Genetic Profile of ACE (I/D) (rs4646994) Single Nucleotide polymorphism Among Sample of Egyptian patients with Alzheimer Disease in Upper Egypt

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Abstract

Background: Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by impaired memory and progressive cognitive and behavioral decline. Angiotensin converting enzyme (ACE) was suggested to have a role in inhibition of Aβ peptides accumulation with formation of plaque *in vitro*. The role of ACE (I/D) genotypes regarding AD development and severity is questionable.

Objectives: to assess the role of ACE (I/D) single nucleotide polymorphism (SNP) as a possible genetic risk factor for AD occurrence and for prediction of the disease severity.

Patients and Methods: This case- control study was carried out in the Neuropsychiatry Department, Qena University Hospital during the period between March 1st 2019 and February 28th 2020. The study included 50 AD patients and 50 healthy age, sex and education matched controls. All cases underwent clinical assessment using Mini Mental State Examination (MMSE), Advanced medical imaging with computed tomography (CT) or magnetic resonance imaging (MRI) of the brain. Genetic analysis for ACE (I/D) (rs4646994) was done using conventional PCR with primers without restriction enzyme.

Results: Mean age of the included patients was 70.1 ± 9.35 years with female predominance (60%). About 46% of patients had mild disease, 42% had moderate disease and 12% had severe disease based on MMSE assessment tool. Diabetes had higher frequency among AD group (30%). ACE homozygous DD genotype had higher frequency (OD=35.9; 95%CI= [2.8-440.2]) and D allele was significantly commoner among AD group than control group (OD=2.13; 95% CI= [1.05-3.2]), (P < 0.05 for all). However, no statistically significant differences in relation to degree of dementia and ACE (I/D) genotypes were recorded. Although homozygous DD genotype and D alleles had higher frequency among severe AD group (P>0.05).

Conclusion: an evidence of significant association between homozygous ACE (DD) and D allele among sample of AD patients in Upper Egypt. However, there is lack of significance association of ACE (I/D) SNP in prediction of disease severity.

Keywords: ACE (I/D) (rs4646994); Alzheimer disease; Egypt; Single nucleotide polymorphism; Conventional PCR; Mini mental state examination.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease marked by progressive cognitive and behavioral impairment as well as memory loss. It represents 60–70% of dementia cases and is considered the most prevalent type. By 2030, there will be 75 million dementia sufferers worldwide, and by 2050, there will be 131 million. Low- and middle-income nations are predicted to see the largest increases in dementia cases. **(Vogt et al., 2023)**.

The two main pathological hallmarks of AD are the extracellular deposition of plaques formed of the β-amyloid peptides (Aβ) and the intracellular neurofibrillary tangles of the microtubule binding protein tau. The membrane-bound amyloid precursor protein (APP) produces proteolytic by-products called Aβ peptide, which are continuously metabolized in the brain. Any defects in Aβ breakdown or removal will cause its accumulation and deposition, which will then trigger an inflammatory response, cause neuritic damage, accumulate hyper phosphorylated tau protein, create fibrillary tangles, and result in neuronal malfunction and cell death. **(McGirr et al., 2020)**.

Numerous pieces of evidence suggested that ACE played a role in AD etiology. ACE was a crucial player in the breakdown of Aβ being a membrane-bound zinc metalloprotease. Being a component of the renin-angiotensin system (RAS), Angiotensin Converting Enzyme (ACE) promotes the synthesis of Angiotensin II (Ang II) from Angiotensin I (Ang I) **(Meghana et al., 2023)**.

Chromosome 17q23 is the location of ACE gene. A 287-bp insertion/deletion (I/D) variant of the ACE gene's intron 16 (rs4646994) is the most prevalent polymorphism. The I/D genotype is thought to influence the amount of ACE expressed in plasma, cells, and tissues **(Fekih- Mrissa et al., 2017)**.

Herein, we explored the possibility of ACE (I/D) (rs4646994) genetic polymorphisms as association genetic risk factor of AD and predictor of disease severity.

Patients and methods

Study design and approval

This Case-control cross sectional study was conducted in the Neuropsychiatry Department, Qena University Hospital during the period between March 1st 2019 and February 28th 2020.

Study population and grouping

The current study included 50 AD patients (study group) and 50 healthy age and sex matched participants (control group). Patients were categorized according to MMSE score into mild, moderate, and severe AD disease **(Eftychios et al., 2021)**. Sample size was calculated by G-power program with α. Error = 0.05 and power 80% (type 1 error) and it was equal to 50 patients.

