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Full Length Research Paper

Lack of association between two *ACE* gene polymorphisms (rs4291 and Alu I/D) and late onset Alzheimer's disease

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Alzheimer's disease (AD) is a prevalent disorder and the most common cause of dementia in elderly populations. Genetic and environmental factors together play a role in developing late onset Alzheimer's disease (LOAD). According to the recent published papers, *ACE* is one of the candidate susceptibility genes for LOAD. In this study, allele and genotype frequencies for rs4291 and rs1799752 polymorphisms of *ACE* gene, for 100 Iranian patients, affected with AD and 100 healthy controls were compared using Chi-square test. No statistically significant differences were found in genotype and allele frequencies of rs4291 and rs1799752 polymorphisms between our LOAD patients and controls. The pair-wise haplotype analysis of rs4291 -240 A/T and rs1799752 Alu I/D polymorphisms were also performed, but no significant associations were identified.

Key words: *ACE*, Alzheimer's disease, Iranian, association, polymorphism.

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in elderly populations (Hardy, 1997). AD is a heterogeneous and multifactorial neurodegenerative disorder (Iqbal and Grundke-Iqbal, 2000) which is clinically characterized by progressive loss of memory and cognitive abilities, disorientation to time and place, problems with language and changes in personality (Rocchi et al., 2003).

Neuropathological signs of AD are neurofibrillary tangles and amyloid plaques in the medial temporal lobe structures and cortical areas of the brain (Masters and Beyreuther, 2006).

AD, based upon the age at onset before or after about 60 years, is categorized into two types called early onset or pre-senile and late onset or senile form of thedisease, respectively. According to literature, early onset AD is inherited as an autosomal dominant trait. However, the most Alzheimer's cases are sporadic with senile form. Genetic and environmental factors cooperate with each other in developing late onset Alzheimer's disease (LOAD) that constitutes the most population of Alzheimer's patients worldwide (Rocchi et al., 2003).

Over the last decades, genetic association studies have been used to suggest numerous underlying genes for Alzheimer's disease. Mutations in *APP*, *PSEN1* and *PSEN2* genes have been suggested as a cause of AD in the early onset patients (Goate et al., 1991; Sherrington et al., 1995; Levy-Lahadet al., 1995).

Inheritance of *APOE* ε4 allele has been identified to be a risk factor for both early and late onset forms of Alzheimer's disease (Saunders et al., 1993; Farrer et al., 1997). On the other hand, a few other genes including *ACE* have been considered as candidate susceptibility genes for LOAD.

ACE gene, at locus 17q23, consists of 25 exons, encodes angiotensin converting enzyme. This functional protein removes two amino acids from the carboxy

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Abbreviations: AD, Alzheimer's disease; **LOAD,** late onset Alzheimer's disease

Table 1. Genetic variations of *ACE.*

Marker name	Map position	Genetic location	Minor allele
rs4291	58,907,926	Promoter SNP	T
rs1799752	58,919,636	Intron	I

terminal of angiotensin I and converts it to angiotensin II which is an effective vasopressor (OMIM, 106180). *ACE* is expressed in many tissues, including vascular endothelial cells, renal epithelial cells, and testicular Leydig cells (Ramaraj et al., 1998). *ACE* is a pivotal enzyme for blood pressure regulation and its level in the blood has an association with cardiovascular risk (Breteler, 2000).

In this study, we analyzed two genetic polymorphisms of *ACE* including rs4291 and rs1799752. These two polymorphisms have been reported to have an association with LOAD. Their criteria are shown in Table 1. rs4291 A/T is a promoter single nucleotide polymorphism located at -240 bp from the initiation codon of the gene. rs1799752 or *Alu* I/D variation is based on the insertion or deletion of a 287 base pair *Alu* sequence in the intron 16 of *ACE* gene.

Previous suggestions of the probable association of some genetic variations with AD and also their conflicting results in different populations encouraged us to investigate the association of LOAD with rs4291 and rs1799752 allele distributions and also pair-wise haplotype analysis of these two polymorphisms through a case-control study among Iranian population.

In this genetic epidemiological study, the frequencies of *ACE* A and T alleles for rs4291, I and D alleles for rs1799752 were compared and pair-wise haplotype analysis of rs4291 and rs1799752 were also performed in independent case and control populations.

