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ORIGINAL PAPER

Genetic Influences on Alzheimer's Disease: Evidence of Interactions Between the Genes *APOE, APOC1* and *ACE* in a Sample Population from the South of Brazil

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Abstract Alzheimer's disease is a complex neurodegenerative disorder. Several genes have been suggested as Alzheimer's susceptibility factors, the apolipoprotein E (APOE) gene being an established susceptibility gene and the genes coding angiotensin-converting enzyme (ACE) and apolipoprotein C1 (APOC1) being considered possible candidate genes for the disease. The objective of this study was to investigate the association of ACE and APOC1 gene polymorphisms with susceptibility to Alzheimer's disease and dementia in general, both alone and combined with the APOE gene. Forty-seven patients with dementia in general (35 of them with Alzheimer's disease) and 85 controls were investigated. The haplotypes *E*3/−317*ins* and *E*4/−317*ins* of APOE/APOC1 genes were significantly more frequent in the groups with Alzheimer′s disease and dementia in general ($P < 0.001$). The frequency of the *ACE*ins* allele was also greater in the groups with Alzheimer's disease and dementia in general ($P = 0.022$; $P = 0.045$), but genotype frequencies were only different in groups without the *E*4/−317*ins* haplotype ($P = 0.012$ for Alzheimer's disease; $P = 0.04$ for dementia). Our data point to important genetic interactions involved in these diseases.

Keywords Gene interactions · Alzheimer's disease · APOC1 · ACE · APOE

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Introduction

Although the etiology of Alzheimer's disease is not yet fully understood, epidemiological and molecular evidence suggests that many factors influence the pathology, including genes and the environment, and for this reason it is classified as a multifactorial disease. Investigations are being conducted with populations from all around the world in order to identify genetic risk factors, but the only risk factor that has been confirmed to date, including the Brazilian population, is the *E*4* allele (rs429358) of the apolipoprotein E (APOE) gene [1, 2]. As with any risk factor in isolation, confirmation of the presence of the *E*4* allele is not in itself sufficient to provide accurate information about susceptibility to Alzheimer's disease.

A candidate gene for Alzheimer's disease codes for apolipoprotein C1 (APOC1), which is the only apolipoprotein other than apoE that is produced in cerebral tissues. Its role is also apparently related to neuronal plasticity through redistribution of lipids to axons and regeneration of Schwann cells [3]. Although the function of apoC1 at the cerebral level is not yet well understood, it is known that apoC1 levels are elevated in the hippocampus of Alzheimer sufferers and apoC1 has been suggested to interact with apoE in lipid metabolism at the cerebral level [4]. Studies have detected associations between insertion/deletion (ins/del) polymorphisms of 4 bp at position −317 in the promoter region of the APOC1 gene (rs11568822) and

Alzheimer's disease. However, because the APOC1 gene is located in the same cluster as the APOE gene, some authors have suggested that the association detected with the *−317*ins* allele is nothing more than a previously confirmed relationship with APOE, due to the linkage disequilibrium between the two genes [5–7]. While the association between Alzheimer's disease and the APOE gene has already been confirmed in Brazilian populations [2, 8], APOC1 has not yet been investigated in this population.

Angiotensin-converting enzyme (ACE) is a component of the renin-angiotensin system, which produces angiotensin II (ANG II). In addition to playing a role in neurogenic hypertension, ANG II also acts as a nonclassical neurotransmitter activating neurons in the brain [9]. Additionally, the ACE enzyme also appears to be involved in breaking down the β-amyloid protein, preventing it from aggregating and forming the senile plaques that are characteristic of Alzheimer's disease [10]. A polymorphism in intron 16 of the ACE gene, consisting in insertion/deletion (*ins/del*) of a sequence of 287 base pairs (rs1799752), has been associated with ACE concentrations in blood. In different populations, it has been observed that genotypes that are carriers of the **ins* allele are at increased risk of Alzheimer's disease, although some authors have contradicted this finding and others have failed to detect any association with Alzheimer's disease [9, 11–14]. The influence of the ACE gene on Alzheimer's disease has never been investigated in Brazilian populations.

