The effects and interactions of APOE and APH-1A polymorphisms in Alzheimer disease

Burcu ACAR ÇİNLETİ, Nilgül YARĐIMCI, Zübeyde AYTÜRK, Atilla İLHAN, Gülhan KAYA,
Muradiye ACAR, Emine Rabia KOÇ, Esra GÜNĐÜZ*, Mehmet GÜNĐÜZ*

1Department of Neurology, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
2Department of Neurology, Faculty of Medicine, Gazi University Ankara, Turkey
3Department of Medical Imaging Techniques, Vocational School of Health Sciences, Turgut Özal University, Ankara, Turkey
4Department of Medical Genetics, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
5Department of Neurology, Faculty of Medicine, Balıkesir University, Balıkesir, Turkey
6Department of Otolaryngology, Faculty of Medicine, Turgut Özal University, Ankara, Turkey

Received: 04.07.2014 • Accepted/Published Online: 25.05.2015 • Printed: 30.10.2015

Background/aim: Alzheimer disease (AD) is characterized by the accumulation of senile plaques composed of amyloid β-peptide, which is derived from β-amyloid precursor protein through degradation by β-secretase and γ-secretase complexes. One of the major components of γ-secretase complex, anterior pharynx-defective-1 (APH-1), is responsible for the activity of the γ-secretase complex. In this study, we searched for not only the most known common genetic risk factor, APOE, but also the APH-1a gene polymorphism in AD patients in a Turkish population.

Materials and methods: In this study, 49 AD patients and 45 healthy controls were included. The genetic polymorphisms and allele frequencies of APOE and APH-1a were investigated. Patients were evaluated for behavioral, cognitive, and functional domains by detailed neurocognitive tests, and comparison between the above-mentioned polymorphisms and disease severity was made.

Results: Although there was an increased tendency of the APO ε4 allele in the AD group, no statistically significant difference was detected either in APOE or APH-1a polymorphisms, not suggesting a strong susceptibility to the development of AD.

Conclusion: While searching for the pathogenesis of AD in order to develop novel diagnostic as well as therapeutic approaches, analysis of other genes with a possible role in AD is warranted.

Key words: Alzheimer disease, APOE, APH-1A, polymorphism, biomarker

1. Introduction
Alzheimer disease (AD) is the most frequent form of dementia, characterized by progressive impairment in multiple cognitive domains. AD is a major public health issue and places a considerable burden on society. The clinical diagnosis of AD cannot be determined by laboratory tests. These tests are important mainly in identifying other possible causes of dementia that must be excluded before the diagnosis of AD is made (1). Its definite diagnosis is possible with postmortem neuropathological study (2). The following classification is used for AD: possible AD, probable AD, and definite AD. While the first two categories are intended for clinical use, the third is intended for research purposes (3). Early diagnosis and intervention in early stages play important roles during the course of the disease as well as in efficacy of the treatment. The etiology of the sporadic form of AD remains largely unknown. Recent evidence has suggested that genetic and environmental interactions may play a decisive role in the development and progression of the disease (4). In terms of age of onset, AD is classified into early-onset AD (EOAD, onset at <65 years), accounting for 1%–5% of all cases, and late-onset AD (LOAD, onset at ≥65 years), accounting for >95% of the patients. EOAD, also known as familial AD, is generally associated with a more rapid rate of progression and a number of different single-gene mutations on chromosomes 21, 14, and 1. Mutations in the amyloid precursor protein (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2) genes have consistently been associated with early-onset familial AD (5). The single-gene mutations directly responsible for EOAD do not seem to be involved in LOAD. The causes of LOAD are not completely understood, but they are suggested to be influenced by a combination of genetic, environmental, and lifestyle factors.
APOE is the best-known gene to be associated with LOAD, yet it represents only a small ratio of the genetic factors, and probably many other genetic polymorphisms are involved in onset of the disease (6). APOE is the major lipoprotein within the central nervous system, where it is synthesized by astrocytes. APOE plays an important role in lipid metabolism (7). Injuries in the central and peripheral nervous system structures cause an increase of APOE expression (8). The APOE protein exists in three major isoforms: E2, E3, and E4. The APOE4 isofrom, a key risk factor for AD, is cytotoxic, and it damages the cytoskeleton, increases the production of amyloid β-peptide, and affects mitochondrial function in neuronal cells (9).

