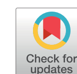
Gene Reports 14 (2019) 65–71

Contents lists available at ScienceDirect

Gene Reports

journal homepage: www.elsevier.com/locate/genrep

Association analysis of ApoE gene polymorphisms among Egyptian patients with Alzheimer's disease



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ARTICLE INFO

Keywords:
ApoE gene polymorphisms
Alzheimer's disease
Egyptian population

ABSTRACT

Background: Alzheimer disease (AD) is one of the leading causes of dementia among elderly. It is a progressive brain disorder associated with unusual behaviors, personality changes, and irreversible decline in thinking ability. Epsilon 4 allele of apolipoprotein E (ApoE) is considered as a major risk factor for AD in several populations.
Aim: We investigated APOE polymorphisms in a group of Egyptian patients with Alzheimer's disease.
Subjects and methods: We analyzed APOE polymorphisms in 53 Alzheimer patients who were 60 years or older and the study included a control group of 100 individuals. For APOE genotyping, we used conventional PCRs followed by Restriction Fragment Length Polymorphism (RFLP) analysis. Additionally, we performed Real time PCR analysis to confirm the results of RFLP and apply a more accurate method that is fast, simple, and cost-effective.
Results: We observed that E4 allele was associated with 28.3% of Alzheimer patients as E3/E4 genotype (22.6%) and E4/E4 (17.0%), while we detected it in only 4% of the control group as E3/E4 genotype (8%). E3 allele has been shown in 69.8% of Alzheimer patients and in 93.5% of the control subjects. We have detected E2 allele in 18.9% of the cases and in 25% of the control group. RT-PCR analysis showed the exact patterns of genotype as determined by RFLP.
Conclusion: There is a significant association between ApoE4 isomer and Alzheimer dementia in the Egyptian patients.We recommend conducting further studies to screen larger number of patients and confirm that ApoE4 could be considered as a reliable molecular marker for late onset Alzheimer disease (LOAD) among Egyptian elderly.

Abbrevations

AD	Alzheimer disease
ApoE	apolipoprotein E
R	arginine
CDR	Clinical Dementia Rating Scale
CI	confidence interval
r	correlation coefficient ()
Ct	cycle threshold
C	cysteine
dNTP	deoxynucleotide
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
DMSO	dimethyl sulfoxide
E4	Epsilon 4
EDTA	ethylenediaminetetraacetic acid
HDL	high density lipoprotein
LOAD	late onset Alzheimer disease
MgCL	magnesium chloride
µg	micro gram
µl	microliter
µM	micromolar
mM	millimolar
MMSE	Mini-Mental State Examination
MoCA-Basic	Montreal Cognitive Examination Basic
MNDs	motor neuron diseases
ng	nano gram
NRC	National Research Centre (Cairo, Egypt)
OR	odds ratio
p	p. value
PCR	rolymerase chain reaction
Rs(No)	reference SNP
RFLP	restriction fragment length polymorphism

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https://doi.org/10.1016/j.genrep.2018.10.017
Received 31 May 2018; Received in revised form 13 October 2018; Accepted 29 October 2018
Available online 16 November 2018
2452-0144/ © 2018 Published by Elsevier Inc.

SNP	single nucleotide polymorphism
SD	standard deviation
SPSS	statistical package for the social sciences
UCSC	University of California, Santa Cruz (genome browser)
VLDL	very low density lipoprotein

1. Introduction

Alzheimer's disease (AD) is a progressive brain disorder with a serious impact on humans. AD is the most prevalent form of dementia, a common term for memory loss and cognitive problems. AD is characterized by the presence of amyloid-β aggregations (National Institute on Aging, 1997). AD begins with limited failure in memory and gradually becomes more severe and associated with confusion, poor intellectual abilities, occasional seizures, and several serious complications (Bird and Miller, 2005). In Egypt, AD has been reported as the most prevalent form of dementia, accounting for 51.2% of all hereditary cases (Tallawy et al., 2012). AD is categorized into early-onset familial AD (onset before age 60–65 years) and late-onset familial AD (onset after age 60–65 years) (Smits et al., 2015). With the lack of specific therapy for AD, the treatment is directed toward slowing disease progression. The strategy for treatment depends mainly on identifying AD carriers at early stages (Humpel and Hochstrasser, 2011). Therefore, precise diagnostic methods are of high demand such as looking for strongly associated molecular biomarkers. Several markers have been studied and linked to the AD particularly apolipoprotein E (ApoE) which has showed significant association in different populations (Huynh and Mohan, 2017).