MATERIALS AND METHODS

Clinical materials

100 Iranian patients, affected with AD and 100 Iranian normal persons, without family history of AD, as control, were studied. The age of onset for patients was 74.28±7.7 years, and the cases and controls were matched by sex, age, ethnic background, and geographic areas. Our patients were collected from the dementia outpatient clinic of the Iranian Alzheimer Association (IAA), a member of the Alzheimer's Disease International (ADI) and also from a few nursing homes in Tehran, Iran. All patients met standard diagnostic criteria for AD. Cases had been corroborated to have Alzheimer's disease according to neurological and neuropsychological testing, including the Mini-Mental State Examination [MMSE] or Folstein test (Folstein et al., 1975) accomplished by neurologists in IAA. Our controls were 100 non-demented elderly individuals. None of them had clinical history of neuropathological or psychiatric disorders in their families. All the patients or their legal guardians and all the controls, signed informed consent document, and the study was approved by the Ethics Committee of the Hospital before it was commenced.

Genotyping

A blood sample was collected from each patient and control. In the next step, genomic DNA was extracted from white blood cells using a salt-out procedure (Miller et al., 1988). All cases and control samples were evaluated for rs4291 and rs1799752 polymorphisms. Genotypes for rs4291 were also determined by an allele specific PCR protocol and 186 base pair products were observed by electrophoresis assay. Forward and reverse specific primers for allele A were: 5'-ACTGCCGGGTCCCCATCTTGA and 5'-GCTTCCTCCTCCGCTCCAGAG. Allele T specific primers were 5'-ACTGCCGGGTCCCCATCTCCT and 5'-GCTTCCTCCTCCGCT-CCAGAG. Since SNP rs4291 is located in a GC rich region, betaine (1 mM) was used to increase amplification. The accuracy of 186 base pair PCR products for some samples was checked by bidirectional sequencing. For determination of genotype of the rs1799752 polymorphism, two PCR series were performed. In the first reaction, primers that flank I/D polymorphism were used to amplify DNA from each subject (5´- CTGGAGACCACT CCCATCCTTTCT and 5´-GATGTGGCCATCACATTCGTCAGA). The resulting PCR products included 490 base pair and 190 base pair bands for I and D alleles, respectively. Due to the fact that this reaction frequently fails to detect the insertion allele, a second PCR reaction was performed which specifically amplifies the allele containing the insertion primers (5´- CTGGGATTA CAGGCGTGATACA and 5´-TTGATG AGTTCCACGTATTTCG). The resulting PCR product was a 259 base-pair fragment. The data from both reactions were used to establish the rs1799752 genotype.

Statistical analysis

All statistical calculations were performed using Statistical Package for Social Science, version 17.0 (SPSS, Chicago, USA). Genotype and allele frequencies for rs4291 and rs1799752 were compared between case and control groups by using Chi-square analysis. Whenever appropriate, the observed number of each genotype was compared with the expected for a population in the Hardy-Weinberg equilibrium by using a goodness of fit x^2 test. Differences were considered significant if the p value was less than 0.05.

RESULTS AND DISCUSSION

The main finding of our study was the lack of association between *ACE* rs4291 and also rs1799752 polymorphisms with the risk of developing AD in our Iranian group of LOAD patients. According to the results shown in Tables 2 and 3 and Figures 1 and 2, no statistically significant differences were found in genotype and allele frequencies of rs4291 and rs1799752 polymorphisms between our LOAD patients and controls.

The pair-wise haplotype analysis of rs4291 -240 A/T and rs1799752 *Alu* I/D polymorphisms were also

Table 2. Genotyping.

Variable	Genotype			Allele	
	AA	AT	TT	A	T
Cases (n = 100)	31	50	19	112	88
Controls (n = 100)	38	43	19	119	81

$x^2 = 1.23$, $p = 0.5$; alleles: $x^2 = 0.54$, $p = 0.4$.

Table 3. Genotyping.

Variable	Genotype			Allele	
	DD	ID	II	I	D
Cases (n = 100)	32	49	19	87	113
Controls (n = 100)	43	37	20	77	123

$x^2 = 0.741$, $p = 0.6$; alleles: $x^2 = 1.02$, $p = 0.3$.

analyzed and the results are shown in Table 4. No significant association between various haplotypes of these two sites and LOAD was observed ($P = 0.19$).

