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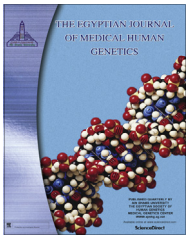

ORIGINAL ARTICLE

Association of insertion–deletion polymorphism of ACE gene and Alzheimer's disease in Egyptian patients

Omayma M. Hassanin [a,*], **Maha Moustafa** [b], **Tarek M. El Masry** [c]

[a] *Clinical Pathology, Molecular Biology Department, Medical Research Center, Ain Shams University, Egypt*
[b] *Biochemistry Department, Faculty of Sciences, Ain Shams University, Egypt*
[c] *Department of Clinical Pathology, Ain Shams University Hospitals, Egypt*

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KEYWORDS

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Abstract *Introduction:* Alzheimer's disease (AD) is a progressive, neurodegenerative disease. Many studies proposed an association of the insertion (I)/deletion (D) polymorphism (indel) in intron 16 of the gene for angiotensin I-converting enzyme (ACE) on chromosome 17q23 with Alzheimer's disease. *ACE* indel and related haplotypes associated with AD risk have reduced plasma ACE whereas protective genotypes have elevated ACE.

Object: To investigate whether there is a correlation between polymorphisms of the ACE I/D locus gene and AD in Egyptian patients and to determine whether there is a difference in ACE activity in the plasma of clinically diagnosed AD patients.

Methods: Subjects of this study are 84 dementia patients diagnosed as having Alzheimer's disease, 45 males and 39 females aged 65 ± 7 years from the Geriatric Department at Ain-Shams University Hospitals and 86 individuals as non dementia controls, 44 males and 42 females aged 63 ± 6 years.

All subjects were genotyped for the common insertion/deletion polymorphisms for ACE gene locus, and ACE plasma activity assay was measured for AD patients.

Results: There was statistically significant difference in the frequency of the ACE insertion/deletion alleles between the cases and controls where the I allele distribution in AD cases and controls was 74% vs. 15%, and the I/I genotype frequency was 60% vs. 5%, respectively. They both reached a statistical significance range (I allele frequency: OR = 3.714, 95% CI 1.311–10.523, $p < 0.01$; I/I

Abbreviations: AD, Alzheimer's disease; ACE, angiotensin converting enzyme; I/D, insertion/deletion; Aβ, amyloid β; LOAD, late-onset Alzheimer's disease

* Corresponding author. Tel.: +20 1004019109. fax: +2024190555.
E-mail address: omhassanin@yahoo.com (O.M. Hassanin).

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genotype frequency: OR = 3.18 95% CI 2.33–4.33, $p < 0.01$). But no significant difference in ACE plasma level was found between different genotypes in our AD patients.

Conclusions: Our present study supports the hypothesis of implication (I allele) of ACE gene polymorphism in the development of AD. On the other hand, we did not find significant difference in plasma ACE activities when compared with different studied genotypes.

1. Introduction

Dementia will increase exponentially in coming years, and the number of patients suffering from dementia is predicted to double every 20 years to 81.1 million by 2040 in the world [1]. This increasing prevalence underlines the necessity for antecedent biomarkers in order to have more accurate diagnosis and treatment [2].

Alzheimer's disease (AD) is the most over diagnosed and misdiagnosed disorder of mental functioning in older adults. Part of the problem, is that many other disorders show symptoms that resemble those of AD. The crucial difference, however, is that many of these disorders – unlike AD – may be stopped, reversed, or cured. Based on these findings, clinical diagnosis of AD has been referred to as "a diagnosis by exclusion", and one that can only be made in the face of clinical deterioration over time. There is no specific clinical test or finding that is unique to AD. Hence, all disorders that can bring on similar symptoms must be systematically excluded. The "classical" senile plaques and the neurofibrillary tangles seen in an AD brain at autopsy typically are the only definitive diagnosis of the disease [3].