ApoE is a substantial glycoprotein for lipid metabolism (Mahley, 1988). The human ApoE gene is located on chromosome 19 (Das et al., 1985). There are three co-dominant isomers of ApoE including ApoE2, ApoE3, and ApoE4. These three proteins differ at the amino acid residues 112 and 158. Both amino acids are cysteine (C) in ApoE2 or arginine (R) in ApoE4, while ApoE3 has 112C and 158R (Emi et al., 1988). These amino acids have significant impact on the lipid binding activities of the ApoE since ApoE2 and ApoE3 bind preferentially to HDL, whereas E4 prefers VLDL (Saito et al., 2003). The pathogenesis of different diseases has been linked to the presence of certain ApoE isomer. For example, ApoE4 is identified as a major risk factor for the cardiovascular disorder and AD. However ApoE2 confers the protection against the development of AD and it is associated with familial type III hyperlipoproteinemia (Roses et al., 1994). Recently, genotyping ApoE becomes of interest to identify people with risk for developing heart disease or who are susceptible for Alzheimer (Santos et al., 2017).

Different methods have been described for genotyping ApoE. The primary method is the restriction fragment length polymorphism (RFLP) (Zivelin et al., 1997). Several laboratories have introduced other effective and less time consuming methods such as mass spectrometry (Srinivasan et al., 1998), Amplification Refractory Mutation System-PCR (Donohoe et al., 1999), Simple Sequence Specific Primer-PCR (Pantelidis et al., 2003), Real Time PCR, and Fluorescent Resonance Energy Transfer (Rihn et al., 2009).

In the current study, we study the correlation between certain genotypes of ApoE and elderly Egyptians suffering from Alzheimer. We used RFLP and RT-PCR analysis to determine the allele frequency of the different ApoE isomers and do genotype analysis. We identified significant correlation between AD and ApoE4 allele. We observed that E4 allele is represented by E4/E4 genotype in AD patients only or by E3/E4 in patients and non-AD petients.

2. Subjects & methods

The research proposal was revised and approved by the ethical committee at the National Research Centre (NRC), Cairo, Egypt. (Ethical Approval Number: 16011). An informed consent was obtained from patients and/or their guardian.

3. Study design and population

The study is a descriptive, case-control study with consecutive sample. Patients were recruited from the Memory Clinic of Old Age Psychiatry Unit, Psychiatry and Addiction Hospital, Faculty of Medicine, Cairo University while controls were recruited from Cairo University Geriatric Social Centre. The study included 53 patients, 60 years old or older of both genders. For AD diagnosis, we followed the diagnostic and statistical manual of Mental Disorders (DSM-5) criteria (APA, 2013) of Major Neurocognitive Disorder. To confirm AD diagnosis, we used Clinical Dementia Rating Scale (CDR): disease score of 1 or more/3 classified as major neurocognitive disorder following Washington's University CDR-assignment algorithm (Morris, 1993). For further confirmation, we used Mini-mental State Examination and Montreal Cognitive Assessment.

All Patients requested cognitive assessment and fulfilled the inclusion criteria, were invited to participate in the study. Patients and the responsible care giver or legal guardian were asked to give an informed written consent. The patients were then subjected to the usual assessment procedures, neuropsychological tests, and the necessary laboratory and brain radiological investigations to verify the diagnosis. The study included the patients with clear evidence of gradual and steadily progressive decline in memory and learning and associated with impairment in either executive, visuospatial, or language. A consultant psychiatrist attending the clinic at the time of assessment reviewed the diagnosis. We excluded patients with MNDs due to other etiologies or with mixed pathology. We excluded, patients with uncorrected visual or auditory deficits, terminal organ failure, comorbid medical or mental condition or those who use drug and/or substance that may interfere with cognitive testing.

The control group included 100 elders who responded to an advertisement processed through Cairo University Geriatric Social Center requesting volunteers to participate in a study about AD. The center offers a range of social, recreational and sportive activities to elderly living in Cairo. We included subjects who were not having any memory complaints, or difficulties in the activities of daily practices, and were not clinically depressed or suffering from a mental or general medical conditions or receiving drugs known to impair cognition at the time of assessment. Additionally, control subjects were required to achieve a normal score in both MMSE and CDR.

