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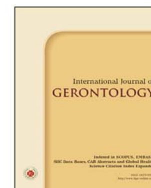

Original Article

The Association of *APOE* Genetic Polymorphism and Environmental Factors with Alzheimer's Disease

Zohreh Harati [a], Ali Javadpour [b], Raheleh Masoudi [a *], Syedeh Leila Abtahi [a]

[a] *Department of Biology, College of Sciences, Shiraz University, Shiraz, Iran,* [b] *Shiraz Geriatric Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran*

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SUMMARY

Background: There are several environmental and genetic risk factors contributing to late onset Alzheimer's disease (AD). Two genetic risk factors for AD are ε4 allele of *APOE* and Brain Derived Neurotrophic Factor (BDNF) Val66Met polymorphisms. The objective of this research is to investigate the association of *APOE* and BDNF polymorphisms, some clinical and environmental factors either independently or in combination, with risk of AD in a population from the south west of Iran.
Methods: 66 AD patients and 74 healthy controls were included in this case-control study. Participants were genotyped for the polymorphisms using Allele specific PCR.
Results: The frequency of ε3, ε2, and ε4 alleles of *APOE* gene was 87.8, 6.8, and 5.4%, respectively. Frequencies were 84.5 and 15.5% for G and A allele of *BDNF* polymorphism, respectively. When each factor was applied as univariate, there was significant association between some factors including depression, history, smoking, education, and ε4 allele carrier, with risk of developing AD. In multiple analysis, there was an association of APOE gene and smoking/history with risk of AD. However, when all factors were considered together, the effect of *APOE* polymorphism on the risk of AD was no longer significant. A correlation between depression and ε4 allele was observed in this study.
Conclusion: It can be concluded that environmental factors may have significant role in developing AD. In addition, a correlation between depression and *APOE* gene may suggest that individuals with depression and ε4 allele are at greater risk for developing AD compared to people lacking depression and/or ε4 allele.

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1. Introduction

Alzheimer's disease (AD) causes memory loss and cognitive impairment in elderly people.

Mutation in genes coding *amyloid precursor protein* (*APP*), *presenilin 1* (*PSEN1*), and *presenilin 2* (*PSEN2*) can lead to amyloid beta deposition and early onset AD (EOAD).[1,2]

Late onset AD (LOAD) occurs after age 60–65 resulting from complex interaction between genetic and environmental factors.[3]

Polymorphism in *Apolipoprotein E* (APOE) gene is the most important genetic risk factor for LOAD.[2] Three major alleles of *APOE* gene are formed as the result of two common polymorphisms, rs 7412 and rs429358 which encode three isoforms of the APOE polypeptide, APOE2 (Cys at position 112,158), APOE3 (Cys and Arg at position 112 and 158, respectively), and APOE4 (Arg at position112 and 158).[4,5] The difference between amino acids alters the binding affinities of these isoforms to low density lipoprotein receptor (LDLR) and LDLR-related protein (LRP). Levels of plasma LDL are higher in APOE4 phenotype.[6-8]

Human Brain Derived Neurotrophic Factor (*BDNF*) is involved in neuronal growth, differentiation, survival, synaptic plasticity, and neurogenesis.[9] One of the functional polymorphisms in the *BDNF* gene is rs6265 with a G/A substitution at position 196 in the coding region which leads to a valine to methionine substitution at codon 66 of BDNF prodomain (val66met).[10] This polymorphism decreases BDNF secretion which in turn can lead to memory impairments and loss of hippocampal volume.[9,11,12]

Some environmental factors such as exposure to pesticides, hypertension, high cholesterol levels in middle age, smoking, and depression can also increase risk of AD.

The aim of this research is to examine the independent and combinational effect of the common *APOE* and *BDNF* polymorphisms, and some of the clinical and environmental factors on the risk of AD in a population from the south west of Iran. Association between *APOE* polymorphism and AD has been studied in some areas of Iran.[13-15] However, *BDNF* polymorphism and its possible association with AD have not been investigated in Iran, yet.

