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ORIGINAL ARTICLE

High APOE epsilon 4 allele frequencies associated with Alzheimer disease in a Tunisian population

Afef Achouri Rassas · Hela Mrabet Khiari · Sondes Hadj Fredj · Safa Sahnoun · Hend Batti · Nouria Oudiaa Zakraoui · Aroua Cherif · Nadia Anane · Nadia Ben Ali · Taieb Messaoud · Amel Mrabet

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Abstract The goal of the study was to examine the Apolipoprotein E (*APOE*) genotypes in a Tunisian sample of patients with Alzheimer disease (AD) and normal controls, and to compare the results with the findings from the literature. A hospital-based case–control study of two groups (58 patients with AD, 71 controls) was conducted. Patients received a detailed clinical history, neurological examination, neuropsychological testing and brain imaging. A neurological examination and the Arabic version of the Mini-Mental State Examination were made for controls. Genotyping was performed using the PCR restriction fragment length polymorphism (PCR–RFLP) method. There were no statistical differences in age ($p = 0.05$) and gender ($p = 0.046$) between the two groups. The *APOE* ε4/4 genotype was over represented in the AD group in comparison with the controls (13.3 vs. 2.8%). A significant increased risk of AD among *APOE* ε4 allele carriers was observed. The odds ratio for the association of AD patients with homozygous and heterozygous ε4 allele was, respectively, 5.40 (1.35–21.48) and 2.90 (1.27–6.62). Our results in addition to previously published genetic studies suggest that AD disease is multifactor in origin. Ethnicity, genetic and environmental factors contribute to AD risk in different ethnic groups.

A. A. Rassas (✉) · H. Mrabet Khiari · H. Batti · N. O. Zakraoui · A. Cherif · N. Anane · N. Ben Ali · A. Mrabet
Neurological Department, Charles Nicolle Hospital, Boulevard du 9 Avril, 1006 Tunis, Tunisia
e-mail: achouriafef@yahoo.fr

H. Mrabet Khiari · A. Mrabet
Faculté de Médecine de Tunis, Tunis, Tunisia

A. A. Rassas · S. Hadj Fredj · S. Sahnoun · T. Messaoud
Biochemistry and Molecular Biology Laboratory, Hôpital d'Enfants, Tunis, Tunisia

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Introduction

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disease, considered as one of the main causes of dementia [1]. The majority of large-scale genome-wide association studies have demonstrated that Apolipoprotein E (*APOE* gene; APOE protein) genotype is the major risk factor for late-onset AD [2, 3]. ApoE, a lipid transport protein in the plasma and central nervous system, may contribute to AD pathology by acting through both Aβ-dependent and -independent pathways [4]. Three common isoforms exist. The ε2 allele is suggested to have a protective effect against the development of AD, whereas ApoE4 is associated with the increasing risk of AD and the lowest age of onset, and apoE3 with intermediate risk and age of onset.

Some prospective and retrospective data showed a lack of correlation between the APOE ε4 allele and cognitive impairment [5–8], suggesting a high level of genetic and heterogeneity in AD patients of different countries and ethnic groups. The aim of this study was to examine the *APOE* genotypes in a Tunisian sample of patients with AD and normal controls, and to compare the results with findings from other regions of the world.

Methods

Participants

The study was designed as a hospital-based case–control study. It was conducted from January 2009 to June 2010 in

the Neurological Department of Charles Nicolle Hospital, Tunis, Tunisia, according to the Declaration of Helsinki Principles and the guidelines for Good Clinical Practice, and approved by the Local Ethics Committee. Written informed consent was obtained from the patients or from their legal guardians before participation into the study.

Determination of clinical diagnosis

All the patients received a detailed clinical history, neurological examination, neuropsychological testing, brain MRI and routine blood analyses. Validated, reliable and standardized neuropsychological tests in conformity with the cultural standards of the country and with normative data scores exist in Tunisia since 1998 and were used in the study. It included the Mini Mental State Examination, the Alzheimer's disease Assessment Scale Cognitive subscale, Frontal Assessment Battery, Geriatric Depression Scale, Instrumental Activities of Daily Living scale, and the Clinical Dementia Rating [9–14].