In view of the above, the objective of this study was to investigate the influence of polymorphisms of the APOC1 and ACE genes on dementia in general and on possible or probable Alzheimer's disease in a sample from southern Brazil. The relationships were investigated both alone and combined with the APOE susceptibility gene.

Experimental Procedures

Sample

All patients were tested with the Mini-Mental State Examination (MMSE) and met DSM-IV criteria for dementia. Thirty-five patients with possible or probable Alzheimer's disease were recruited in the cities of Porto Alegre, Caxias do Sul, and Novo Hamburgo (all cities are located in Rio Grande do Sul, Brazil's southernmost state). In addition to patients with Alzheimer's disease, 12 further dementia patients, who had been excluded from the Alzheimer's disease group based on the exclusion criteria published by Mckhann et al. [15], were also enrolled in the study. The entire sample of 47 patients was defined as the "general dementia" group, and this sample contained the 35 patients defined as "Alzheimer's patients", i.e., the two patient samples are not separate groups, but one includes the other.

The control group was composed of 85 people recruited from the general population of Porto Alegre, aged >60 years and with no reported neurological disease, but who had not been assessed for dementia. All participants and patients' caregivers received information about the study and provided informed consent. The study protocol was approved by the Research Ethics Committee of Universidade Feevale, Brazil.

Laboratory Methods

A 5-ml sample of peripheral blood was collected and DNA was extracted using the method described by Lahiri and Nurnberger [16]. The rs 1799752 polymorphism consists in a 287 bp insertion/deletion polymorphism in intron 17 of the ACE gene. This variant was identified by genotyping with PCR and electrophoresis in 1.5% agarose gel, using the same primers and under the same conditions as described by Franken et al. [17]. In the presence of insertion allele (or **ins* allele), the expected PCR product size was 477 bp, while in the presence of deletion allele (or **del* allele) the amplified product size was 190 bp.

The presence of two single-nucleotide polymorphisms (SNPs) in exon 4 of the APOE gene (rs 429358 and rs 7412) raises the possibility of three alleles, namely *E*2*, *E*3,* and *E*4*. These SNPs lead to an amino acid change in two sites of the apoE protein (Cys112Arg and Arg158Cys), and the alleles are named after the set of these two SNPs, that is, *E*2* allele contains 112Arg and 158Arg, *E*3* allele contains 112Cys and 158Arg, and *E*4* allele contains 112Cys and 158Cys. These variants were determined using PCR and cleavage with restriction enzyme *Hha* I, and the fragments were detected through electrophoresis in 8% acrylamide gel, using the same primers and under the same conditions as described by Maekawa et al. [18].

A 4 bp insertion/deletion polymorphism in the promoter region (rs11568822, located at 317 bp before the transcription start site) of the APOC1 gene was genotyped by PCR and cleavage with restriction enzyme *Hinc* II. In the presence of insertion allele (or −317**ins* allele), the PCR product contains the restriction enzyme site and the restriction fragment of interest is then cut, while in the presence of deletion allele (or −317**del* allele) this site is not present and the PCR product remains intact. Fragment size was detected by electrophoresis in 2.5% agarose gel, the same primers and conditions described by Nillesen et al. [19] were used.

Statistical Methods

Differences between the frequencies of each genotype in controls and patients were compared using the Chi-square

test function available in SPSS 15.0. InStat version 3.6 was used to compare the frequencies of alleles and haplotypes. Analysis of residuals was conducted when the chi-square test detected significant differences. The aim of this *post-hoc* analysis was to demonstrate which study group contributed most significantly to the final test results. This analysis was conducted using PEPI, version 4.0 [20]. To test whether the influence of a certain variant was present in the general sample and whether this influence was context-dependent, the same statistical approach was repeated in different subsamples, according to genetic profile. Bonferroni correction was not used because of the small sample size. This choice was based on the conceptual nature of a multifactorial disease, for which there are always several potential influences, and because of that, when one single possible factor is tested, its isolated influence is expected to be small. Since Bonferroni correction controls only the probability of false positives, the correction ordinarily comes at the cost of an increased probability of producing false negatives. A careful review of the literature reveals that a large number of studies do not use this statistical correction, especially when testing new gene markers or investigating new gene–gene or gene-environment interactions. Thus, any data determined by multiple comparisons in subsamples without Bonferroni correction should be interpreted with caution, because of possible false positives. However, this type of approach may indicate genetic possibilities and should be explored in further investigations.