Clinical assessment and Data collection

Patients' demographic and clinical data included age, sex, residence, dominant hand, common reported complaints and associated medical diseases were recorded. Diagnosis of Alzheimer disease: was done according to DSM-5 diagnostic criteria (**American Psychiatric Association; 2013**). Mini-Mental State examination was done to determine disease severity. It is a tool for cognitive screening that offers a brief, objective assessment of cognitive function. It can be used to demonstrate cognitive impairment, assess how severe the problem is, and monitor cognitive decline over time. The MMSE is the brief cognitive test that is most frequently used in clinical and research settings. Multiple cognitive domains are evaluated by the MMSE, including language, visual construction, orientation, repetition, verbal memory, attention, and calculation. It only requires counting the right answers to determine a test taker's score. Any score of 24 or higher (out of 30) according to interpretations denotes normal cognition. Scores below this range can suggest cognitive impairment that is mild (19–23 points), moderate (10–18 points), or severe (9 points) (**Arevalo et al., 2021**). Using new cut off points for MMSE became now available for both educated and low educated geriatric people. The new cut-off scores are $\leq$ 22 for subjects > 9 years education and $\leq$ 21 for illiterate subjects and those with <9 years education (**Andrews et al., 2019**).

Advanced medical imaging with computed tomography (CT) or magnetic resonance imaging (MRI) of the brain used to exclude other cerebral pathology or subtypes of dementia (**Mendez, 2006**). Moreover, it may predict conversion from prodromal stages (mild cognitive impairment) to Alzheimer's disease (**Schroeter , 2009**). By using one of the following devices in Qena University Hospitals:

- CT Brain: using 64- multi-slices CT- Siemens- Germany.
- MR brain: using 1.5 Tesla MRI set- Philips- USA.

Blood samples and genetic analysis of ACE (I/D) (rs4646994) SNP

Five mls venous blood were withdrawn on EDTA containing tubes from every included subject and were kept at -80 ^{0}C till time of their analysis. The G-spinTM total DNA extraction kit (iNtRON Biotechnology, Inc., Korea) used to extract DNA from whole EDTA blood samples in accordance with the manufacturer's instructions. The extracted DNA was kept at -80 °C for subsequent genetic investigation.

Genetic analysis for ACE (I/D) (rs4646994) was done using conventional PCR with primers without restriction enzyme using the following primers' sequences:F5′CTGGAGACCACTCCCATCCTTTCT3′;R5′GATGTGGCCATCACATTCGTCAGAT-3′ (**Zmorzynski et al.,2019**) as follow: 25 µl of the PCR master mix solution (catalog no. 25028, iNtRON Biotechnology, Korea) were added to 1 µl of forward primer, 1 µl of reverse primer, 21 µl of nuclease-free water, and 2 µl of the extracted DNA. The PCR condition used for genotyping was as follows : the mixture was heated for 5 min at 94^{0}C, then 35 cycles of amplification, including denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s, and elongation at 72 °C for 30 s, within the course of a 5-minute heating period. At 72°C, the final extension took 5 minutes. Utilizing a Biometra thermal cycler (Germany, serial number 2603204), the PCR reactions were carried out. After measurement of DNA integrity, agarose gel (2%) to be stained with 5 µl of ethidium bromide was used were the PCR products were loaded. An electric field was applied to the gel for 15-20 min with 120 volts for DNA. The gel then was removed carefully from the chamber and put in the gel documentation system for visualization of the bands under UV light. Fragments of 190 or 490 bp were used to distinguish between D- and I-alleles, respectively. The existence of two