The following assessment protocol was used to evaluate both patients and control subjects:

1- Semi-Structured Clinical Interview: We interviewed the candidates at the Psychiatry Department, Faculty of Medicine, Cairo University. We recorded demographic data, habits of medical importance, medical and family history, and the results of the present state examination as well general medical and neurological records.

2- Mini-Mental State Examination (MMSE): The MMSE is a brief, quantitative measure of cognitive status in adults and elderly. Although, it was initially formulated by Folstein et al. (1975), it is considered as standard cognitive assessment in clinical situations because of easy administration and concise nature. The MMSE has demonstrated validity and reliability with both adults and geriatrics in clinical and research settings including, residential facilities, and hospitals. In our study, we used MMSE to confirm the diagnosis. Subjects scoring 24 or more were classified as cognitively normal, those with score of 18–23 were considered suffering from mild impairment while 10–17 score used for moderate impairment, and 9 points as an indicator for severe impairment (Mungas, 1991). We purchased The MMSE from Psychological Assessment Resources (PAR). We gave illiterate or patients with < 4 years of education 2 free points to adjust the cut-off (Crum et al., 1993; Wrobel and Farrag, 2008).

3- Montreal Cognitive Examination Basic (MoCA-Basic) - Arabic

Table 1
Demographic and clinical characteristics with AOR (95%CI) of controls and cases.

Descriptive statistics		Control group N = 100	Cases group N = 53	OR (95%CI)	P value
Age (years)	60–65	57(57.0%)	18(34.0%)	1(reference)	0.01*
	Over 65	43(43.0%)	35(66.0%)	2.58(1.29–5.15)	
	Mean ± SD (Range)	65.9 ± 5.0 (60–77)	70.5 ± 7.5 (60–89)		
Gender	Male	24(24.0%)	24(45.3%)	1(reference)	0.01*
	Female	76(76.0%)	29(54.7%)	2.62 (1.29–5.33)	
Marital status	Single	1(1.0%)	1 (1.9%)	1(reference)	0.3
	Married	51 (51.0%)	19 (35.8%)	0.37 (0.02–6.26)	0.4
	Divorced	7 (7.0%)	4 (7.5%)	0.57 (0.03–11.85)	0.7
	Widowed	41 (41.0%)	29 (54.7%)	0.7 (0.04–11.78)	0.8
Education	Yes	96 (96.0%)	22 (41.5%)	1(reference)	0.001**
	No	4 (4.0%)	31 (58.5%)	33.82 (10.82–105.72)	
	Mean ± SD (years)	13.1 ± 4.03	9.5 ± 3.9		
Residence	Rural	7 (7.0%)	24 (45.3%)	1(reference)	0.001**
	Urban	93 (93.0%)	29 (54.7%)	10.99 (4.29–28.13)	
Living status	Alone	18 (18.0%)	3 (5.7%)	1(reference)	0.03*
	With others	82 (82.0%)	50 (94.3%)	3.66 (1.03–13.05)	
Smoking	Yes	8 (8.0%)	16 (30.2%)	1(reference)	0.001**
	No	92 (92.0%)	37 (69.8%)	4.97 (1.96–12.61)	
Hypertension	Normal	47 (47.0%)	30 (56.6%)	1(reference)	0.4
	Controlled	47 (47.0%)	19 (35.8%)	0.96 (0.25–3.68)	0.9
	Uncontrolled	6 (6.0%)	4 (7.5%)	0.6 (0.15–2.39)	0.5
Diabetes (DM)	Normal	70 (70.0%)	38 (71.7%)	1(reference)	0.6
	Controlled on oral	17 (17.0%)	4 (7.5%)	0.43 (0.14–1.38)	0.1
	Controlled on insulin	10 (10.0%)	7 (13.2%)	1.29 (0.45–3.66)	0.6
	Uncontrolled on oral	0 (0.0%)	2 (3.8%)	–	0.9
	Uncontrolled on insulin	3 (3.0%)	2 (3.8%)	1.23 (0.19–7.67)	0.8
Cardiac	Normal	88 (88.0%)	47 (88.7%)	1(reference)	0.9
	IHD	8 (8.0%)	5 (9.4%)	1.17 (0.36–3.78)	0.7
	Arrhythmias	3 (3.0%)	0 (0.0%)	–	0.9
	P cardiomyopathy	1 (1.0%)	1 (1.9%)	1.87 (0.12–30.62)	0.6
Family history of Alzheimer's	Negative	85(85.0%)	40 (75.5%)	1(reference)	0.1
	Positive	15 (15.0%)	13 (24.5%)	1.84 (0.8–4.23)	

Age, sex, Marital, Education, Residence,living status, smoking, Hypertension, DM, Cardiac, Renal, Hepatic andfamily historyare represented as number and percent, While the Education yearsis represented as Mean and SD.OR: Odds Ratio; CI: Confidence Interval; *P value ≤ 0.05 significant while **P value ≤ 0.01highly significant.
The P. value depending on the logistic regression model.

