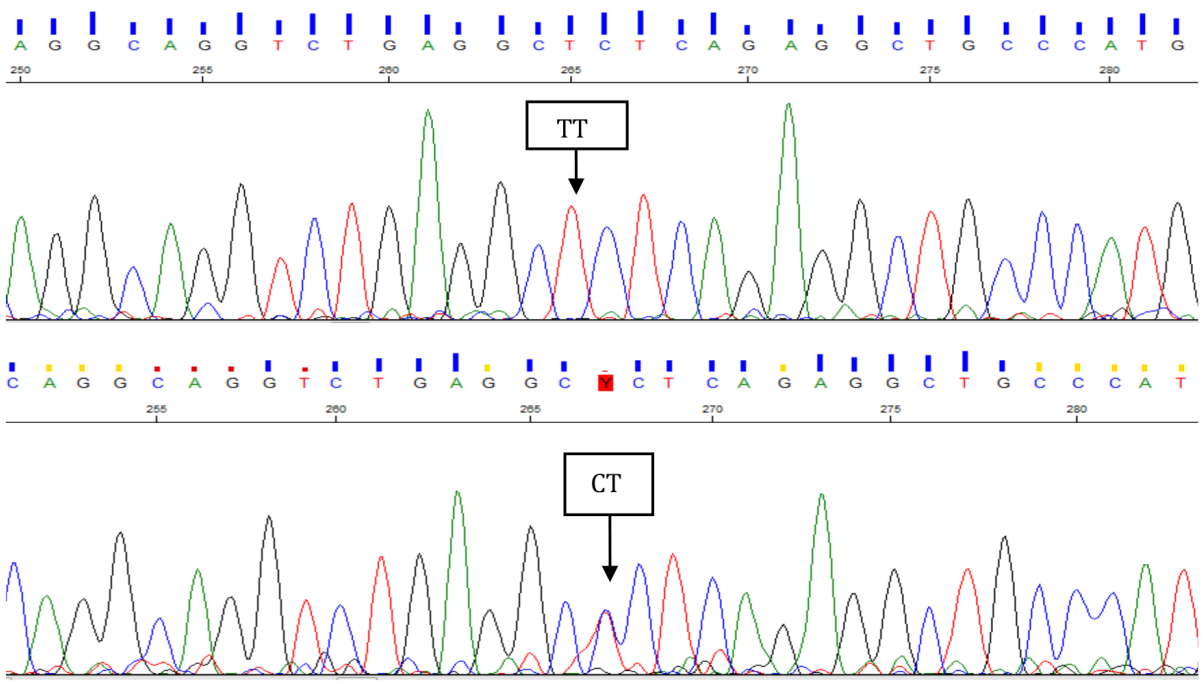

Figure 7. *BIN1* gene rs744373 polymorphism.

Table 1. *APOE* and *BIN1* allele distributions in AD and control groups.

Groups	*APOE* allele						*BIN1* allele			
	E3/E3	E4/E4	E2/E3	E2/E4	E3/E4	Total	TT	CC	TC	Total
AD patients	31 (58.5%)	3 (5.7%)	4 (7.5%)	0 (0%)	15 (28.3%)	53 (100%)	35 (66%)	3 (5.7%)	15 (28.3%)	53 (100%)
Controls	38 (67.9%)	0 (0%)	10 (17.9%)	1 (1.8%)	7 (12.5%)	56 (100%)	34 (60.7%)	3 (5.4%)	19 (33.9%)	56 (100%)
Total	69 (63.3%)	3 (2.8%)	14 (12.8%)	1 (0.9%)	22 (20.2%)	109 (100%)	69 (63.3%)	6 (5.5%)	34 (31.2%)	109 (100%)

Table 2. Distribution of *APOE* gene E4 and E2 allele frequencies.

Groups	*APOE* allele							
	E4 allele	Others	Total	P-value	E2 allele	Others	Total	P-value
AD patients	18 (34%)	35 (66%)	53 (100%)		4 (7.5%)	49 (92.5%)	53 (100%)	
Controls	8 (14.3%)	48 (85.7%)	56 (100%)	P = 0.016*	11(19.6%)	45 (80.4%)	56 (100%)	P = 0.067*
Total	26 (23.9%)	83 (76.1%)	109 (100%)		15 (13.8%)	94 (86.2%)	109 (100%)	

*Pearson chi-square.