Diagnostic accuracy is lower at early and presymptomatic stages of AD (10). It is believed that the development of AD takes place over a long prodromal period, approximately 20 years, but this is difficult to determine because of the absence of biomarkers that reliably determine the absence of AD takes place over a long prodromal period, approximately 20 years, but this is difficult to determine because of the absence of biomarkers that reliably determine the onset of presymptomatic disease before the onset measurable cognitive impairments. Because intervention with disease-modifying therapies for AD is likely to be the most efficacious treatment method before the occurrence of significant neurodegeneration, there is a crucial need for biomarker-based tests that enable an accurate and early diagnosis of AD (11,12). Moreover, such tests could also provide improved monitoring for AD progression, evaluation of new treatment strategies, and enhanced discrimination of AD cohorts with specific subsets in clinical trials. The ideal biomarker for AD should be reliable, noninvasive, simple to use, and inexpensive (11).

The neuropathological hallmarks of AD are the presence of senile plaques, neurofibrillary tangles, and neuronal loss (13). Amyloid β-peptide, the major component of senile plaques that accumulates in the brains of AD patients, is a prooxidant and increases the formation of reactive oxygen radicals (14). Amyloid β-peptide is derived from β-amyloid precursor protein (APP) through sequential cleavage by the β-secretase and γ-secretase complex (15). β-Secretase is identified as the beta-site APP-cleaning enzyme (16) and the γ-secretase-mediated process includes presenilin (PS), nicastrin (Nct), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (17). APH-1, a key factor in γ-secretase activity, forms a stable subcomplex with nicastrin and contributes to the stabilization of the γ-secretase complex. Therefore, variations in the APH-1 gene sequence may increase the risk of AD by altering the γ-secretase activity. APH-1a is the primary isofrom of APH-1 in mammals (18). There are some studies about PS and Nct of the γ-secretase complex (19,20), but APH-1a has not been investigated thoroughly in AD except for a few studies (21–23). Moreover, there is no study investigating APH-1a polymorphism and AD risk in a Turkish population.

In regard to this knowledge, we tested the hypothesis that polymorphic alterations in APH-1a exons and nearby intronic regions can play an important role in AD pathogenesis. We also searched for APOE polymorphisms and matched them with APH-1a polymorphisms to analyze whether there is an increased risk for AD. To the best of our knowledge, this is the first such study in the literature in a Turkish population.

2. Materials and methods
2.1. Patients and clinical evaluation
Forty-nine patients diagnosed clinically with probable AD according to the Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (1) and 45 healthy controls were included in the study. Being younger than 65 years, existence of another neurodegenerative disease and/or a history of a previous cerebrovascular disease, and having any other neurological, psychiatric, or medical condition interfering with cognition were chosen as exclusion criteria for the case group. The study protocol was approved by the ethics committee of the Turgut Özal University Medical Faculty.

After obtaining informed consent, detailed medical history, neurological examination, routine blood tests, and computed tomography and/or magnetic resonance imaging of the brain were performed for each patient.

2.2. DNA isolation
Genomic DNAs were isolated from whole blood by phenol-chloroform extraction assay.

2.3. DNA amplification
To study polymorphisms rs429358 T>C and rs7412 C>T, 303 bp of the APOE gene was amplified. The primers of the APOE gene for PCR amplification were modified from a previous report as follows: 5'-CGGGCACCGGTGTCCAAAGGAG-3'(forward) and 5'-CTGGTGGAACAGGGCCGCGT-3' (reverse) (24).