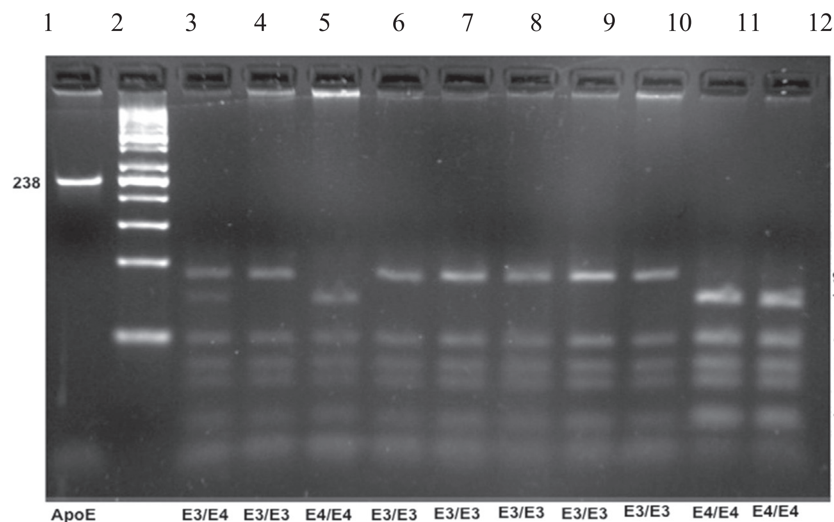

Fig. 1. Analysis of ApoE genotyping. Amplified ApoE fragment digested with HhaI and visualized on 4% gel electrophoresis. The figure shows different genotype for ApoE as indicated for different AD patients. Lane 1: PCR product of 238 bp for ApoE exon4 fragment Lane 2: shows the marker. Lane 3: the heterozygous allele E3/E4. Lane 4 & 6–10: the homozygous allele E3/E3. Lane 5, 11 &12: the homozygous allele E4/E4.

version: The MoCA-Basic is composed of 9 subtests covering executive functions, attention, memory, orientation and visuospatial functions. A score of 26/30 or above is presumed normal although results might be affected by low educational levels (Saleh et al., 2018). The translated Arabic version is identical to the original version with no cultural or linguistic modifications. A well-trained clinical psychologist performed both the MMSE and MoCA-B on the same day while he was not aware of the diagnosis or CDR score.

4- Clinical Dementia Rating Scale (CDR): The CDR is a semi-structured clinical interview for determining the severity of neurocognitive disorders. It includes questions for the patient and the informant (care giver) covering memory, orientation, judgment, problem solving, community affairs, daily activities, and personal care. Next, we generated an overall score to categorizes the level of impairment as described before: 0 = no impairment, 0.5 = questionable, 1 = mild impairment, 2 = moderate impairment, 3 = severe impairment (Morris, 1993). We used CDR to confirm the diagnosis of MNC and

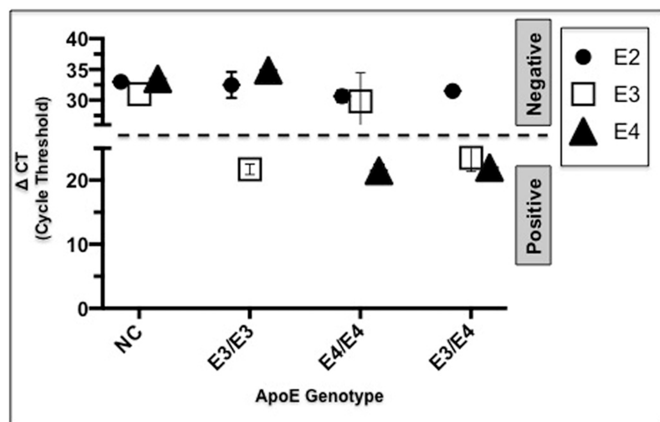

Fig. 2. Amplification plots (ApoE Genotype vs. Ct). Real Time PCR System of the three different APOE haplotypes in 35 CE patients and negative controls (NC).