bands at 490 bp and one band at 190 bp indicate the heterozygous ID

genotype**(Fig.1 and 2)**.
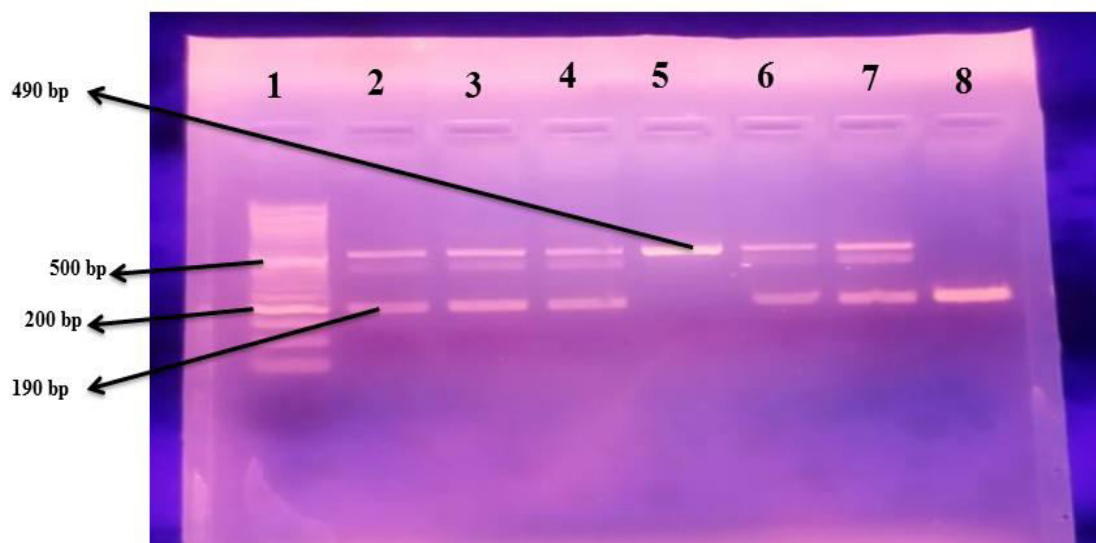
Fig.1. Gel electrophoresis of ACE rs4646994 (I\D) SNP utilizing the conventional PCR method, numbers refer to lanes. Lane 1 shows 50 bp DNA ladder; Lanes 2,,3,4,6,7 are heterozygous (ID) genotypes with 190 and 490 bp bands; Lanes 5 is homozygous (II) genotype with 490 bp band; Lane 8 is homozygous genotype (DD) with 190 bp band.
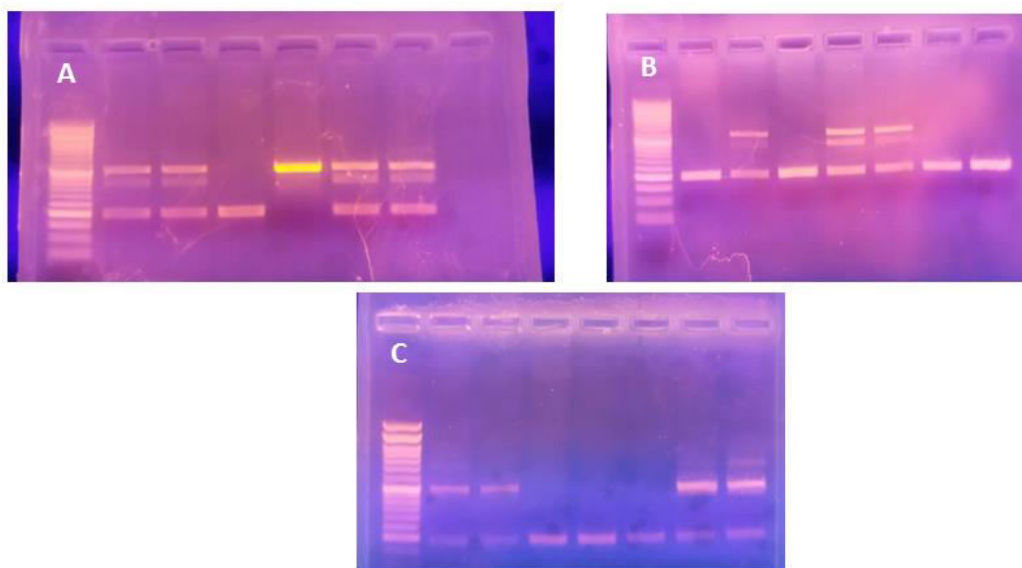
Fig.2. Gel electrophoresis of ACE rs4646994 (I\D) SNP by the conventional PCR method. First lane in each image (A, B & C) showing 50 bp DNA ladder; Other lanes represent various ACE rs4646994 (I\D) genotypes as previously described.

Ethical approval

This study has been approved by the Local Ethics Committee of Faculty of Medicine, South Valley University, Qena, Egypt. Written informed consent was obtained from each participant. The study was conducted in accordance to the Declaration of Helsinki.