2. Methods

The AD group included 66 patients (47% females; mean age, 76.12 ± 7.31 years, range, 62–91 years) diagnosed by a neurologist according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Re-

* Corresponding author. Department of Biology, College of Sciences, Shiraz University, Shiraz, Iran.
E-mail addresses: rmasoudi@shirazu.ac.ir (R. Masoudi)

lated Disorders Association (NINCDS-ADRDA) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). The Alzheimer's criteria of NINCDS-ADRDA describe eight cognitive domains impairment including, memory, language, problem solving, attention, constructive abilities, perceptual skills, orientation and functional abilities. The control group included 74 subjects (43.2% females; mean age, 73.88 ± 6.92 years, range, 61–96 years) with no cognitive impairment according their medical history and general examination. Diabetes or depression was taken into account when the subject was taking medication. All participants were from the south west of Iran. The proposal of this study was approved by the Shiraz University ethical committee.

Blood samples were taken after a formal letter of consent was signed by the participant of this study or their legal guardians. Boiling Method was applied in order to extract the genomic DNA from each blood sample.[16]

2.1. APOE genotyping

Multiplex T-ARMS PCR (tetra-amplification refractory mutation system) was performed with approximately 20 ng of human genomic DNA in a reaction containing 1X Hot Start master mix (Amplicon) with 1.5 mM $MgCl_2$ and 10% DMSO. Six primers (Table 1, Fig. 1) for two SNPs (0.1 μM FO, 0.5 μM RO, 0.5 μM FI-1, 0.5 μM RI-1, 0.2 μM FI-2, and 0.5 μM RI-2)[17] were used in a total volume of 20 μL reaction.

To validate the results of multiplex T-ARMS, in two T-ARMS PCR reactions, about 35 ng of human genomic DNA, 8% DMSO, 1X of HotStart master mix, and four primers (0.1 μM FO, 0.5 μM RO, 0.75 μM FI-1, and 0.5 μM RI-1 at codon 112; 0.1 μM FO, 0.5 μM RO, 0.2 μM FI-2, and 0.5 μM RI-2 at codon 158) were applied in a total volume of 10 μL.

The PCR steps were as follow: 15 min at 95 °C for enzyme activation, 35 cycles of the following steps: 30 s at 95 °C, 30 s at 68 °C, and 30 s at 72 °C, and a final extension at 72 °C for 7 min.

The PCR products were run on 2% agarose gels containing 0.5 μg/mL ethidium bromide and visualized under UV light (Fig. 2).

2.2. BDNF genotyping

ARMS PCR for genotyping rs6265 polymorphism in *BDNF* gene was accomplished with approximately 40 ng of human genomic DNA, in a reaction with 1X Hot Start master mix. Four primers (Table 1, Fig. 3) (1.5 μM P1, 1.25 μM P2, 0.75 μM P3 and 1.25 μM P4) were used in a total volume of 25 μL in a single reaction tube.[18]

The amplification was carried out as follows: enzyme activation step at 95 °C for 15 min, 35 cycles of the following steps:94 °C for 45 s, 62.5 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C

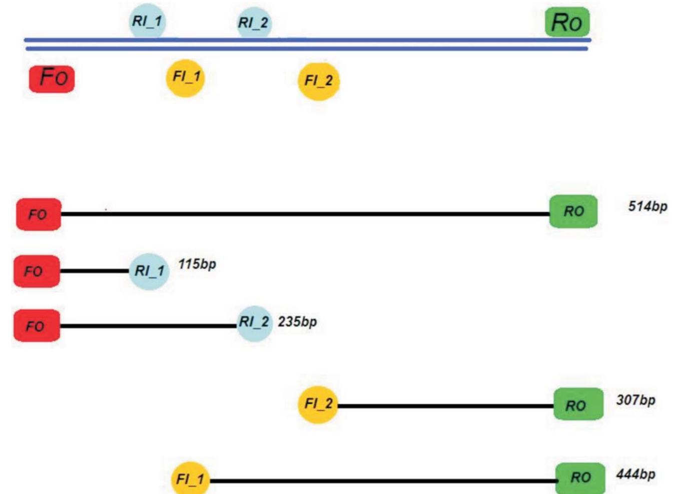

Fig. 1. Diagram of multiplex T-ARMS PCR for *APOE* genotyping. Outer primers (FO and RO) amplified 514 bp product, and the combinations of each inner primer (FI-1, FI-2, RI-1, and RI-2) and outer primer in two SNP sites produced specific amplicons illustrated in the figure.