Alzheimer's disease (AD) is a progressive, neurodegenerative disease characterized clinically by gradual loss of memory and pathologically by neurofibrillary tangles and amyloid plaques in the brain. Currently, the apolipoprotein E ε4 allele is the only broadly recognized genetic risk factor for late-onset AD (LOAD) in most populations. Much attention has been focused on the connection between angiotensin I converting enzyme and AD [4].

Angiotensin-converting enzyme (ACE) is an endopeptidase that consists of two catalytic domains and is normally expressed by endothelial, epithelial and neuronal cells [5]. It exists in both membrane-bound (ACE) and soluble (sACE) forms, the latter is produced by the action of an as yet unidentified zinc metalloprotease ('ACE secretase') which cleaves mature, membrane-bound ACE at a juxtamembranous extracellular domain to release the large extracellular part of the enzyme [6,7]. The traditional view of the function of ACE relates to the renin-angiotensin system (RAS) pathway, within which ACE catalyzes the formation of the vasoconstrictor octapeptide angiotensin II (Ang II) from its non-vasoactive precursor angiotensin I (Ang I) and is also responsible for cleavage and inactivation of the vasodilator bradykinin [8]. The net result is vasopressor activity, which can be blocked by ACE inhibitors – a standard treatment for hypertension [9]. More recently ACE has been shown to cleave amyloid-β (Aβ), the accumulation of which is central to the pathogenesis of Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA). ACE-mediated cleavage of Aβ has been demonstrated in vitro [10], ex vivo [11] and in some [12] but not in all [13] recently studied animal models of AD.

Several studies show that an insertion/deletion (I/D) polymorphism in the ACE gene is associated with increased plasma level of ACE. Studies showing association between the I/D polymorphism and cardiovascular disease risk, and evidence suggesting cardiovascular risk factors promote AD are consistent with the idea that ACE might play a role in AD via a cardiovascular mechanism [14].

Angiotensin converting enzyme (ACE) plays a key role in the renin-angiotensin system (RAS) pathway. The actions of the RAS have been extensively studied in the periphery, particularly the role of Ang II in hypertension. However, it is now recognized that nearly all organs of the body have their own local paracrine-like RAS, with organ-specific actions [15]. The actions of Ang II within the central nervous system are of increasing interest in the context of Alzheimer's disease (AD). Ang II inhibits the release of acetylcholine (ACh) and has a pro-inflammatory effect [16].

A polymorphism in the gene coding for angiotensin I-converting enzyme (ACE) was identified by Rigat et al. in 1990 [17]. The polymorphism is due to a 287 bp fragment in the ACE gene in chromosome 17. The fragment is present in the insertion (I variant) and absent in the deletion (D variant), which results in the three genotypes: Homozygotes II and DD and heterozygotes DI. The genotype accounts for approximately half of the variance in the circulating ACE level and from the II to the DD genotype the presence of each D allele is associated with an additive effect on ACE activity (50% higher in the DD compared with the II genotype) [18].

The relationship between ACE genotypes, in particular DD, and the occurrence of cardiovascular and renal diseases has therefore been the focus of several studies in the past decade [19].

In 1999, Kehoe et al. [20], proposed the first study that reported an association between Alzheimer's disease and the insertion (I)/deletion (D) polymorphism (indel) in intron 16 of the gene for angiotensin I-converting enzyme (ACE), and the D allele is associated with raised plasma levels of the enzyme [21]. While Lehmann et al. [22], found that I positives, that is, DI and II, were at increased risk of Alzheimer's disease.

The ACE gene (*ACE*) has been featured now as one of the top susceptibility genes for AD [20,22]. According to the meta-analysis database of AD candidate genes listed on Alzgene (www.Alzgene.org), *ACE* indel and related haplotypes associated with AD risk have reduced plasma ACE whereas protective genotypes have elevated ACE [23]. Other studies have observed reduced ACE activity in CSF from AD patients [16].