Statistical analysis

Statistical analysis was conducted using SPSS (version 21, Chicago, IL, USA). Chi-square test was used to compare

categorical data. The studied SNP was followed the Hardy- Weinberg equilibrium. Analysis of variance (ANOVA) was used to compare normally distributed quantitative data between more than 2 groups. Risk factors of occurrence AD were analyzed using logistic regression analysis. P-value < 0.05 was considered statistically significant

Results

Demographics and Clinical data among study group

Mean age of AD patients in the current study was 70.1 ± 9.35 years with female predominance (60%) and most of patients came from rural areas (80%) and were right-handed (82%). AD group had higher frequency of diabetic patients (30% vs. 14%; p= 0.04) and smoking (26% vs. 0%; p<0.001) than control group. The main reported complaints were difficulty in remembering and poor concentration (100%) followed by problems in finishing daily tasks, confusion with time and place and withdrawal from work (54%). Other complaints of low frequencies are language problems, poor judgement in decisions and mood changes (6%).

All patients were assessed using MMSE and had mean value 17 ± 5.8. Accordingly, 46% of patients had mild disease, 42% of patients had moderate disease and 12% of patients had severe disease. No statistically significant differences exist among the 3 groups as regard age, age at time of diagnosis and dominant hand. Higher female patients were reported in moderate and severe group (mild, moderate and severe: 43.5%, 66.7% and 100%respectively) with higher

percent of male patients among mild dementia (mild, moderate and Severe dementia: 56.5%, 33.3%and 0% respectively) with statistically significant difference (p= 0.03). However no statistically significant differences was found between the studied group as regard associated medical disorders. As regard common reported complaints, difficult in remembering and poor concentration were reported by all patients in the 3 groups. Problems in finishing daily tasks, confusion with time and place and withdrawal from the work were reported more by patients with moderate or severe disease with statistically significant differences (p< 0.001). Language problems, poor judgement in decisions and mood changes were reported by all patients with severe disease with statistically significant difference (p< 0.001).

Genetic profile of ACE gene (I/D) (rs4646994) among the study groups

ACE (I/D) showed statistically significant differences as regard genotypes distribution between study and control group, with higher frequency of DD (40% vs. 20%) but lower frequency of DI (50% vs. 60%) and II (10% vs. 20%) genotypes among study group than control group (p= 0.04). D alleles showed a frequency among study group more than control group with statistically significant difference (65% vs. 50%; p= 0.03), (OR=1.8; 95%CI= 1.05-3.2) **(Tables 1and 2)**. In addition to DD genotype and D allele, diabetes mellitus was also a significant risk factor for developing AD (p value = 0.04) (OR=4.14; 95% CI= 1.07-16.02, **Table. 2).**

Table 1. Genetic differences between Alzheimer and control groups

Variables	Study group (n= 50) No. (%)	Control group (n= 50) No. (%)	*P value
ACE genotypes - DD - DI	 20 (40%) 25 (50%)	 10 (20%) 30 (60%)	 **0.04**

- II	5 (10%)	10 (20%)	
ACE alleles			
- D	65 (65%)	50 (50%)	**0.03**
- I	35 (35%)	50 (50%)	

Chi square test for categorical data; ***** Level of significance < 0.05. ACE: angiotensin-converting enzyme, D: deletion and I; insertion.

Table 2. Multivariate analysis of risk factors for Alzheimer disease

Variables	B estimate	95% confidence interval		Odds ratio	*P value
		Lower	Upper		
Residency Rural vs. urban	1.02	0.7	10.5	2.77	0.13
Diabetes	1.4	1.07	16.02	4.14	**0.04**
ACE genotype:					
DD vs. II	3.6	2.8	440.2	35.9	**0.005**
DI vs. II	2.4	1.01	136	11.75	**0.04**
DD vs. DI	1.11	0.8	11.2	3.03	0.09
ACE D allele	2.13	1.05	3.2	1.8	**0.03**

* Level of significance < 0.05; ACE: Angiotensin converting enzymes

Genetic profile of ACE gene (I/D) (rs4646994) in terms of AD severity

As regard ACE genotypes, homozygous DD mutation had higher frequency among severe disease group with no statistically significance among the 3 groups (mild vs. moderate vs. severe: 47.8% vs. 28.6% vs. 50%; with p= 0.2). Also, no statistically significant differences exists between the 3 groups regarding ACE alleles frequencies (p= 0.16) **(Table. 3).**