Table 2
APO-E Allele frequency in cases and controls.

APOE		Control group N = 100	Cases group N = 53	P. value
Genotype	E2/E2	0(0.0%)	0(0.0%)	NA
	E2/E3	5(5.0%)	2(3.8%)	0.7
	E2/E4	0(0.0%)	0(0.0%)	NA
	E3/E3	87(87.0%)	30(56.6%)	0.04*
	E3/E4	8(8.0%)	12(22.6%)	0.02*
	E4/E4	0(0.0%)	9(17.0%)	0.001**
Alleles	E2	5 (0.0250)	2 (0.0189)	0.7
	E3	187 (0.9350)	74 (0.6981)	0.03*
	E4	8 (0.0400)	30 (0.2830)	0.001**

ApoE genotype is represented as number and percent, while ApoE Allele is represented as number and Allele frequency. **P* value ≤ 0.05 significant; **P value ≤ 0.01 highly significant.

Table 3
Cognitive tests in cases and controls.

Cognitive tests		Control group N = 100	Cases group N = 53	P value
Mini-Mental State Examination (MMSE)		29.07 ± 1.2	16.85 ± 6.7	0.001**
Montreal Cognitive Assessment (MoCA-B)		25.4 ± 3.07	9.5 ± 5.3	0.001**
Clinical dementia rating (CDRS)	Negative (0)	91 (91.0%)	0 (0.0%)	0.001**
	Nearly mild (0.5)	9 (9.0%)	0 (0.0%)	
	Mild (1)	0 (0.0%)	19 (35.8%)	
	Moderate (2)	0 (0.0%)	26 (49.1%)	
	Severe (3)	0 (0.0%)	8 (15.1%)	

MMSE and MoCA-Bare represented as Mean and SD whereas the P. value depending on (t) test, while CDRS is represented as number and percent whereas the P. value depending on x^2 test. *P value ≤0.05 significant; **P value ≤0.01 highly significant.

rate the severity (Nasreddine et al., 2005).

5- Molecular tests:
DNA extraction: We collected EDTA-anticoagulated venous blood samples from all participants. We purified genomic DNA using QIAmp extraction Kit (qiagen) according to the manufacturer's instructions. The quality and quantity of DNA was determined using Nanodropper 2000 (ThermoScientific).
APOE Polymorphisms genotyping: APOE genotypes were performed by polymerase chain reaction followed by restriction fragment length polymorphism analysis as previously described (Geesje et al., 1995). Leukocyte DNA was amplified by PCR using oligonucleotide primers F (5′-GCGGGCCCCGGCCTGGTACAC-3′) and R (5′-GACGC GGGCACGGCTGTCCAAGGA-3′). To amplify exon four of ApoE, we used 100 ng of the isolated DNA, 0.2 mM dNTP, 0.4 μM of each primer, 1.5 mM $MgCl_2$, 7% DMSO, and 1 U ThermoDNATaq (EP0402). We optimized the PCR conditions as following: Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 2 min. The final amplicon extension was performed at 72 °C for 5 min. PCR products were visualized by electrophoresis on 2% agarose gel containing 5 μg ethidium bromide to confirm successful amplification. The amplified PCR product then was digested with 10 U of the restriction enzyme Hha1 for 15 min at 37 °C. The digested PCR products were separated on 4% agarose gel at 120 V for I hr and were analyzed using gel documentation system (Bio-Rad) (Geesje et al., 1995).