Indeed after *APOE*, the only widely accepted susceptibility gene for late-onset AD [24], *ACE1* is probably the strongest candidate susceptibility gene for AD. The Aβ degradation hypothesis would explain this on the basis that differences in *ACE1* genotype influence ACE levels and activity and these, in turn, affect Aβ accumulation and toxicity. In most

published studies of ACE protein levels and enzyme activity in human brain tissue, ACE was found to be elevated in AD [13].

Several studies have shown ACE to be capable of degrading Aβ in vitro. Variations in *ACE* were associated with differences in CSF Aβ level [22], and some animal data suggested that Aβ level was increased by administration of ACE inhibitors. It is not well documented whether ACE inhibitor use in humans influences Aβ accumulation. However, some studies revealed a correlation between ACE inhibitor administration and LOAD, where about 39.53% of the LOAD patients were administering ACE inhibitors while only 18.6% of controls did [2].

We aimed to investigate whether there is a correlation between polymorphisms of the ACE I/D locus gene and AD in Egyptian patients. We also aimed to determine whether there is a difference in ACE activity in the plasma of clinically diagnosed AD patients.

2. Materials and methods

2.1. *Patients and controls

This study was conducted in the Medical Research Center at Ain-Shams University hospitals, on a total of 84 dementia patients diagnosed as having Alzheimer's disease from the Geriatric Department at Ain-Shams University Hospitals, (45 males and 39 females aged 65 ± 7 years) and 86 individuals as non dementia controls, (44 males and 42 females aged 63 ± 6 years).

All subjects included in this study were subjected to full history taking, thorough clinical examination, and ACE I/D polymorphism typing and ACE plasma level was measured for AD patients.

The work was done after taking acceptance of all patients and controls to share in the study as well as acceptance of ethics committee of the University. The works have been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2.2. *ACE I/D genotype detection

DNA was extracted from whole blood using a QIAamp Blood mini-prep Kit (QIAGEN, Germany) according to manufacturer's instructions.

Genomic DNA (300 ng) was amplified in a final volume of 50 μl, containing 10 mM TRIS pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each dNTP, 1 μM of each primer and 2U Taq polymerase (all reagents from MBI Fermentas, St. Leon-Rot, Germany). Details of *ACE D/I* genotype determination have been described according to Lindpaintner et al. [25]. The differentiation between D and I alleles in heterozygous samples was done according to the method described by Lindpaintner et al., and originally described by Shanmugam et al. [26].

We used an optimized primer pair to amplify the *D* and *I* alleles, resulting in 319-bp and 597-bp amplicons, respectively (hace3s, 5′ GCCCTGCAGGTGTCTGCAGCATGT3′; hace3as, 5′GGATGGCTCTCCCCGCCTTGTCTC3′). The

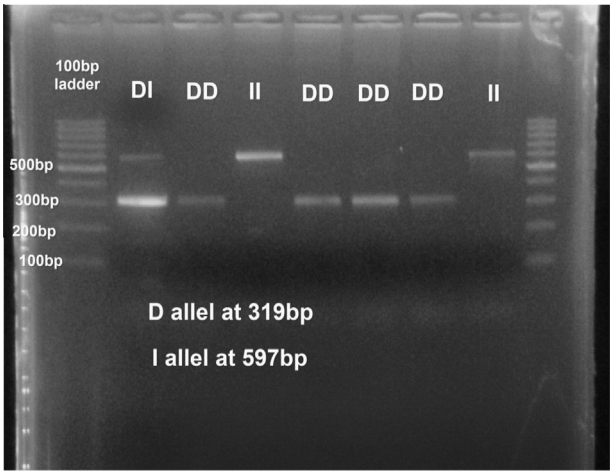

Figure 1 Shows a 2% Agarose gel analysis of amplified Polymerase chain reaction (PCR) to detect ACE insertion/deletion polymorphisms showing: Lane 1: 100 bp ladder Marker. Lane 2: heterozygous (I/D), Lanes 3, 5, 6 and 7 are homozygous for the deletion allele (D/D). Lanes 4 and 8 are homozygous for the insertion allele (I/I).