Because APOE and APOC1 genes are closely located at chromosome 19, their alleles are not independently inherited, but rather form a haplotype. This term can be defined as the set of alleles in different genes along a chromosome. When the distance between two genes is too short, there is a complete "linkage disequilibrium" between them (D value is equal to '1'), which means that alleles in different genes are always inherited together. The greater the distance between two genes, the greater the possibility of crossing over between them, and in this case, the two genes would not be in complete linkage disequilibrium (D value lower than '1'). ARLEQUIN, version 2000 [21], was used to calculate the degree of linkage disequilibrium between two genes ("D" value) in this study. Haplotypes cannot be directly determined in subjects heterozygous for both investigated genes as in homozygous individuals. Therefore, it is necessary to estimate haplotype frequencies by the maximum likelihood method, which in the present study was performed using MLOCUS, version 2.0 [22].

Results

Table 1 describes the frequencies of each allele and haplotype of APOE/APOC1 gene cluster in each group. In addition to comparing controls against patients with possible or probable Alzheimer's disease (p^b), comparisons were also made between controls and the group with dementia in general (p^a), which includes both patients with Alzheimer's disease and patients with dementia due to other causes (primarily cerebral vascular accidents). Significant differences were observed in the frequencies for all three genes when controls were compared with Alzheimer patients alone. The same APOE and APOC1 gene alleles were the rarest type in both groups, although twice as many patients were *E*4* allele carriers, when compared with controls ($P = 0.04$). The difference between groups was even more pronounced when prevalence rates for the APOC1 *−317*ins* allele were compared, with a significantly higher frequency among patients ($P < 0.001$). Regarding the ACE gene, the **ins* allele was rarer among controls, but more common than the **del* allele among Alzheimer patients ($P = 0.022$). When the general dementia group was compared with the control group, the same trends were observed for all three genes, but differences in *E*4* allele frequencies were no longer significant. These associations were stronger among homozygous than heterozygous subjects for the three studied variants (data not shown).

Haplotype frequencies for the APOE/APOC1 cluster observed in each of the three groups are also shown in Table 1. An analysis of linkage disequilibrium for the entire sample demonstrated that the *E*3* allele was significantly linked with the *−317*del* allele ($D' = 0.637$; $P < 0.0001$) and that the *E*4* allele was linked with the *−317*ins* allele ($D' = 0{,}898$; $P < 0.0001$). There was no significant linkage between the *E*2* allele and APOC1 alleles ($P = 0.98$). The two most common haplotypes were *E*3/−317*del* and *E*4/−317*ins* in all three samples, and the haplotype frequencies *E*3/−317*ins* and *E*4/−317*ins* were significantly greater in the Alzheimer's disease group compared with the control group, and the same was true for the general dementia group (for both groups, $P < 0.001$). The presence of these two risk haplotypes, both in homozygotes and heterozygotes, was analyzed for all three groups by comparing the frequency of carriers (Table 2). This analysis revealed that there were at least six times more patients with the *E*3/−317*ins* haplotype in both patient groups than in the control group ($P = 0.004$ and $P = 0.007$). Although the frequency of carriers of the *E*4/−317*ins* haplotype was also elevated, differences achieved no statistical significance.