To study polymorphism analysis of the APH-1a gene at rs3754048 C>G, 427 bp of the genomic region was amplified using the primers 5'-CTGGTGGAACAGGGCCGCGT-3' (forward) and 5'-CTGGTGGAACAGGGCCGCGT-3' (reverse) (22). 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 APH-1a genes. In the reaction Eppendorf tubes, the total volume used in gradient PCR was 25 µL with 10X buffer, MgCl₂, dNTP, primers, H₂O, Taq polymerase, and DMSO. The DNA samples were
added at the end of all procedures. Total gradient PCR mix consisted of 10X buffer (2.5 µL), MgCl2 (2.5 µL), dNTP (0.5 µL), forward primer (0.5 µL), reverse primer (0.5 µL), Taq polymerase (0.2 µL), DMSO (1 µL), H2O (15.3 µL), and DNA (2 µL) for APOE and APH-1a 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 (denaturation), 71 °C for 30 s (annealing), and 72 °C for 1 min (hybridization), and final extension at 72 °C for 5 min. Reaction conditions for APH-1a involved the same procedures for APOE except the annealing temperature (67 °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 the APOE gene rs429358 T>C and rs7412 C>T polymorphisms, a 303-bp PCR product was fragmented into 91-, 83-, 72-, and 48-bp fragments using CFO1 (Hhal) enzyme (New England Biosystems, USA). The enzyme sliced the C base from the region of the GCGC sequence with recognition sequence of 5’..RGGCY’...3’ , 3’ …Y’CGCGR…5’ .

For enzymatic digestion of the APH-1a gene rs3754048 and rs7412 T>C polymorphisms, a 303-bp PCR product was fragmented into 192- and 235-bp fragments or left uncut by 2% agarose gel electrophoresis. However, for RFLP 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 1).

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).

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).

2.8. APH-1a PCR electrophoresis results
To evaluate PCR products of the investigated region, samples were run on 2% agarose gel, with a fragment of 303 bp in length, and visualized in a UV transilluminator.

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).

2.9. Sequence confirmation
After detecting the presence of PCR products, certain reference samples underwent DNA sequencing and APH-1a and APOE genes were confirmed. PCR products were purified using Sephadex column purification after sequencing by PCR amplification. Samples were analyzed by the Applied Biosystems 3130 genetic analyzer.

2.10. Statistical analysis
SPSS 11.5 was used for the statistical analysis of the data. The Kolmogorov–Smirnov test was used to detect the accuracy of the normal distribution of continuous and discrete variables. The descriptive statistical methods applied for the continuous and discrete variables were standard deviation, mean, and median, while case numbers and percentages were applied for categorical variables. Statistical significances of mean values among groups were tested with Student’s t-test. Differences
between groups based on the median values were tested with the Mann–Whitney U test. Pearson's chi-square test, Fisher's exact test, and likelihood ratio tests were used to compare categorical variables. The relationship between the continuous and discrete variables among themselves was tested with the Spearman's correlation test. Odds ratio and confidence intervals (95% confidence interval) were examined to assess the susceptibility of APH-1a and APOE genotypes and alleles to the risk of AD. P < 0.05 was considered significant.

3. Results
3.1. Demographic data and comorbid conditions in patient and control groups
A total of 49 patients diagnosed with AD (30 women and 19 men; average age of 78.5 ± 6.8 years) and 49 healthy participants (27 women and 18 men; average age of 71.9 ± 8.7 years) were examined. Fifteen (30.6%) patients had a family history of AD. Members of the control group had no family history of AD.

3.2. Allele distributions of APOE and APH-1a genes
APOE allele distribution of patient and control groups is shown in Table 1, while APH-1a allele distribution is shown in Table 2. The most frequent allelic distribution was ε3/ε3 in both groups (57.1% and 64.4%, respectively). The frequency of carrying at least one ε4 allele was found to be 32.7% in the patient group and 17.8% in the control group (P > 0.05). The ε2 allele frequency distribution in the patient group was 6.1% and it was 10% in the control group. The ε3 allele frequency distribution in the patient group was 75.5% and it was 80% in the control group (P > 0.05). The ε4 allele frequency distribution in the patient group was 18.4% and it was 10% in the control group (P > 0.05).
No statistically significant difference was observed between the patient and control groups in terms of prevalence of ε4 allele, but a tendency was detected between ε4 allele presence and AD (P > 0.05) (Table 1).