Table 3. Differences between Alzheimer disease degrees of severity according to ACE gene and alleles

	Mild (n= 23) No. (%)	Moderate (n= 21) No. (%)	Severe (n= 6) No. (%)	*P value
ACE genotypes				
- DD	11 (47.8%)	6 (28.6%)	3 (50%)	
- DI	12 (52.2%)	11 (52.4%)	2 (33.3%)	0.2
- II	0 (0%)	4 (19%)	1 (16.7%)	
ACE alleles				
- D	34 (73.9%)	23 (54.8%)	8 (66.7%)	0.16
- I	12 (26.1%)	19 (45.2%)	4 (33.3%)	

Chi- square test; *****Level of significance < 0.05; ACE: Angiotensin converting enzymes

Discussion

Alzheimer's disease is the most prevalent kind of dementia and typically affects ages above 60-65. Amyloid beta (Aβ) plaques are believed to be the main factor behind the disease progression **(Zverova, 2019)**. ACE was crucial in the breakdown of Aβ, Results from earlier research on the relationship between ACE genotypes and the prevalence and severity of Alzheimer disease yielded inconsistent findings **(Fekih- Mrissa et al., 2017)**.

The current study included AD and control groups which were matched in age and sex distribution. The percent of

females were higher than males in the present study.

Like our study, previous studies compared the ACE genotypes among AD patients in comparison to control group did not find statistically significant age and sex differences (**Durmaz et al., 2019; Tadokoro et al., 2020; Xu et al., 2021**).

On the other hand, **Ramadan et al. (2019)** conducted their study on 53 Egyptian AD patients and were compared with 100 healthy control participants. He reported higher mean age of AD group than control group with significant difference. He also reported higher male predominance among AD group than females. **El Shamieh et al. (2018)** found that female patients were higher significantly among AD group with statistically significant difference when 83 AD Lebanese patients were compared to 80 control participants, but he did not report significant age differences. **Saddiki et al. (2020)** demonstrated that AD patients had higher mean age than control group with statistically significance. Also, he reported that female patients had higher predominance. **Lucatelli et al. (2011) also** found higher mean age among AD group more than control group and showed statistically significant difference (P< 0.001).

The current study showed that most of patients in AD group came from rural areas (80%) with statistically significant difference between rural versus urban (p= 0.029). In agreement with the current study, **Ramadan et al. (2019)** reported that most AD patients were resident of rural areas. On the other hand, **Deng et al. (2015)** had no significant differences between AD and control groups as regard residency. **Abner et al. (2016)** found higher prevalence of Alzheimer disease among urban areas, but he referred the difference to that older patients living in rural areas might face barriers in diagnosis of AD.

The present study demonstrated low MMSE scores among AD patients.

This came in concordance with (**Ramadan et al., 2019**) who reported lower MMSE among AD group. Also, (**Xie et al., 2022**) demonstrated lower MMSE among AD group than control group.

Saddiki et al. (2020) similarly reported lower mean values of MMSE among AD group. (**Tadokoro et al. 2020**) showed that AD patients had mean 20.7 ± 6.6 which is significantly lower than control group (29.0 ± 1.1).

The main findings in this current study were the existence of significant association between homozygous D/D ACE genotype, D alleles and incidence of Alzheimer disease. However, a significant association between ACE genotype and disease severity was not obvious.

The current results showed statistically significant differences between both groups as regard ACE genetic polymorphisms with higher incidence of DD genotype (study vs. control: 40% vs. 20%; OR: 35; p= 0.04) and D alleles (study vs. control: 65% vs. 50%; OR: 1.8; p= 0.03) among Alzheimer disease patients. In agreement with the current study, **Wang et al. (2006)** demonstrated that DD homozygotes are found to have increased risk of AD. Another Tunisian study showed that the D/D genotype was overly represented in the AD group if compared with the controls (61.2 vs. 38.9 %). The frequency of D allele was higher in the patients' group, and the difference was statistically significant (p = 0.001). Other I/D and I/I genotypes (OR 0.433, 95 % CI 0.227–0.826, p = 0.01; OR 0.311, 95 % CI 0.108–0.895, p = 0.03, respectively) represent a susceptibility protective factor for AD (**Achouri et al., 2016**).