Table 3. Distribution of *APOE* and *BIN1* gene allele frequencies with respect to sex.

	Study groups			*APOE*		
	AD patients	Control	P-value	E4 allele	E2 + E3 allele	P-value
Female	29 (54.7%)	36 (64.3%)		12 (18.5%)	53 (81.5%)	
Male	24 (45.3%)	20 (35.7%)	P = 0.309*	14 (31.8%)	30 (68.2%)	P = 0.108*
Total	53 (100%)	56 (100%)		26 (23.9%)	83 (76.1%)	
	BIN1					
	CC+CT	TT	P-value			
Female	26 (65%)	39 (56.5%)				
Male	14 (35%)	30 (43.5%)	P = 0.385*			
Total	40 (100%)	69 (100%)				

*Pearson chi-square.

gene, which is the most crucial biomarker indicating the risk of AD during the aging of healthy individuals. We also investigated the rs744373 polymorphism of *BIN1*, one of the most important candidate genes recently identified in GWASs for AD. The present study is the first study to investigate *BIN1* polymorphism as well as these two polymorphisms together in Turkey. Findings obtained in this study can be summarized as follows:

1. AD patient and control groups were similar in terms of sex distribution and family history for AD.

2. The frequency of *APOE* E4 allele was higher in the AD patient group, while the frequency of the E2 allele was higher in the control group.

3. The E4/E4 genotype was detected in the AD patient group, while this genotype was not observed in the control group.

4. The prevalence of ischemic heart disease was significantly higher in the control group. Since the case number in our study is relatively low, this finding needs to be confirmed with further studies.

Table 4. Distribution of frequencies of comorbid conditions*.

Comorbid conditions		AD patients	Control	Total	P-value
Ischemic cardiac disease		10 (38.5%)	16 (61.5%)	26 (100%)	P = 0.035§
High cholesterol level		2 (6.7%)	10 (29.4%)	12 (18.8%)	P = 0.020§
Hypertension		39 (79.6%)	31 (70.5%)	70 (75.3%)	P = 0.308§
Diabetes		15 (30.6%)	11 (25.6%)	26 (28.3%)	P = 0.593§
History of stroke		7 (14.3%)	8 (18.2%)	15 (16.1%)	P = 0.610§
Triglyceride	High	8 (26.7%)	10 (29.4%)	18 (28.1%)	P = 0.807§
	Normal	22 (73.3%)	24 (70.6%)	46 (71.9%)	
Family history of AD		5 (25%)	4 (66.7%)	9 (34.6%)	P = 0.138¶

§Pearson chi-square.
¶Fisher's exact test.
*Some patients and control subjects had more than one disease. In the control group, there were 20 participants without any disease, 14 participants with only one disease (11 hypertension, 1 ischemic heart disease, 1 history of stroke, 1 triglyceride), 8 participants with two diseases (1 high cholesterol + hypertension, 1 high cholesterol + ischemic heart disease, 1 ischemic heart disease + diabetes, 3 ischemic heart disease + hypertension, 2 diabetes + hypertension), 6 participants with three diseases (2 history of stroke + diabetes + hypertension, 1 ischemic heart disease + history of stroke + hypertension, 2 triglyceride + hypertension + high cholesterol, 1 triglyceride + hypertension + ischemic heart disease), and 8 participants with four or more diseases (1 diabetes + hypertension + high cholesterol + triglyceride, 2 diabetes + hypertension + ischemic heart disease + history of stroke, 2 hypertension + high cholesterol + triglyceride + ischemic heart disease + history of stroke, 2 hypertension + high cholesterol + triglyceride + ischemic heart disease + diabetes, 1 hypertension + high cholesterol + triglyceride + ischemic heart disease + history of stroke + diabetes). In the patient group, there were 11 patients without any other diseases, 18 patients with only one disease (17 hypertension, 1 diabetes), 14 patients with two other diseases (4 diabetes + hypertension, 1 high cholesterol + hypertension, 1 triglyceride + hypertension, 4 ischemic heart disease + hypertension, 2 history of stroke + hypertension, 2 triglyceride + diabetes), 6 patients with three diseases (3 diabetes + hypertension + triglyceride, 1 ischemic heart disease + history of stroke + hypertension, 1 ischemic heart disease + diabetes + hypertension, 1 history of stroke + diabetes + hypertension), and 4 patients with four or more other diseases (2 diabetes + hypertension + ischemic heart disease + history of stroke, 1 hypertension + ischemic heart disease + history of stroke + triglyceride, 1 diabetes + hypertension + ischemic heart disease + triglyceride + high cholesterol).