To validate the results of the conventional PCR genotyping, we randomly selected 35 samples positive for the APOE polymorphisms (rs7412 and rs429358; codon 112 and 158, respectively). We further analyzed the samples by RT-PCR using the LightCycler480 Real Time PCR System (Roche). We tested each sample for the presence of the different isoforms of ApoE gene. Here, we used previously designed primers specific, for ApoE_112C: (5′-CGGACATGGAGGACGTGT), ApoE_112R: (5′-CGGACATGGAGGACGTGC), ApoE_158C: (5′-CTGGTA CACTGCCAGGCA), and ApoE_158R (5′- CTGGTACACTGCCAGGCG) (Calero et al., 2009). Briefly, each PCR reaction mixture contained the following: 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.6 μM of each primer and 50 ng of genomic DNA to amplify an ApoE fragment of 173 bp. Each isoform of ApoE was targeted by using the appropriate pairs of primer as following: ApoE2, (ApoE_112C and ApoE_158C), ApoE3 (ApoE_112C and ApoE_158R) and ApoE4 (ApoE_112R and ApoE_158R). Reaction mixture without DNA served as a negative control. All the reactions were run in duplicate. The PCR amplification conditions were set as following: initial activation for the AmpliTaq Gold DNA Polymerase at 95 °C for 10 min, followed by 40 cycles with a denaturation step at 95 °C for 15 s, and annealing/extension at 62 °C for 1 min. Amplification was performed on the LightCycler480 Real Time PCR System (Roche). The presence of certain ApoE isomer was determined by differential amplification of the three specific reactions for E2, E3, or E4 alleles on the basis of their Ct values (absolute quantitation method). Ct values lower than 25 indicated a positive amplification reaction, whereas Ct values higher than 25 was considered as a negative amplification. For example, an ApoE E3/E4 heterozygous yielded Ct values for ApoE3 amplification and that of ApoE4 significantly lower than that for ApoE2. In contrast, an ApoE E3/E3 homozygous showed a Ct value for ApoE3 amplification significantly lower than that obtained for ApoE2 or ApoE4 (Calero et al., 2009).

Statistical analysis: We analyzed the data using Microsoft Excel 2010 and (SPSS version 24.0) (SPSS IBM., Chicago, IL). Continuous normally distributed variables were represented as mean ± SD. With 95% confidence interval, and using the frequencies and percentage for categorical variables; the results with a p value < 0.05 were considered statistically significant. To compare the means of normally distributed variables between groups, the Student's *t*-test was performed. $\chi 2$ test or Fisher's exact-test was used to determine the distribution of categorical variables between groups. We used Spearman's rank correlation coefficient (r) to show the correlation between the different parameters. Effect modifications were evaluated by stratification, and statistical interaction was assessed by including main effect variables and their product terms in the logistic regression model.

4. Results

Distinct patterns for ApoE genotyping by RFLP. We selected a total

Table 4
Correlations between Apo-E allele frequency and cognitive tests.

Correlation parameters	MMSE[1]		MoCA-B[2]		CDRS[3]		Apo-E	
	r	P value	r	P value	r	P value	r	P value
Family history of Alzheimer's	−0.141	0.083	−0.095	0.24	0.184*	0.023	0.115	0.157
MMSE[1]			0.843**	0.001	−0.858**	0.001	0.239**	0.003
MoCA-B[2]	0.843**	0.001			−0.878**	0.001	0.285**	0.001
CDRS[3]	−0.858**	0.001	−0.878**	0.001			−0.280**	0.001
ApoE	−0.359**	0.001	−0.381**	0.001	0.381**	0.001		
Duration of illness (years)	−0.838**	0.001	−0.832**	0.001	0.950**	0.001	−0.290**	0.001

1-MMSE = Mini-Mental State Examination. 2-MoCA-B = Montreal Cognitive Assessment-Basic. 3-CDRS = Clinical Dementia Rating Scale. r = Correlation coefficient, * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

Table 5
Alzheimer risk according to *Apo* Genotype.

ApoEgenotype	Control N = 100	Cases N = 53	Univariate OR (95%CI)	P value	AOR(95%CI)	P value
E2/E2	0(0.0%)	0(0.0%)	_	NA	_	NA
E2/E4	0(0.0%)	0(0.0%)	_	NA	_	NA
E3/E3	87(87.0%)	30(56.6%)	1(reference)		1(reference)	
E2/E3	5(5.0%)	2(3.8%)	1.34 (0.25–7.16)	0.5	1.16 (0.21–6.29)	0.8
E3/E4	8(8.0%)	12(22.6%)	3.37 (1.28–8.86)	0.01*	4.35 (1.62–11.66)	0.003**

OR:/Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence Interval; *P value ≤0.05 significant; **P value ≤0.01 highly significant.
N·B: E3/E3 is used as a reference genotype for detecting the univariate (OR) Odds Ratio and AOR: Adjusted Odds Ratio regarding to control and patient groups.