Clinical diagnoses of AD were determined by a consensus diagnostic conference, of neurologists and neuropsychologists, using all available information and after at least 12 months of follow-up. Dementia diagnosis was established using Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) criteria [15]. Clinical AD diagnoses were established using National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for Possible or Probable AD [16].

A sample of cognitively intact subjects was recruited from one primary care clinic. A detailed clinical history, neurological examination and the Arabic version of the Mini-Mental State Examination (MMSE) were made for all these subjects [9]. They were included in our study if no personal or familial psychiatric or cognitive impairment history and no alcohol or drug abuse were reported, if neurological examination was normal and if the MMSE score was above 26 points. We considered these subjects as normal controls (NC).

Molecular methods

Genomic DNA was extracted from peripheral blood leukocytes by the phenol/chloroform [17] protocol and the salting out [18] procedure. Genotyping was performed using the PCR restriction fragment length polymorphism (PCR–RFLP) method, DNA was amplified by utilizing a PCR thermal cycler along with oligonucleotide primers APOE-sens: (5′CGGGCACGGCTGTCCAAGGAG3′) and APOE-reverse: (5′CACGCGGCCCTGTTCCACGAG3′). Each amplification reaction contained 250 ng of genomic DNA, 20 pmol of each primer, 10% dimethylsulfoxide, 200 μM of each dNTP, and 0.25 μl of *Taq* DNA polymerase in a final volume of 50 μl. Reaction conditions included denaturation for 30 s at 94°C, annealing for 30 s at 65°C, and extension for 30 s at 72°C, for 30 cycles. The PCR products were digested with *Cfo*I, and the fragments were separated by electrophoresis on a 20% polyacrylamide non denaturing gel. After electrophoresis, the gel was treated with ethidium bromide for 30 min, and DNA fragments were visualized by UV illumination. APOE genotypes for the case and control groups were determined in a blinded fashion by scoring for a unique combination of fragment sizes, as described by Hixon and Vernier [19]. Digestion gives various combinations of fragment sizes: E2/E2, 91 and 83 bp; E3/E3, 91 and 48 bp; E4/E4, 72 and 48 bp and a mixed genotype: E2/E3, 91, 83, and 48 bp; E3/E4, 91, 72 and 48 bp; E2/E4, 91, 83, 72 and 48 bp [19].

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS).The Student *t* test was used for continuous variables, allele and genotype distributions in patients and controls were compared using Chi square test. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated as strength of association between alleles or genotypes and AD. A p value less 0.05 was considered statistically significant.

Results

The assessments were performed on 71 controls and 58 patients with AD, originating from several regions of Tunisia. The mean age (SD) of the normal controls and AD subjects was 69 (15.18) and 73 (9.09) years, respectively. There were no statistical differences in age ($p = 0.05$) and gender ($p = 0.046$) between the two groups. There were no significant differences in any of the mean values of blood chemistry including triglycerides and cholesterol between the demented and control groups. In the AD group, 95% of our patients were illiterate with a history of hypertension in 45% of the cases or of diabetes in 30% of the cases. The mean age of onset of the AD disease was 66.5 years. The mean follow-up duration of AD in our department was 5 ± 3 years.

APOE genotype for AD cases and controls

More than 75% of our total sample carried the APOE ε3/ε3 and ε3/ε4 genotypes, with ε3/ε3 genotype as the most common genotype. Only one control had the rare ε2/ε2 genotype.

Table 1 Genotypic distribution for the ApoE polymorphism in the Tunisian population

	AD group ($n = 44.96$) (%)	Control group ($n = 55.04$) (%)	p value
Genotype			
E4/E4	13.3	2.8	$\chi^2 = 13.43$, $p = 0.02$
E4/E3	35	18.3	
E4/E2	1.7	1.4	
E3/E3	41.7	69.0	
E3/E2	8.3	7.0	
E2/E2	0	1.4	
Allele			
E4	31.65	12.65	$\chi^2 = 4.69$, $p = 0.03$
E3	63.35	81.65	
E2	5	5.6	

Table 1 shows the distribution of each genotype and the allele frequencies. The distributions of allele frequencies in AD patients and controls were in the Hardy–Weinberg equilibrium.