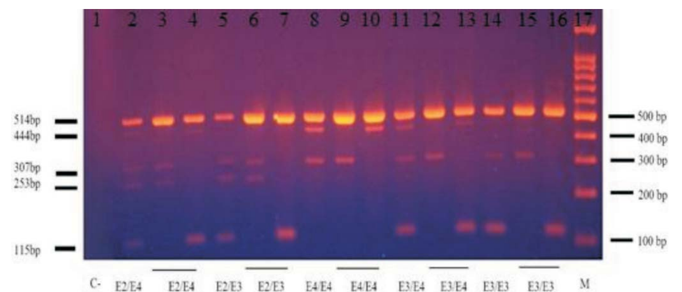

Fig. 2. T-ARMS PCR confirms multiplex T-ARMS PCR for APOE genotyping. For each genotype, the first lane from left indicates multiplex T-ARMS PCR and the next two lanes show the results of T-ARMS PCR. Lane 1 is negative control. T-ARMS PCR involved separate amplifications with the respective primers for codon 112 (lanes 4, 7, 10, 13, and 16) and codon 158 (lanes 3, 6, 9, 12, and 15) sites, while multiplex T-ARMS PCR combined all primers for the two SNP sites (lanes 2, 5, 8, 11, 14). Lane 17 is 100 bp molecular marker and lane 1 represents negative control.

Table 1
Primers used for Multiplex T-ARMS PCR *APOE* genotyping, *BDNF* val66met (rs6265) allele specific PCR, and *BDNF* (rs6265) PCR-RFLP.

Multiplex T-ARMS PCR *APOE* genotyping	Primers	5′→3′ Sequence
Common outer primers	FO	ACTGACCCCGGTGGCGGAGGA
	RO	CAGGCGTATCTGCTGGGCCTGCTC
Inner primers at codon 112	FI-1	GGCGCGGACATGGAGGACG**g**G***C***
	RI-1	GCGGTACTGCACCAGGCGGCC**t**C***A***
Inner primers at codon 158	FI-2	CGATGCCGATGACCTGCAGA**c**G***C***
	RI-2	CCCGGCCTGGTACACTGCCAG**t**C***A***
BDNF val66met (rs6265) allele specific PCR		
	P1 (forward)	CCTACAGTTCCACCAGGTGAGAAGAGTG
	P2 (reverse)	TCATGGACATGTTTGCAGCATCTAGGTA
	P3 (G allele specific)	CTGGTCCTCATCCAACAGCTCTTCTAT**a**A**C**
	P4 (A allele specific)	ATCATTGGCTGACACTTTCGAAC**c**C**A**
BDNF (rs6265) PCR-RFLP		
	Forward	GAGGCTTGACATCATTGGCT
	Revers	CGTGTACAAGTCTGCGTCCT

The boldface lowercase letters are a deliberate mismatch, and the boldface uppercase letters are an allele-specific mismatch.

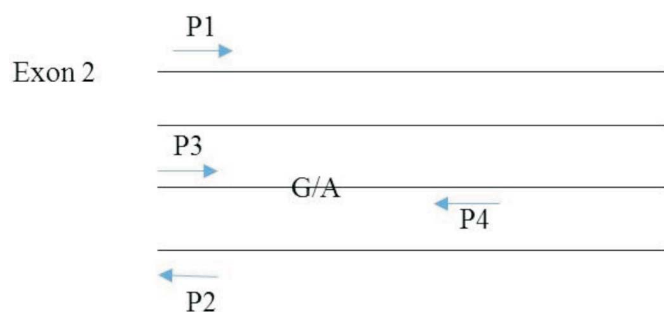

Fig. 3. Tetra primer positions relative to the G/A substitution at codon 196 in exon 2 are shown by arrows at the 5′ end of each primer. P1 and P2 = control primers, P3 = G allele-specific primer and P4 = A allele-specific primer. 401 bp band represents the control amplicon, whereas the G and A allele-specific bands are represented by the 253 and 201 bp amplicons.