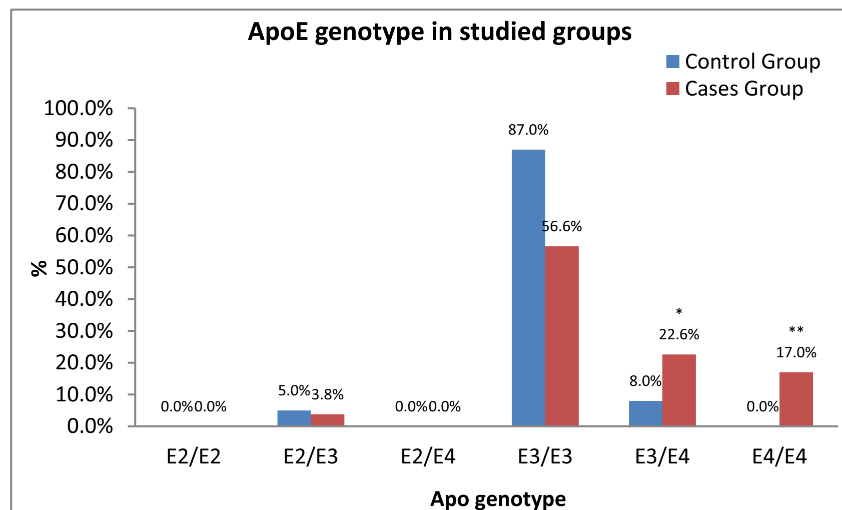

Fig. 3. ApoE genotypes in studied groups.

of 153 individuals to participate in the study. The participants were 60–89 years old, the age of control group was 65.9 ± 5.0 years and clinically diagnosed AD patients were 70.5 ± 7.5 years. We observed that The Alzheimer's dementia patients were significantly older (p = 0.01) and less educated (p = 0.001) than the control group. Table 1 outlines the demographic and clinical characteristics of participants. To determine the different genotypes of ApoE associated with AD in Egyptian patients, we performed RFLP analysis. First, we generated an amplified PCR product of 238 bp fragment representing the fourth exon of ApoE. This DNA fragment contains five *Hha*I sites (GCGC) (Kontula et al., 1988). In addition, *Hha*I enzyme cleaves the fragment if a cleavage site generated at codon 112 or 158, which means a codon encodes arginine instead of cysteine. The restriction map of each ApoE isomer shows a distinct pattern; ApoE2: 91, 83, 38, and 19 bp; ApoE3: 91, 48, 38, 35, and 19 bp; and ApoE4: 72, 48, 38, 35, and 19. We tested each DNA sample for the occurrence of the possible six different genotypes of ApoE (E2/E2, E3/E3, E4/E4, E2/E3, E2/E4, or E3/E4). We observed that each genotype had a distinguished restriction fragment pattern (Fig. 1). The genotype E3/E3 shows fragments of 91, 48, 38, and 35 bp. Whereas E4/E4 genotype lacks the 91 bp fragment and 72 bp is formed instead. E4/E3 genotype showed the three small fragments in addition to the 91 bp and 72 bp fragments. The E2/E2 genotype is characterized by the presence of the 91 bp, 83 bp, and 38 bp fragments (Dallinga-Thie et al., 1995).

In agreement with previous reports, we observed a remarkable association between ApoE4 and patients with Alzheimer disease. We observed that 22.6% of Alzheimer cases had E3/E4 genotype while E4/E4 was detected in 17.0% of the patients. These results indicate that E4 allele is associated with 28.3% of Alzheimer patients. On the other hand, E4 allele has been detected in 4% of the control group and represented by E3/E4 genotype (8%). Moreover, E3 allele has been shown in 69.8% of the cases as E3/E3 genotype (56.6%) and E3/E4 (22.6%). However, E3 allele frequency was among 93.5% of the control subjects and E2 allele has been showed in 18.9% of the cases as E2/E3 genotype (3.8%) and in 25% of the control group as E2/E3 genotype (5%).

To analyze the genotype of ApoE in a more accurate and fast method, we used Real time PCR to confirm the results of RFLP. In order to measure in real time, the DNA amplification, the double-stranded DNA fluorescent dye, SYBR Green, was used for APOE genotyping. Using a primer specific for each isomer, we were able to discriminate between the different APOE haplotypes on the basis of their Ct values. The presence of certain ApoE isomer was determined by differential amplification of three specific reactions for E2, E3, or E4 alleles. In agreement with RFLP results, The Ct value near that of the negative control means the absence of the isomer but the Ct value, which is significantly lower, indicates the presence of the tested allele.