The same approach shown in Table 2 was repeated in order to test whether this gene would produce context-dependent genetic effects. The groups were divided into four different subgroups, as follows: (1) carriers versus non-carriers of the *E*4/−317*ins* haplotype, (2) carriers versus non-carriers of the *E*3/−317*ins* haplotype, (3)

Table 1 Characteristics and allele/haplotype frequencies across study groups

	General dementia	Alzheimer's disease	Controls	p[a]	p[b]
n	47	35	85		
Age	75.2 ± 4.6	74.7 ± 4.1	68.3 ± 6.0	<0.001	<0.001
Min/max age	61/87 years		60/84 years		
APOE (rs429358/7412)[c]					
E*2	6.5	5.7	5.9		
E*3	71.7	70*	82.9*	0.07	**0.04**
E*4	21.8	24.3**	11.2**		
APOC1 (rs11568822)[c]					
−317*del	59.6	57.1	83.5	**<0.001**	**<0.001**
−317*ins	40.4	42.9	16.5		
APOE/C1[c] haplotypes					
*E*2/−317*del*	0	0	0.6	**<0.001**	**<0.001**
*E*3/−317*del*	**59.7***	**57.1***	**82.4**		
*E*4/−317*del*	0	0	0.6		
*E*2/−317*ins*	6.5	5.7	5.3		
*E*3/−317*ins*	**12.0***	**12.9***	**1.8**		
*E*4/−317*ins*	**21.7****	**24.3****	**9.4**		
ACE (rs1799752)[c]					
*del	39.4	35.7	53.7	**0.045**	**0.022**
*ins	60.6	64.3	46.3		

The entire sample of 47 patients was defined as the "general dementia" group, and this sample contained the 35 patients defined as "Alzheimer's patients", i.e., both patient samples are not separate groups, but one includes the other

ACE angiotensin-converting enzyme, *APOC1* apolipoprotein C1, *APOE* apolipoprotein E; p[a], comparison between controls and patients with general dementia; p[b], comparison between controls and patients with Alzheimer's disease

[c] Values are shown as percentage

* Analysis of residuals $P < 0.05$

** Analysis of residuals $P < 0.01$

Table 2 Analysis of risk haplotypes in different study groups

	General dementia		Alzheimer's disease		Controls		p[a]	p[b]
	n	%	n	%	n	%		
*E*3/−317*ins*								
Carriers	9	19.6	7	20	3	3.6	**0.004**	**0.007**
Non-carriers	37	80.4	28	80	81	96.4		
*E*4/−317*ins*								
Carriers	15	32.6	13	37.1	16	19	0.09	0.06
Non-carriers	31	67.4	22	62.9	69	81		

The entire sample of 47 patients was defined as the "general dementia" group, and this sample contained the 35 patients defined as "Alzheimer's patients", i.e., both patient samples are not separate groups, but one includes the other; p[a], comparison between controls and patients with general dementia ($P < 0.001$); p[b], comparison between controls and patients with Alzheimer's disease

carriers versus non-carriers of the *APOC1 −317*ins* allele, and (4) carriers versus non-carriers of the *APOE E*4* allele. Comparisons of the frequencies of ACE genotypes were analyzed again for each subset, and although all subsets were smaller than the entire sample, significant results were detected in certain genetic contexts. The ACE **ins/*ins* genotype was significantly more common only among patients without the *E*4/−317*ins* haplotype ($P = 0.04$ for general dementia and $P = 0.012$ for Alzheimer's disease), whereas among carriers of this haplotype, no significant difference was detected. When the sample was divided according to presence or absence of the *−317*ins*

allele of the APOC1 gene, the same tendency was observed only among non-carriers of the allele ($P = 0.038$ for general dementia and $P = 0.032$ for Alzheimer's disease), whereas among carriers no significant difference was detected. The same was true when the sample was stratified according to presence or absence of the *E*4* allele: the influence of the ACE gene was significant only in the subset of non-carriers of *E*4* ($P = 0.029$ for general dementia and $P = 0.011$ for Alzheimer's disease).

Discussion

This study investigated two candidate genes for Alzheimer's disease (APOC1 and ACE), in combination with a well-established susceptibility gene for this disease (APOE). The allele frequencies of these three loci were within the range of variation reported by other authors, particularly in populations with European ancestry [4, 7, 17].