The study of rs1799752 or I/D polymorphism has been of interest in genetic research on LOAD. Such study has been performed among different populations around the world and conflicting results have been obtained. Literature review revealed the association between *ACE* I/D polymorphism and incidence of LOAD in 14 studies (Alvarez et al., 1999; Cheng et al., 2002; Crawford et al., 2000; Helbecque et al., 2009; Hu et al., 1999; Kehoe et al., 1999, 2003; Kölsch et al., 2005; Mattila et al., 2000; Narain et al., 2000; Ning et al., 2010; Richard et al., 2001; Wang et al. 2006; Wang et al., 2006) which shows positive association. However, there was no association between this polymorphism and LOAD in 20 studies (Buss et al., 2002; Camelo et al., 2004; Carbonell et al., 2003; Chapman et al., 1998; Cousin et al., 2009; Keikhaee et al., 2006; Lehmann et al., 2005; Lendon et al., 2002; Meng et al., 2006; Monastero et al., 2002; Myllykangas et al., 2000; Nacmias et al., 2007; Palumbo et al., 1999; Panza, 2002; Perry et al., 2001; Scacchi et al., 1998; Seripa et al., 2003; Wakutani et al., 2002; Wehr et al., 2006; Zuliani et al., 2001) showing negative association.

According to several biochemical researches, N-terminal catalytic domain of *ACE* has the ability of degrading of amyloid-β (Aβ) peptide *in vitro*. It has been observed that *ACE* inhibitors can block this effect of the enzyme. It seems that the low level of *ACE* might result in the increase of the accumulation of A*β* peptide as a hallmark of LOAD (Hemming and Selkoe, 2005; Sun et al., 2008). On the other hand, rs1799752 was found to be strongly associated with the level of circulating *ACE* (Rigat et al., 1990). As a result, this insertion deletion polymorphism might have a link with incidence of LOAD. However, *in vivo* studies in mice (Eckman et al., 2006; Hemming et al., 2007; Wang et al., 2007) and in human

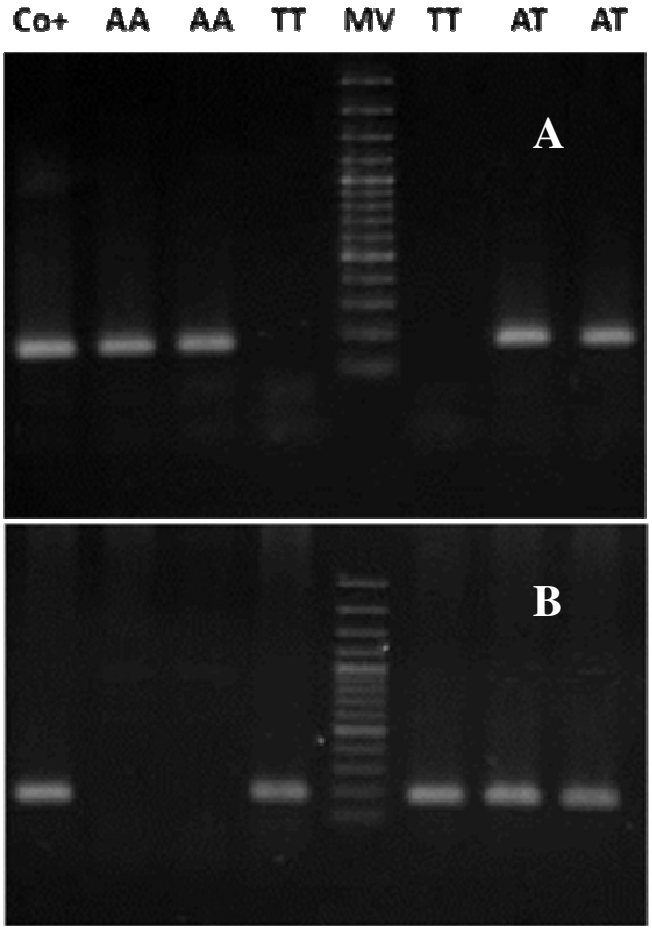

Figure 1. Electrophoresis assay for PCR products by allele specific primers; **A**) allele A and **B**) allele T.

(Lendon et al., 2002; Minerset al., 2009) have shown no change in brain A*β* peptide levels according to *ACE* availability. This finding disagrees with the association of insertion/deletion polymorphism rs1799752 with A*β* peptide level in brain.

Our results show that there was no statistical significant association between rs1799752 and LOAD among Iranian population. Our results confirm the previous study on this polymorphism in an independent Iranian population of LOAD cases (Keikhahee et al., 2006). It appears that the whole genetic and biochemical findings about rs1799752 insertion/deletion polymorphism do not completely support each other.