The D allele frequencies of patients having mild cognitive impairment (MCI) in the Chinese population were considerably more than control group. Additional findings suggested that the ACE D-allele may have an impact on cognitive dysfunction and serum ACE levels, suggesting a potential link between

the development of MCI and Alzheimer's disease **(Zhang et al., 2012).** D-allele is therefore believed to boost ACE activity in humans, increasing the likelihood of AD development. **Nimral et al. (2011)** reported that ACE DD genotype and D alleles were commoner among AD patients but within Hardy- Weinberg equilibrium as no statistically significant difference was found. In another Tunisian study, In a Tunisian population, the D allele and the D/D genotype of the ACE I/D polymorphism were linked to a higher chance of developing AD. **(Fekih- Mrissa et al., 2017)**.

In contrary to the current study, another Egyptian study on 95 AD patients reported higher frequencies of homogenous II genotype and I allele among AD patients with statistically significant difference in comparison to control group **(Hassanin et al., 2014). Nissar et al. (2021)** reported significant difference between both groups as regard ACE polymorphisms but with higher frequency of DD genotype among control group (61.5%) than Alzheimer disease group (32.3%) (p= 0.004). He reported also higher frequency of I allele among AD group. Previous research revealed that the I Allele and ID genotype are linked to an increased risk of Alzheimer's disease, while the DD genotype was protective against Alzheimer disease. **(Shanmugam et al., 1993; Yang et al., 2017)**.

Wang et al. (2014) in their meta-analysis including 48 studies reported controversial results against the current results as they found a higher frequency of I/D ACE genotype among AD group than DD genotype with high I allele predominance. The I allele could be the more risky allele because it has been shown in another study to cause a decrease in ACE levels, whereas the D allele was predicted to protect against disorders like Alzheimer's **(Lucatelli et al., 2011). El Shamieh et al. (2018)** in his study on Lebanese patients found that patients with ACE II and ID polymorphism had higher

risk for AD with significant differences between AD and control groups as regard ACE II, ID genotypes and I allele **(p= 0.006). Durmaz et al. (2019)** found that ACE DD genotype had higher frequency among AD group but within Hardy-Weinberg equilibrium (no significant difference in comparison to control group).

The International Genomics of Alzheimer's Project's (IGAP) largest genetic study on AD, which included information from 35,274 AD cases with clinical and autopsy-diagnosed AD and 59,163 controls, confirmed that none of the ACE variants previously under investigation showed a significant association with late-onset Alzheimer's disease **(Kunkle et al., 2019). Guzel et al. (2022)** reported that ACE-1 protein level was lower in homozygous (II) individuals compared to homozygous (D/D) with higher risk for AD When compared to I/D and D/D genotypes, people with the I/I genotype had considerably larger (p=0.008) parenchymal Aβ load in the frontal cortex.

The discrepancies in racial, ethnic, geographic, sample size, and patient selection criteria are the most likely causes of the results difference, where the effect of genotype is reversed most likely due to different linkage to a functional SNP.

As regard ACE genotypes relation to disease severity, no statistically significant differences was obtained between the stages of AD regarding DD, ID and II frequencies. Also, no significant differences were reported as regard ACE alleles. In agreement with the current study, a Danish study did not demonstrate an association between ACE gene polymorphism and cognitive dysfunction as reflected by MMSE **(Frederiksen et al., 2003)**. On the other hand, previous Tunisian study stated that severe disease group had higher frequency of DD genotype (77%) and D allele (88%) with statistically significant difference to mild and moderate disease groups (p< 0.001) **(Fekih- Mrissa et al., 2017). Richard et**

al. (2000) found that when DD carriers were compared to I/D subjects as a reference, they had the lowest cognitive scores and had a higher prevalence of cognitive decline.

A Greek study showed that homozygous DD genotype was associated with lower MMSE and severe disease form of AD (**Richard et al., 2000**). **Stewart et al. (2004)** reported aggressive disease in patients homozygous DD for ACE genetic types. In contrast, according to research by Chou et al. (2017), AD patients who are homozygous for the I allele have a faster rate of decline than do people with other ACE genotypes as assessed by the MMSE.

Conclusion

There is significant association between homozygous D/D genotype and D allele in ACE gene (rs4646994) and the incidence of Alzheimer disease. While there is a protective value for I alleles against Alzheimer disease as it had higher frequency among healthy control group with statistically significant differences. There were no statistically significant association between ACE genotype SNPS (rs4646994) and Alzheimer severity.

Study limitations

Small sample size was the main limitation of the study so larger scale studies to confirm findings for the present work can required.

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