We used RT-PCR to investigate the genotypes of 10 cases and compare the results with that of RFLP. We set three different reactions for each sample in a duplicate manner and included a negative control without DNA. We observed Ct value of 33–35 with the negative control which we used as an indicator for the absence of the isomer (Fig. 2). However, the Ct value for the present ApoE isomer (as shown by RFLP) ranged between ~20 and ~23. For instance, an ApoE E3/E4 heterozygous yielded Ct values for amplification of ApoE3 or ApoE4 significantly lower ($\Delta Ct \geq 10$ cycles) compared to ApoE2. Whereas, an ApoE E3/E3 homozygous showed a Ct value for ApoE3 amplification significantly lower than that obtained for ApoE2 or ApoE4. We did not determine Ct values in non-expected range (lower than 15). In summary, Ct values lower than 25 indicated a positive amplification reaction, whereas Ct values higher than 25 were considered as a negative amplification. This strategy meets the sensitivity and the threshold set-up defined for Light Cycler 480 Real Time PCR System. The genotypes of all the samples, determined by RT-PCR, showed the exact pattern that was determined by RFLP analysis (Table 2).

The statistical analysis shows a significant difference between the control group and AD cases as determined by cognitive tests which was observed in the MMSE, MoCA-B, and CDRS ($p = 0.001$). The frequency of Apo-E4 allele was associated with lower cognitive scores denoting the severity of dementia (MMSE $p = 0.003$, MoCA-B, $p = 0.001$ & CDR, $p = 0.001$). Moreover, Apo-E4 allele was associated with longer duration of Alzheimer's dementia ($p = 0.001$). These correlations are outlined in Tables 3 and 4. Overall, Apo-E genotyping analysis showed that the risk of Alzheimer's dementia was strongly associated with Apo E3/E4 with adjusted odds ratio of 4.35 (CI = 1.62–11.66) and p value of 0.003 (Table 5 and Fig. 3).

5. Discussion

In the present study, we shed light on the association between ApoE alleles and Alzheimer's disease in Egyptian elderly. We identify ApoE4 as an important genetic marker for late onset AD, which is in agreement with results for Polish AD patients (Limon-Sztencel et al., 2016). Moreover, we observed that ApoE3 is a common haplotype among non-Alzheimer people while ApoE2 is less common. Several molecular markers have been described to screen and diagnose AD particularly ApoE4 (Anderson, 2013). Although, great efforts have been spent for biomarker identification and analysis, disease complications enforce continuing the biomarker search and drug development. Moreover, different methods have been described for ApoE genotyping targeting the accuracy, simplicity, and low-cost (Zhong et al., 2016).

Analysis of AD among Egyptians shows that people over 65 years have high probability to suffer from AD (OR is 2.58), which is similar to what have been reported before for Chinese patients (Mengying et al., 2014). We demonstrated that women are more susceptible to the disease than men with OR value of 2.62. Similar to Limon-Sztencel's report in 2016, we observed that the education is a significant protective factor since uneducated patients showed OR value of 33.82. The demographic data indicates that urban dwellers are at higher risk of suffering from AD than rural patients with OR value of 10.99, which is in line with Guodong et al. (2017).

Clinical examinations showed highly significant results for the cognitive tests of MMSE, MoCA-B, and CDRS ($p = 0.001$). The high frequency of Apo-E4 allele was associated with low cognitive scores and severe dementia (MMSE $p = 0.003$, MoCA-B, $p = 0.001$ & CDR, $p = 0.001$). Moreover, we observed significant association between Apo-E4 allele and prolonged Alzheimer's dementia ($p = 0.001$), which is similar to previous Chinese study (Montufar et al., 2017).

6. Conclusion

We determined a significant association between ApoE4 isomer and Alzheimer's dementia among Egyptian patients. Further studies with larger number of Alzheimer patients is recommended to confirm that ApoE4 could be considered as a reliable molecular marker for late onset Alzheimer disease (LOAD) among Egyptian elderly subjects.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

This work was entirely supported by National Research Centre Grant P101305 (Egypt). We would also like to thank everybody participated in this study from the Memory Clinic of Old Age Psychiatry Unit, Psychiatry and Addiction Hospital, Faculty of Medicine, Cairo University and from Cairo University Geriatric Social Centre.

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