for 7 min. The products were separated on 2% agarose gels containing 0.5 μg/mL ethidium bromide and visualized by UV light (Fig. 4).

To verify our results, PCR-RFLP was carried out to detect *BDNF* polymorphism. Following PCR reaction (40 ng of genomic DNA) using primers in Table 1,[19] 5 μL of PCR product was digested with the restriction enzyme *HinfI*. The digestion products were electrophoresed on 2.5% agarose gels (data not shown).

2.3. Statistical analysis

Hardy Weinberg equilibrium for the alleles was evaluated using a χ^2 test. Univariate and multiple logistic regression were applied in order to examine the association of genetics, clinical and environmental factors with AD. All variables were categorical. Factors with p value less than 0.05 were entered into multiple analysis, backward-stepwise model. In multiple analysis, $p < 0.05$ was considered statistically significant. Pearson Chi-Square was applied to determine the correlation between *APOE* and depression in AD. All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA; version 23).

3. Results

The number of participants, mean age, and number of females are shown in Table 2. There was no significant difference between AD and control groups regarding age (p = .064) and sex (p = .393).

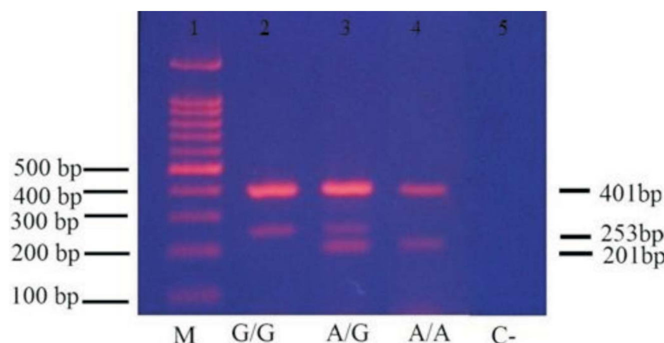

Fig. 4. The result of ARMS PCR for *BDNF* genotyping. Lane 1 is molecular marker. Lane 5 is negative control. rs6265 genotyping by ARMS-PCR. Lane 1, 100-bp molecular marker, lane 2, val/val genotype, lane 3, val/met, lane 4, met/met genotype and lane 5 negative control. 401 bp band represents the control amplicon, whereas the G and A allele-specific bands are represented by the 253 and 201 bp amplicons.

3.1. Association of some clinical and environmental factor with AD

Table 3 shows the results from univariate logistic regression analysis. Individuals with a first-degree relative affected by AD showed higher risk of developing AD (OR = 15.88, p = .009). Depression also showed significant association with AD (OR = 14.5, p < 0.00). Although studies repoerted that diabetes increases the risk of cardiovascular disease and AD,[20] no significant difference between AD patients and control groups was detected in terms of being affected by diabetes (p > 0.05). A significant increase was observed in the risk of AD for smokers (OR = 4.6, p = 0.002). Illiterate people were at higher risk of developing AD compared to individuals who had more years of formal education. (OR = 12.6, p < 0.00). Some data were missing in the control group of all above variables.

3.2. Association of genetic polymorphisms with AD

The distribution of *APOE* alleles and related genotypes is presented in Table 4. No deviation was observed from Hardy-Weinberg equilibrium for these polymorphisms. ε3ε4 genotype was significantly higher in AD patients versus controls (p = 0.015) with 3.5 fold increase in the risk of developing AD for carriers of this genotype (95% CI = 1.28–9.79, OR = 3.535). The difference in ε4 allele frequency was significant (p = 0.016) between cases and controls (95% CI = 1.22–6.85, OR = 2.886). Carriers of ε4 allele showed 3.5 fold increase in the risk of developing AD (OR = 3.6, p = 0.008) compared to non-carriers.