thermocycling procedure (9700 apparatus, Applied Biosystem, USA.) consisted of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, and extension at 72 °C for 2 min, repeated for 35 cycles, followed by a final extension at 72 °C for 7 min. After the addition of 5 μl of a glycerol-based loading buffer (bromophenol blue), 7 μl of the mixture was loaded onto a 1.5 percent submarine agarose slab (BIOMETRA, Germany) containing 40 mM TRIS acetate, 2 mM EDTA, and 1 μg of ethidium bromide per milliliter of solution and fractionated according to size of 100 bp molecular weight marker (Fermentas, St. Leon-Rot, Germany). The amplification products of the *D* and *I* alleles were identified at 319-bp and 597-bp amplicons, respectively by 300-nm ultraviolet transillumination as distinct bands as shown in Fig. 1.

2.3. *ACE plasma activity assay

Blood for ACE plasma activity assay was collected from AD patients only, into heparinized tubes and centrifuged and the plasma was stored at −70 °C until assay. To establish ACE activity, a colorimetric method, using Fortress diagnostics kit was adopted. Based on hydrolysis of furanacryloyl-L-phenylalanylglycylglycine (FAPGG) to FAP and Glycylglycine and was quantified by measuring the decrease in absorbance at 340 nm. (Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BT41 1QS (United Kingdom).

2.4. Statistical analysis

Statistical analyses were carried out with SPSS 18.0 software for Windows. Comparisons of the allele and genotype frequencies between groups were assessed using X^2 test analyses. The 95% confidence interval (CI) and Odds ratio (OR) were used to estimate the risk for developing AD. The criterion for significance was set at $p < 0.05$ for all the tests.

3. Results

The *ACE* genotype and allele distribution are shown in Tables 1 and 2. According to our data there was a significant difference in allele and genotype frequency between AD patients and controls.

It was observed that the frequency of DD genotype was low (26%) in AD patients as compared to controls (85%) with $p < 0.05$ while there was no statistically significant difference between the AD group and the control group in ID heterozygote frequency which was 10% in controls and 13% in the AD group with $p = 0.427$. On the other hand, the II genotype had a higher frequency in the AD group (60%) as compared to controls (5%) with a statistically significant difference $p < 0.01$.

The ACE allele frequencies in both groups shown in Table 2, revealed that the D allele was found to be predominant among normal subjects (95%) while the I allele was predominant among the AD group (74%).

The I allele distribution in AD cases and controls was 74% vs. 15%, and the I/I genotype frequency was 60% vs. 5%, respectively. They both reached a statistical significance range at $p < 0.01$.

No significant relation was observed between the ACE gene polymorphism when compared with age and gender of the studied population (data not shown).

Furthermore, plasma level of ACE in patients with I/I genotype was lower than those with D/D or D/I genotypes, but was not statistically significant as shown in Table 3.

Table 1 Genotype frequencies at the ACE I/D locus in AD and control groups.

	AD No. (%)	Controls No. (%)	*p*-value	Odds ratios (95% CI)
DD (%)	22 (26%)	73(85%)	<0.05*	1.24 (1.10–3.56)
ID (%)	11 (13%)	9 (10%)	0.427	0.36 (0.20–0.64)
II (%)	51 (60%)	4 (5%)	<0.01*	3.18 (2.33–4.33)

* $P < 0.05$ is statistically significant.

Table 2 Allele frequencies at the ACE I/D locus in AD and control groups.

	AD	Controls	*p*	Odds ratios (95% CI)
D allele (%)	33 (39%)	82 (95%)	<0.05*	1.765 (1.268–4.185)
I allele (%)	62 (74%)	13 (15%)	<0.01*	3.714 (1.311–10.523)

* $p < 0.05$ is statistically significant.