5. No significant difference was observed between patient and control groups in terms of prevalence of hypertension, diabetes, history of stroke, or triglyceride levels.

6. The number of individuals with high cholesterol levels was significantly higher in the control group; however, there was no statistically significant relationship between *APOE* genotypes and cholesterol levels.

7. In both groups, the prevalence of hypertension was high among cases with the *APOE* E4 allele, while no significant difference was observed for diabetes, stroke history, high cholesterol, triglyceride levels, and ischemic heart disease.

8. There was no significant relationship between the frequencies of all *APOE* genotypes and triglyceride levels, stroke history, and ischemic heart disease in either group.

9. No statistically significant difference was observed between patient and control groups in terms of the frequencies of *BIN1* alleles.

10. There was no relationship between *BIN1* genotypes and family history of AD in either group.

11. The prevalence of hypertension was significantly lower in individuals with the C allele of the *BIN1* gene in both groups; however, no significant relationship was observed between C allele frequency and diabetes, high cholesterol, triglyceride levels, stroke history, and ischemic heart disease.

12. The relationships between *APOE* and *BIN1* alleles and MMSE scores in AD patients were given in the present study for the first time in the literature (Table 7). This is important because MMSE scores take into consideration when doctors decide if drugs will help AD patients in neurology and psychiatry clinics. It is possible to compare the allele distribution and MMSE scores in Table 7.

Obtained findings suggest that there is a strong relationship between the E4 allele of *APOE* and AD, while there is no significant relationship between the rs744373 polymorphism of the *BIN1* gene and AD. Several different

Table 5. The relationships of *APOE* genotype and *APOE* E4 and other gene allele genotypes with certain comorbid conditions.

Comorbid conditions		*APOE* (columns a)							*APOE* E4 and others (columns b)			
		E3/E3	E4/E4	E2/E3	E2/E4	E3/E4	Total	P-value	E4 allele	Other alleles	Total	P-value
Ischemic cardiac disease	Positive	18 (69.2%)	0 (0%)	3 (11.5%)	0 (0%)	5 (19.2%)	26 (100%)	P = 0.754*	5 (19.2%)	21 (80.8%)	26 (100%)	P = 0.444*
	Negative	40 (59.7%)	3 (4.5%)	9 (13.4%)	1 (1.5%)	14 (20.9%)	67 (100%)		18 (26.9%)	49 (73.1%)	67 (100%)	
	Total	58 (62.4%)	3 (3.2%)	12 (12.9%)	1 (1.1%)	19 (20.4%)	93 (100%)		23 (24.7%)	70 (75.3%)	93 (100%)	
High cholesterol (for columns a only) / Hypertension (for columns b only)	Positive	10 (83.3%)	0 (0%)	1 (8.3%)	-	1 (8.3%)	12 (100%)	P = 0.652*	21 (30%)	49 (70%)	70 (100%)	P = 0.040*
	Negative	34 (65.4%)	1 (1.9%)	6 (11.5%)	-	11 (21.2%)	52 (100%)		2 (8.7%)	21 (91.3%)	23 (100%)	
	Total	44 (68.8%)	1 (1.6%)	7 (10.9%)	-	12 (18.8%)	64 (100%)		23 (24.7%)	70 (75.3%)	93 (100%)	
History of stroke	Positive	10 (66.7%)	0 (0%)	3 (20%)	0 (0%)	2 (13.3%)	15 (100%)	P = 0.767*	2 (13.3%)	13 (86.7%)	15 (100%)	P = 0.508**
	Negative	49 (62.8%)	3 (3.8%)	9 (11.5%)	1 (1.3%)	16 (20.5%)	78 (100%)		20 (25.6%)	58 (74.4%)	78 (100%)	
	Total	59 (63.4%)	3 (3.2%)	12 (12.9%)	1 (1.1%)	18 (18.4%)	93 (100%)		22 (23.7%)	71 (76.3%)	93 (100%)	
Triglyceride (for columns a only) / Diabetes (for column b only)	Positive	13 (72.2%)	0 (0%)	2 (11.1%)	-	3 (16.7%)	18 (100%)	P = 0.920*	4 (15.4%)	22 (84.6%)	26 (100%)	P = 0.229*
	Negative	31 (67.4%)	1 (2.2%)	5 (10.9%)	-	9 (19.6%)	26 (100%)		18 (27.3%)	48 (72.7%)	66 (100%)	
	Total	44 (68.8%)	1 (1.6%)	7 (10.9%)	-	12 (18.8%)	64 (100%)		22 (23.9%)	70 (76.1%)	92 (100%)	
Family history of AD	Positive	5 (55.6%)	1 (11.1%)	0 (0%)	-	3 (33.3%)	9 (100%)	P = 0.286*	4 (44.4%)	5 (55.6%)	9 (100%)	P = 0.188**
	Negative	12 (70.6%)	0 (0%)	2 (11.8%)	-	3 (17.6%)	17 (100%)		3 (17.6%)	14 (82.4%)	17 (100%)	
	Total	17 (65.4%)	1 (3.8%)	2 (7.7%)	-	6 (23.1%)	26 (100%)		7 (26.9%)	19 (73.1%)	26 (100%)	