The effects of vascular risk factors (diabetes, hypertension, and hyperlipidemia) were investigated. Diabetic patients were 26.5% of the AD group and 26.7% of the control group. Hyperlipidemia was detected in 22.4% of the AD group and 35.6% of the control group. Hypertension was present in 73.5% of the AD group and 73.3% of the control group. Almost 30% of the AD group had a family history of AD.

Allele and genotype frequencies of the APH-1a gene in patient and control groups are shown in Table 2. There was no statistically significant difference for APH-1a CC, GC, or GG genotype prevalence between patients and control subjects (P > 0.05). When allele frequencies were studied, allele frequency of the G allele was 29.3% in the AD group and 27.8% in the control group, whereas allele frequency of the C allele was 70.7% in the AD group and 72.2% in the control group. This did not cause a statistically significant difference (P > 0.05) (Table 2).

Among subjects with APH-1a GG or GC, 30.8% of the patient group and 12% of the control group were found to...
be a carrier of at least one ε4 allele of APOE. Additionally, 69.2% of the AD group was G allele (+) but ε4 (-), whereas 88% of the control group was G allele (+) but ε4 (-). The relationship between APH-1α and APOE alleles was not statistically significant (P > 0.05) (Table 3).

There was no significant relationship between APOE ε4 allele and APH-1α G allele with family history of AD (P > 0.05) (Table 4).

4. Discussion

Identifying individuals at risk of AD, managing controllable risk factors, and ensuring early diagnosis and treatment have gained more importance in recent years (25). The best-known genetic risk factor is the ε4 allele of the APOE gene (26). The mechanisms of the synthesis of APOE in the brain are still not completely known. Many studies performed on AD patients have shown that being a carrier of the ε4 allele of APOE not only increases the risk of AD but also expedites the age of onset of the disease (27). The number, diameter, and intensity of amyloid β plaques are strongly related to the APOE genotype (28).

On the other hand, although the APOE ε4 allele is a potent risk factor for AD, most APOE ε4 carriers do not develop dementia, and about one-half of AD is not APOE ε4-associated while the presence of the ε4 allele has a limited predictive value in asymptomatic persons (29). In the literature, there is a consensus that APOE genotyping should not be used for diagnostic testing (30). This suggests that there are other genetic and environmental factors playing a role in the development of dementia. In our study, there was no patient carrying the ε2/ε4 genotype. There was no person carrying the ε2/ε2 genotype in the control group. The ε3/ε3 genotype is the most frequent genotype in both groups. The frequency of being a carrier of at least one ε4 allele was 32.7% in the patient group, while it was 17.8% in the control group. Although these data suggest the increase of frequency of ε4 allele in the AD group, it did not reach statistical significance. In previous studies, it was demonstrated that the ε3 allele is the most frequently seen genotype in the population, as we confirmed with our study. Two studies in Turkey declared that ε2 allele frequency was higher in the patient group, contradictory to our data (31,32).

Studies have demonstrated that nicastrin, APH-1α, APH-1β, and PEN-2 genes interact with presenilins and are required for the intramembrane proteolysis of APP and other γ-secretase substrates (32–35). Small RNA prevents the formation of multimeric complexes of APH-1α and APH-1β, as well as decreasing the production of amyloid β-peptide (17). Therefore, sequence variations in the APH-1 gene interfere with γ-secretase activity and have a role in determining the risk of sporadic AD. In this study, we tested this hypothesis by analyzing APH-1α for the presence of polymorphisms in a Turkish sample of sporadic AD cases and controls. As shown in Table 3, there was no statistically significant difference in genotype