When comparing the six APOE genotype frequencies among the patients and control groups, a significant difference was observed ($p = 0.02$). The *APOE* ε4/4 genotype was over represented in the AD group as compared to the controls (13.3 vs. 2.8%).

APOE allele frequencies for AD cases and controls

A significant increased risk of AD among *APOE* ε4 allele carriers was observed. The odds ratio for the association of AD patients with homozygous and heterozygous ε4 allele was, respectively, 5.40 (1.35–21.48) and 2.90 (1.27–6.62).

Concerning the gender differences of *APOE* allele frequencies in AD patients, no significant difference was observed in males or females. APOE allele frequencies for males were 6.5% for ε2, 54.3% for ε3, and 39.1% for ε4 and for females were 4.05, 68.95, and 27%, respectively.

Discussion

The incidence of AD increases exponentially with age. In Tunisia, a North African, Arab and Muslim country, percentage of elderly subjects increased from 4.1% on year 1956 to 9.6% on year 2004 [20]. This changing age structure of the Tunisian population markedly affected the occurrence of dementia. Available data of dementia prevalence in Tunisia are obtained from a door to door survey undertaken in 2001 between the Neurological Department of Charles Nicolle Hospital, Tunis, and the Public health Institute [21]. A representative randomized sample of 482 Tunisian general population aged 65 years and more, distributed all across the country. The Arabic version of the MMSE was used to assess the cognitive functions [9]. The dementia prevalence ratio was 3.7% over the age 65 years.

Our sample was relatively limited in size but provided sufficient base data to explore potentially interesting risk factors for AD in Tunisia. For the diagnosis of AD, we used reliable and validated neuropsychological tests, with cut-off scores according to basic education, literacy, sex and age [9–14]. These methods are usually not available in developing countries making the diagnosis of dementia and AD inaccurate [6].

Few studies examining the APOE genotypes in Mediterranean Arab patients with AD exist in the literature. Our results showed significant correlation between ε4 allele frequency and probable AD. In our study, ε4 allele frequency was significantly higher in the AD patients group (31.65%) than in the controls group (12.65%) and possibly constitutes a significant risk factor for AD in urban Tunisians. Our results are in accordance with several previous prospective and retrospective studies reported in Turkish [22], French [23], Canada [24], Iranian [25], Greek [26], Japanese [27], Spanish, and Moroccan [28] populations. In another Mediterranean country near Tunisia, Italy, many other studies from different Italian regions (Sicily, Sardinia, and Apulia) showed similar results with significantly higher frequency of ε4 allele in the AD patients group in comparison with the controls group [29–31]. However, this association is clearly not universal as described by other studies where *APOE* ε4 allele did not constitute a major risk for AD. These studies were conducted in Kenya [32], Yoruba (Nigeria) [33], Bantu (Cameroon), Nilotic Africans [34] and Wadi Ara Arabs in North Israel [5]. Also, some other studies relative to black Americans and American Hispanics populations found similar conclusions [35, 36].

This contradiction in this result shows that the ε4 associated risk for AD may be modified by other genes [37] and a possible environmental implication.

In our study, the distribution number of ε4 alleles differed significantly in heterozygous and homozygous genotypes between AD and controls groups. *APOE* ε4 allele increases the risk for AD in Tunisian population in a dose-dependent manner, similar to the ethnic groups in France [38], Italy [29], Iran [25], Spain [28], Korea [39] and China [40].

It has been estimated that the population-attributable risk for AD caused by APOE ε4 allele ranges from 20 to 70% [41]. Furthermore, the number of APOE ε4 alleles increases from 0 to 2, the risk of developing AD [42]. However, the presence of the ε4 allele is neither necessary nor sufficient to cause AD [43, 44], providing further evidence for the existence of additional factors underlying the genetic risk for AD.

Our study showed no gender based significant differences of *APOE* allele frequencies. This result is similar to the reports of the Nigerian population study [44]. Other studies concerning the Iranian, English, Korean, Chinese, Caucasian, and American populations found a gender difference [39, 40, 45].

In summary, we found high frequencies of the *APOE* ε4 allele. Our results in addition to previously published genetic studies suggest that AD disease is multifactorial in origin, rather than resulting from a single cause [46]. Ethnicity, background, genetic and environmental factors contribute to AD risk in different ethnic groups.

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