*Pearson chi-square.

**Fisher's exact test.

findings were obtained in the present study when compared to previously conducted studies. These differences were probably caused by case series with different case numbers and characteristics, genetic and environmental factors affecting cases, and ethnic origins of cases.

In the pathophysiology of AD, *APOE* interacts with tau protein and amyloid β, and it affects neurofibrillary tangles and senile plaques. In vitro studies revealed that unlike the E3 allele of *APOE*, E4 forms a highly stable complex with amyloid β and the number, diameter, and density of immunoreactive plaques are found to be associated with *APOE* genotype (19). Amyloid β plaques are known to be more common and bigger in individuals carrying the E4/E4 allele compared to those individuals with the E3/E3 allele. In accordance with this, the E4/E4 genotype was detected in the AD patient group, while this genotype was not observed in the control group in the present study.

Table 6. The relationship of *BIN1* gene alleles with certain comorbid conditions.

Comorbid conditions CC + CT		*BIN1*			P-value
		TT	Total		
Family history of AD	Positive	3 (33.3%)	6 (66.7%)	9 (100%)	P = 1.000**
	Negative	6 (35.3%)	11 (64.7%)	17 (100%)	
	Total	9 (34.6%)	17 (65.4%)	26 (100%)	
Hypertension	Positive	17 (24.3%)	53 (75.7%)	70 (100%)	P = 0.000*
	Negative	15 (65.2%)	8 (34.8%)	23 (100%)	
	Total	32 (34.4%)	61 (65.6%)	93 (100%)	
Diabetes	Positive	10 (38.5%)	16 (61.5%)	26 (100%)	P = 0.642*
	Negative	22 (33.3%)	44 (66.7%)	66 (100%)	
	Total	32 (34.8%)	60 (65.2%)	92 (100%)	
Triglyceride	High	9 (50%)	9 (50%)	18 (100%)	P = 0.142*
	Normal	14 (30.4%)	32 (69.6%)	46 (100%)	
	Total	23 (35.9%)	41 (64.1%)	64 (100%)	
High cholesterol level	Positive	4 (33.3%)	8 (66.7%)	12 (100%)	P = 1.000**
	Negative	19 (36.5%)	33 (63.5%)	52 (100%)	
	Total	23 (35.9%)	41 (64.1%)	64 (100%)	
Ischemic cardiac disease	Positive	9 (27.3%)	17 (28.3%)	26 (28%)	P = 0.913*
	Negative	24 (72.7%)	43 (71.7%)	67 (72%)	
	Total	33 (100%)	60 (100%)	93 (100%)	
History of stroke	Positive	3 (9.4%)	12 (19.7%)	15 (16.1%)	P = 0.200*
	Negative	29 (90.6%)	49 (80.3%)	61 (100%)	
	Total	32 (100%)	61 (100%)	93 (100%)	

*Pearson chi-square.
**Fisher's exact test.

Table 7. The frequencies of *APOE* gene E4 allele and *BIN1* gene C allele with respect to MMSE test scores in AD patients.