According to Table 4, no significant difference in the allelic or genotyping distribution of the *BDNF* A196G polymorphism was detected between AD cases and control group.

3.3. Association of genetic polymorphism and environmental factor with AD

In order to calculate the adjusted odd ratios for the association of the genetic and environmental factors with AD, multiple logistic regressions were carried out. Factors from Table 3 with p-value less than 0.05 were applied in multiple analysis. P-value less than 0.05 was regarded as statistically significant in multiple analysis. Carrying ε4 allele and smoking could increase the risk of AD by 8.6 fold (additive effect, no interaction). Having first degree relative affected by AD and being ε4 allele carrier, also increased the risk of AD (OR = 17.6, additive effect). When all environmental and clinical factors (depression, history, education, and smoking) were entered as covariates with carrier ε4 allele, in a backward stepwise model, the effect of ε4 allele in developing AD did not remain evident.

Next, in order to assess whether there is a correlation between any of studied factors with carrier ε4 allele, Pearson Chi-Square were carried out. The only factor showing significant correlation with carrier ε4 allele in developing AD was depression (p = 0.001) (Supplementary data).

Table 2
Demographic characteristics of the participants.

Parameter	Controls	AD	p
No. of subjects	74	66	
Mean age (years)	73.88 ± 6.92	76.12 ± 7.31	.064
Female sex (%)	43.2	47	.393

Table 3
Association of environmental or clinical factors with the risk of AD.

Environmental parameter	Control (%)	Patient (%)	P value	Odd ratio	95% C.I.
First-degree relative with AD (History)	1 (1.81)	15 (22.73)	**0.009**	15.882	2.024–124.625
Depression	3 (4.28)	26 (39.39)	**0.00**	14.517	4.128–51.056
Diabetes	9 (12.88)	15 (22.72)	0.125	2.033	0.821–5.037
Smoking	6 (9.09)	20 (28.57)	**0.002**	4.638	1.727–12.454
Education	6 (10.9)	40 (60.6)	**0.00**	12.564	4.711–33.511

Univariate logistic regression.

Table 4
Genotype and allelic frequency of polymorphic genes and their association with AD.

Polymorphic gene	Genotype/allele	Control (%)	Patient (%)	P value	Odd ratio	95% C.I.
BDNF	GG	55 (74.32)	51 (77.27)	-	-	-
	AA	4 (5.41)	1 (1.51)	0.248	0.270	0.29–2.493
	AG	15 (20.27)	14 (21.21)	0.988	1.007	0.442–2.290
	G	23 (15.5)	16 (12.1)	-	-	-
	A	125 (84.5)	116 (87.9)	0.410	0.750	0.377–1.489
APOE	ε3ε3	57 (77.03)	43 (65.15)	-	-	-
	ε2ε3	10 (13.51)	5 (7.57)	0.481	0.663	0.211–2.081
	ε2ε4	0 (0)	1 (1.51)	1.00	2141	0.00-.
	ε3ε4	6 (8.1)	16 (24.24)	**0.015**	3.535	1.277–9.787
	ε4ε4	1 (1.35)	1 (1.51)	0.844	1.326	0.81–21.797
	ε3	130 (87.8)	107 (81.1)	-	-	-
	ε2	10 (6.8)	6 (4.5)	0.552	0.729	0.26–2.07
	ε4	8 (5.4)	19 (14.4)	**0.016**	2.886	1.22–6.85
Carrier allele A *BDNF*		19 (25.68)	15 (22.72)	0.685	0.851	0.392–1.851
Carrier allele ε4 *APOE*		7 (9.46)	18 (27.27)	**0.008**	3.589	1.390–9.266

Univariate logistic regression.