4. Discussion

The indel polymorphism, consisting of the presence or absence of a 287-bp DNA fragment, has been considered the biomarker of AD, although its validity varies with race. The meta-analyses addressing the relation-ship between ACE indel polymorphism and AD have shown that I allele and I/D genotype are associated with an increased risk of AD, and D/D genotype with reduced risk of AD [27,28]. However, these findings are not consistent with different subgroups of Caucasians, north Europeans and south Caucasians (Mediterranean and Middle East) and within Asians [22]. Another published studies reported no association [29] or association with a particular age group in Caucasians-Americans [30].

Even in Asians, such associations between ACE indel polymorphism and AD were not consistently detected among Japanese and Taiwanese. For Japanese, the ACE I homozygote was associated with the increased prevalence of AD [31] which is in accordance with our results; however the result could not be duplicated, as another study found a significant ethnic difference of the genotype distribution, but failed to replicate the positive association between the I allele and AD [32]. For Taiwanese, the D homozygote and D allele were significantly associated with the increased prevalence of AD [33], which is different from the results in the Japanese study and our study.

Nonetheless, the relationship between ACE and AD is controversial. Several studies have implicated the ID polymorphism and risk of AD [34], while other studies failed to find an association [35]. Moreover, the pattern of association has been confusing, with some studies showing an association with the I allele [36], while others with the D allele [37].

A possible biological explanation for an association between ACE and AD is still unclear. The insertion allele appears to reduce ACE expression, and DD homozygotes and ID heterozygotes have more circulating ACE than II homozygotes: 65%, and 31%, respectively. This increased activity of the D allele may alter Aβ deposition by a direct proteolytic mechanism [38]. Several studies suggest that there may be a relationship between cardiovascular lesions and the severity and progression of AD [39].

We found an association between ACE variants and AD, where inheritance of the II genotype of the *ACE* gene was associated with a higher risk of AD while inheritance of the homozygous DD genotype was associated with a lower risk for AD. The latter observation was confirmed in several meta-analyses [22] and more recently in whole-genome association studies [40].

Several studies have examined the association of ACE I/D polymorphism and ACE plasma concentration and have determined that the D allele or D/D homozygote is associated with increased plasma ACE level [41].

ACE inhibitors seem to provide protection of cognitive function in AD patients. A clinical study has found that using

Table 3 Mean plasma ACE activity of different ACE genotypes in AD patients.

	DD	DI	II	X^2	*P*-value
Plasma ACE activity (U/L) Mean ± SD	56.6 ± 13.7	45.3 ± 10.5	34.4 ± 11.7	1.178	0.333*

* p is insignificant.

brain-penetrating ACE inhibitors could slow the deterioration of cognitive function of these patients, because ACE inhibitors may enhance the release of acetylcholine in human cortex slices and increase cerebral blood perfusion and vasomotor reactivity [42,43].

In our study individuals carrying the *ACE* II 'risk' genotype for AD had no statistically significant differences in plasma ACE activity as compared to samples of heterozygous or homozygous of the 'protective' D allele with intermediate risk.

It is still unclear whether ACE indel polymorphism can be a biomarker of AD. However, it is important to clarify this point not only for the biomarker itself, but also for the emerging alternative therapy provided by the ACE protein. They appear to conflict over the point that using ACE inhibitors can slow the decline of cognitive function of AD patients and in the in vitro study, ACE can directly degrade β-amyloid. The possible benefits of using ACE inhibitors to slow cognitive function may depend on the ACE genotype of the patient, although this lacks definite evidence. These current conflicts and possible benefits of using ACE inhibitors to AD should be clarified in future research.

In conclusion, our present study support the hypothesis of implication (I allele) of ACE in the development of AD. On the other hand, we did not find significant difference in plasma ACE activities when compared with different studied genotypes in AD patients, indicating that measurements of peripheral ACE are not a reliable indicator of ACE brain activity in AD patients.

Conflicts of interest

All authors declare no conflict of interest. There is no financial and personal relationship with other people or organizations that could inappropriately influence this work.

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