MMSE score	APOE			BIN1		
	E4 allele	Other alleles	Total	CC-CT	TT	Total
5–10	0 (0%)	2 (100%)	2 (100%)	1 (50%)	1 (50%)	2 (100%)
10–15	3 (50%)	3 (50%)	6 (100%)	2 (33.3%)	4 (66.7%)	6 (100%)
15–20	4 (40%)	6 (60%)	10 (100%)	3 (30%)	7 (70%)	10(100%)
20–25	7 (38.9%)	11 (61.1%)	18 (100%)	5 (27.8%)	13 (72.2%)	18 (100%)
>25	0 (0%)	3 (100%)	3 (100%)	1 (33.3%)	2 (66.7%)	3 (100%)
Total	14 (35.9%)	25 (64.1%)	39 (100%)	12 (30.8%)	27 (69.2%)	39 (100%)

The E3 allele of *APOE* is the most common form and anticipated as the main form. In the pathophysiology of AD, *APOE* E4 is accepted as a risk factor, while *APOE* E2 is considered a protective factor in some studies (20). It has also been shown that the E2 allele has no protective effect on AD, or was related to a nonsignificantly decreased risk of the disease. Therefore, both alleles (E2 and E3) are not considered as risk factors for AD while E4 has been considered as a high risk factor for AD (21). This conclusion of other studies is confirmed by our findings in the present study.

A number of studies dealing with *APOE* allele frequencies in AD patients revealed that the E2/E2 allele genotype is not observed in AD patients, while the E4/E4 allele is quite rare or absent in controls, similar to the present study (22–26). The comparison of frequencies of *APOE* alleles with the literature is shown in Table 8. Several studies showed significantly higher E4 allele rates in AD patients compared to controls (27–29). Furthermore, a great number of studies from different parts of the world such as Tunisia (17), Germany (30), France (31), Canada (32), Greece (33), Japan (34), three regions of Italy (Sicily, Sardinia, Apulia) (35–37), Iran (38), Spain (39), Korea (40), and China (41) revealed identical results to the present study. Similarly, in the present study the frequency of the E4 allele was significantly higher in the AD patient group compared to controls ($P < 0.05$). Furthermore, there was no E4/E4 genotype in the control group, while it was observed in AD patients, which proves that *APOE* E4 is a susceptibility gene for AD, in accordance with the literature. Unlike a large number of studies from the literature, there are studies claiming that the *APOE* E4 allele is not a risk factor for AD. These studies have dealt with AD patient series from Kenya (42), Nigeria (43), and Africans living in Cameroon and the Nile region (44). Furthermore, these were supported by studies dealing with African Americans and Spanish Americans (45,46). These controversial results might suggest that the AD-associated *APOE* E4 allele could be affected and altered by other active genes and the potential environmental effects as well as ethnic factors (47).

Unlike the present study, certain studies showed the existence of the E2/E2 genotype both in AD patients and controls; however, these studies revealed higher frequency of the E4/E4 genotype in AD patients, in accordance with our study (48,49). Our study shows the effective nature of the E4 allele in the development of AD, similar to the related literature. On the other hand, our study supports the literature indicating the E2 allele as a protective factor for AD.

One of the many risk factors for development of LOAD is the familial history of AD and the E4 allele (50,51). In accordance with the literature, the frequency of the E4 allele of the *APOE* gene was higher in individuals with a family history of dementia in the present study.

There are studies revealing significant correlations between sex and the frequency of the E4 allele (41,52,53). However, the sex distribution between AD patient and control groups was similar ($P = 0.309$) and no significant relationship was observed between sex and the frequency of the E4 allele of *APOE* ($P = 0.108$) in our study. The incidence of AD increases with age, and this statement was supported by our study, as well. A series of studies from North Africa showed that an increase in aged population (from 4.1% in 1956 to 9.6% in 2004) coincided with increased incidence of AD (17,54).

AD is thought to have a multifactorial etiology and the exact mechanism still needs to be explained. Therefore, this study also compared AD patient and control groups in terms of comorbid medical conditions, which might contribute to the development of AD. A significant difference was observed between AD patient and control groups in terms of comorbid ischemic heart disease and high cholesterol level ($P < 0.05$). Furthermore, there was statistically significant correlation between the E4 allele of the *APOE* gene and hypertension ($P < 0.05$). In general, comorbidity describes the effect of all diseases that we

Table 8. The comparison of frequencies of *APOE* alleles with the literature.