4. Discussion

The underlying mechanisms of late onset AD include both genetic and environmental factors. The purpose of this study was to assess the effect of some environmental, clinical and genetic factors, independently or in combination, on the risk of AD. According to our results, when genetic, clinical, or environmental factors are applied as a univariate, there is significant association between depression, first degree relative, smoking, education, ε3ε4 genotype, and carriers of ε4 allele, with the risk of developing AD. Our result is consistent with the results reporting that one first degree family with AD or smoking may increases the risk of AD.[21] In addition, our results confirm that educated people have lower risk for developing AD which can be due cognitive reserve hypothesis or other factors that affect the brain health.[21] In AD cases, depression and other mental health problems are common.[3] Some AD participants in this study had depression for about 20 years. It has been shown that the ε4 form of *APOE* gene, increases the risk of AD.[21] Similar to other findings, our results show that the ε3ε4 genotype and ε4 allele increases the risk of AD about 3.5 folds and 2.9 folds, respectively. In this study, no significant effect of the Val66Met *BDNF* polymorphism on AD was observed. There is controversial evidence about the relationship between AD and rs6265 polymorphism in *BDNF*[10] which could be due to geographical location and ethnical background of the participates in different studies.

According to our multiple analysis, there is an association of *APOE* gene and smoking/history with the risk of AD. However, when all factors are taken into account as covariates, the effect of *APOE* gene variation on the development of AD is not noticeable anymore. It can be concluded that environmental factors may play an important role in developing AD. In addition, a correlation between depression and *APOE* gene is found in the current study. It suggests that people with depression and ε4 allele may have higher risk of developing AD compared to individuals lacking depression and/or ε4 allele.

The frequencies of ε3, ε4, and ε2 alleles of *APOE* in this population are 87.8, 5.4, and 6.8%, respectively. These frequencies are similar to the data from previous studies from Iran[13-15,22] and some studies from Turkish, Greek, Japanese, and Spanish populations[23-26] but are different from data published for American, Finland, and African populations.[27–29] The genotyping for rs6265 polymorphism in *BDNF* is carried out for the first time in Iran and also in Iranian Alzheimer's patients. G and A allele frequencies of rs6265 in *BDNF* is 84.5 and 15.5, respectively, which are similar to the studies carried out in the Italy, Spain and United States.[12,30,31] However, they are different from studies carried out in Japan and China.[32,33]

To sum up, the research reveals the association of a genetic and several environmental factors with the risk of AD in a population from the south west of Iran. Moreover, the importance of clinical and environmental factors in developing AD is shown in this study.

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Conflict of interest

The authors declare no conflict of interest and they are responsible for the content of the paper.

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Supplementary Tables

Table 1

Association of ApoE4 and smoking with the risk of Alzheimer's disease.

Parameter	P value	Odd ratio	95% C.I.
Carrier of ε4 allele	**.008**	3.992	1.432–11.124
Smoker	**.003**	4.629	1.688–12.696

Multiple logistic regression, Backward-stepwise model: APOE and smoking were entered as covariates

Table 2

Association of ApoE4 and history with the risk of Alzheimer's disease.

Parameter	P value	Odd ratio	95% C.I.
Carrier ε4 allele	**.049**	2.854	1.004–8.113
First-degree relative with AD	**.010**	15.064	1.900–119.423

Multiple logistic regression, Backward-stepwise model: APOE and first degree relative were entered as covariates.

Table 3

Association of environmental or clinical factors with the risk of Alzheimer's disease, adjusted results

Parameter	P value	Odd ratio	95% C.I.
History	**0.008**	20.324	2.212–186.725
Education	**0.000**	17.534	5.884–52.257
Depression	**0.002**	13.645	2.606–71.438

Multiple logistic regression, backward-stepwise model including following variable: APOE, History, Education, Smoking, and Depression (detailed information in supplementary data).

Table 4

Correlation between *APOE4* and depression in Alzheimer's disease.

Symmetric Measures

			Value	Approximate significance
Total	Interval by Interval	Pearson's R	.277	.001[c]

[c] Based on normal approximation.

Pearson chi square in order to determine the correlation between *APOE4* and depression in AD.