According to one study, rs4291 A/T influences the levels of toxic A*β* peptide in the cerebrospinal fluid (CSF) (Kehoe et al., 2003). Moreover, rs4291 was reported to be associated with plasma *ACE* levels (McKenzie et al., 2005).

Kehoe et al. (2003) showed that this SNP has the strongest link to incidence of AD amongst *ACE* single nucleotide polymorphisms in a large study on Swedish, Scottish and English independent populations. There are

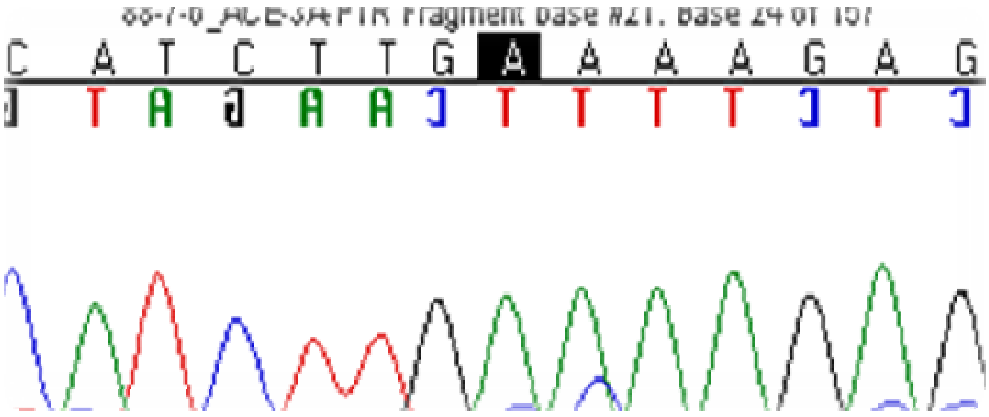

Figure 2. Sequence analysis of the allele A specific PCR product.

Table 4. Pair-wise haplotype analysis for rs4291 and rs1799752 polymorphisms; $x^2 = 6.15$, $p = 0.19$.

Haplotype	Patient (100)	Control (100)
-240 A/T – I/D		
A-I	48	51
A-D	32	43
T-I	7	2
T-D	49	54
Undefined	32	25

A-I haplotypes were detected from AAII, AAID and ATII genotypes; A-D haplotypes from AADD, AAID and ATDD genotypes; T-I haplotypes from TTII, TTID and ATII genotypes; and T-D haplotypes from TTDD, TTID and ATDD genotypes. However, detection of the haplotypes of ATID genotype was not possible.

six other studies, from various populations, reporting an association between LOAD and rs4291 genotype or regarding haplotype structures (Kehoe et al., 1999, 2003; Wang et al., 2006; Edwards et al., 2008; Helbecque et al., 2009; Ghebranious et al., 2010). However, the results of three other separate studies were not consistent with their results. They found no association between LOAD and rs4291 (Meng et al., 2006; Bruandet et al., 2008; Cousin et al., 2009). According to these results, rs4291 were not associated with LOAD in French Caucasian population and also among Israeli Arab community.

We could not detect any statistically significant associations between rs4291 A/T and LOAD among our cases. Our patients were from different regions of Iran and we completely matched our cases and controls in terms of age and gender. We also found no haplotypic association regarding rs4291 and rs1799752 among our cases (Table 4).

There were different reasons which could explain the discrepancies in the results observed in various studies. The negative results may be explained by the fact that the gene(s) responsible for the disease are probably different in various populations and that the epistatic gene-gene interactions or specific multi-loci haplotype may be involved in determining the association.

A recent meta-analytical study on three *ACE* polymorphisms including rs4291 among 10 Caucasian case-control populations has been performed for finding their association with LOAD. According to their statistical results, rs4291 was not associated with LOAD in those Caucasian populations. This research group also found no haplotypic association in their complete dataset (Belbin et al., 2011).

They suggested that *ACE* variants have modest effect sizes, which are likely part of a complex interaction between genetic, phenotypic and pharmacological effects that would be undetected in traditional case-control studies.

Conclusion

Our conclusion is in agreement with the negative association between AD and *ACE* polymorphism. The failure to determine an association between AD and ACE polymorphism in our study may be due to the small size of our sample cohort and a weak association.

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