<table>
<thead>
<tr>
<th>Variables</th>
<th>APH-1α</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G allele (+)</td>
<td>G allele (-)</td>
</tr>
<tr>
<td>Control</td>
<td>3 (12.0%)</td>
<td>5 (25.0%)</td>
</tr>
<tr>
<td>ε4 (+)</td>
<td>22 (88.0%)</td>
<td>15 (75.0%)</td>
</tr>
<tr>
<td>ε4 (-)</td>
<td>8 (30.8%)</td>
<td>7 (35.0%)</td>
</tr>
<tr>
<td>AD</td>
<td>18 (69.2%)</td>
<td>13 (65.0%)</td>
</tr>
</tbody>
</table>

Table 4. Relationship of APOE and APH-1α with family history of AD.

<table>
<thead>
<tr>
<th>Comorbid conditions</th>
<th>APOE</th>
<th>CC</th>
<th>P-value</th>
<th>E4 allele</th>
<th>Other alleles</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family history of AD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>15 (53.6%)</td>
<td>13 (46.4%)</td>
<td>P = 0.686</td>
<td>7 (23.3%)</td>
<td>23 (76.7)</td>
<td>P = 0.172</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (60%)</td>
<td>6 (40%)</td>
<td></td>
<td>7 (46.7%)</td>
<td>8 (53.3)</td>
<td></td>
</tr>
</tbody>
</table>
or allele frequencies of the APH-1a G allele in AD subjects and controls. Our study elucidates how polymorphisms in APH-1a and APOE genes are associated with sporadic AD. The analysis of genotypes and allele frequencies of the APH-1a and APOE genes did not reveal any statistically significant differences among AD cases and controls.

In a previous study investigating the sequence variations in the promoter area of the APH-1a gene, only the 980C/G (rs3754048) single nucleotide polymorphism was found to be associated with AD (36). Regarding this information, we studied 980C/G (rs3754048) polymorphism of the APH-1a gene together with rs429358 and rs7412 polymorphisms of the APOE gene.

It was found that 30.8% of the patient group and 12% of the control group had the concurrence of at least one G allele of APH-1a and at least one ε4 allele of APOE. This did not reach statistical significant difference. In their study, Wang et al. revealed that the GG genotype and G allele frequency of the -980 C/G (rs3754048) polymorphism was increased in the patient group compared with the control group. At the same time, the frequency of APOE ε4 was increased in carriers of the G allele of APH-1a. Wang et al. stated that there is a relationship between the -980 C/G polymorphism of APH-1a and sporadic AD, possibly due to the synergistic interaction between the G allele and APO ε4 (21). Qin et al. reported that the presence of the G allele was higher in the patient group, and therefore they declared that APH-1a polymorphism leads to an increased risk of AD. The possible underlying mechanism is the G allele, which has a higher transcriptional activity causing overexpression of APH-1a (22). In another study conducted by Poli et al., it was concluded that APH-1a and APH-1b did not increase the risk of AD in an Italian population and the disease risk was not connected to the presence of APOE ε4 (23). A study performed by the same group revealed that there is a potential relationship between APH-1b C + 651 T>G polymorphism and the APOE ε4 allele in AD and both gene polymorphisms relatively increase the risk of AD. According to these findings, AD risk increases 3.3-fold in patients positive for APOE ε4 but negative for APH-1b G allele, whereas the risk of AD increases 28.6-fold in patients carrying both alleles. Contrarily, carrying only APH-1b did not cause an increase in the risk of AD (36).

In summary, our study points out that sequence variations in APH-1a and APOE do not affect the risk of sporadic AD in the Turkish population. In addition, our results demonstrate that there is no genetic interaction between APOE and APH-1a alleles. This is the first study to investigate the relationship between APH-1a gene polymorphisms and AD risk in Turkish patients. This study could provide insight for other studies that might be examining the hypothesis in sporadic AD in very large populations in the future.

Acknowledgment
This work was supported by the Scientific Research Fund of Turgut Özal University under project number 2013-04-002.

References
ACAR ÇİNLETİ et al. / Turk J Med Sci