Reference	APOE allele											
	AD patients						Controls					
	E2/E2	E3/E3	E4/E4	E2/E3	E2/E4	E3/E4	E2/E2	E3/E3	E4/E4	E2/E3	E2/E4	E3/E4
(17)	-	35.0%	17%	4.0%	9.0%	35.0%	1%	68.0%	2%	10%	3.0%	16.0%
(18)	-	74.3%	2.9%	5.7%	2.9%	14.3%	-	93.1%	-	3.4%	-	3.4%
(19)	-	63.2%	-	7.4%	-	29.4%	-	71.7%	-	11.8%	0.8%	15.7%
(20)	-	53%	5.0%	1.0%	-	41.0%	-	72.0%	3.0%	7.0%	2.0%	16.0%
Current study	-	58.5%	5.7%	7.5%	-	28.3%	-	67.9%	-	17.9%	1.8%	12.5%

inspected on AD in this study. These results suggest an insight into the relationships between AD, ischemic heart disease, and high cholesterol levels as well as a strong correlation between the E4 allele of the *APOE* gene and hypertension. Because the presence of comorbidity must be taken into account when selecting the diagnosis and treatment plans for AD patients, our findings are worth considering for clinicians who follow patients with AD. According to strong evidence, it has been suggested that AD shares characteristics and possible origins with both cardiovascular disorders and diabetes (55). We did not find such a comorbidity correlation between the occurrence of AD and diabetes.

In the current study, the genotype distribution of *BIN1* in the AD patient group was as follows: 66% for TT, 5.7% for CC, and 28.3% for TC. In the control group it was as follows: 60.7% for TT, 5.4% for CC, and 33.9% for TC. The frequency of the C allele and CC genotype did not show significant difference ($P > 0.05$) between AD patient and control groups, and no significant difference was observed between the groups at all. However, there are previously conducted studies indicating the significance of *BIN1* in the literature (56). Since different genetic variants of *BIN1* might cause an alteration in the way of progression of AD, and only one single nucleotide polymorphism is not sufficient to estimate genetic risks for AD, multilocus genotype patterns might provide a novel analytical approach for genetic risk estimation of AD.

In a study dealing with *APOE* risk-free series, the odds ratio and the population attributable fraction of single nucleotide polymorphisms of *BIN1* were estimated to be 1.2% and 6%, respectively (57). This is partially supported our work. In the literature, *BIN1* expression was shown to change in brains suffering from AD in transgenic mouse models of AD (58). Furthermore, *BIN1* expression was reported to be associated with age of onset and duration of illness (59). In this study we investigated the relation of different genotypes of *BIN1* and certain medical conditions, and we observed a significant relationship between the C allele of the *BIN1* gene and hypertension incidence ($P < 0.001$).

There are very little data regarding the relationship between the *BIN1* gene and neurodegeneration, while there are plentiful data regarding roles of *APOE*, clusterin (*CLU*), and complement receptor 1 (*CR1*) in AD. Therefore, the role of *BIN1* and potential molecular pathways in the pathogenesis of AD need further investigation. Additionally, understanding the *BIN1* roles in other pathways possibly associated with AD might help us to elucidate its role in AD pathogenesis. A recent study showed that *BIN1* increases the risk of AD by interacting with tau protein and by affecting neurofibrillary tangles (60–62). However, further studies are needed to determine the mechanism of interaction between *BIN1* and tau, and neurofibrillary tangles, which might be promising for novel treatment approaches. Additionally, epigenetic regulation in the expression of *BIN1* or methods used for decrease of amyloid β production can be used as new treatments for AD. On the other hand, studies identifying novel molecules and pathophysiological pathways will surely contribute to the development and implementation of more effective treatment procedures.

In conclusion, the obtained results, in addition to the previously conducted genetic studies, showed that AD is not caused by a single etiological factor, but there are multiple risk factors playing roles in its etiology. As a limitation, our study has a small scale. Especially in terms of representing the whole of Turkey, a larger-scale study dealing with patient series from different provinces of Turkey and investigating more candidate genes and polymorphisms should be performed.

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