The influence of the APOC1 gene on Alzheimer's disease has been investigated extensively in an attempt to identify the true role of this gene in the disease. After the first studies investigating this relationship were published, this association was questioned because the APOC1 gene is in the same cluster as the APOE gene, which had already been recognized as the principal gene causing susceptibility to Alzheimer's disease. Several authors have attempted to answer this question by conducting analyses of haplotypes of this cluster. Although no consensus has been reached concerning this issue, it appears that a greater number of studies support the hypothesis that the *−317*ins* allele of the APOC1 gene is a second susceptibility allele, rather than the possibility that this allele is simply inherited together with the *E*4* allele of the APOE gene [4, 23, 24]. Our data also agree with those authors, since they demonstrate that the *−317*ins* allele of the APOC1 gene is more common among patients than controls even in the presence of the *E*3* allele of the APOE gene, which does not cause increased susceptibility to Alzheimer's (Table 2). Indeed, the difference between patients and controls is much more pronounced for the APOC1 gene than for the APOE gene (Table 1). Accordingly, our data indicate that APOE and APOC1 genes affect susceptibility to Alzheimer's disease in a synergistic manner. The apoC1 protein may also play an important role in the pathophysiology of Alzheimer's disease, as already shown for apoE, since it is the only other apolipoprotein found in nerve tissue [4]. Nevertheless, in vitro experiments are needed to uncover how this protein affects the development of Alzheimer's disease.

The role of ACE in the cardiovascular system is well known, and elevated ACE levels have been strongly and consistently associated with increased risk of hypertension and related vascular diseases [25]. Since the **del* allele has been associated with increased ACE levels, this allele has also been associated with these diseases. However, more recently this enzyme has been shown to play a role in neuronal biology. The renin-angiotensin system (RAS) is responsible for a range of effects in the brain, such as the detection of increased ACE concentrations in a number of cortical and subcortical regions of the brain in patients with Alzheimer's disease [11–13]. In addition to ANG II, which is produced by ACE, other members of this system have also been observed in the brain during recent years, including ANG III, ANG VI, ANG(1–7), ACE 2, and receptors named prorenin and Mas; moreover, it has been shown that the metabolites of ANG II act on the AT1, AT2, AT4, Mas, and prorenin receptors [9, 26]. Furthermore, the ACE enzyme also appears to be involved in breaking down the β-amyloid protein, preventing it from aggregating and forming the senile plaques that are characteristic of Alzheimer's disease [10]. Therefore, while high levels of ACE appear to damage the vascular system, they could also protect against neuropathologies.

The **del* allele would therefore be expected to protect against diseases such as Alzheimer's, while the **ins* allele would be the at-risk allele, since it induces a reduction in ACE levels. However, data in the literature regarding this relationship are extremely contradictory, with some studies confirming the influence of the **ins* allele on Alzheimer's disease [11, 27–29] and some reporting non-significant influences [30, 31]; there is even an association between the **del* allele and Alzheimer's [32]. Mirroring this major contradiction, a meta-analysis analyzing more than 6,000 cases found that a significant influence occurred only among heterozygotes, while homozygotes for the **ins* allele were not at increased risk of Alzheimer's disease [14].

A possible reason for this contradiction may lie in the fact that the *ins/del* polymorphism (rs1799752) of the ACE gene is located in an intron. Because this is an apparently non-functional variant, the associations detected could be due to linkage disequilibrium with close variants, such as the SNPs rs4343 (a2350g or Thr776Thr), rs4335 (a > g on intron 15) and especially the rs4291 variant (a > t in the promoter region). Although these variants have been investigated by only a few authors, two recent studies have demonstrated the principal role of polymorphism occurring in the promoter region (rs4291). The **a* allele of this SNP has been associated with an increased risk of Alzheimer's disease among Europeans from five different countries, but the association was only present for carriers of the **a/*ins* haplotype (rs4291 and rs1799752) [30]. In contrast, among North Americans, the **a* allele of rs4291 was associated with an increased risk of Alzheimer's disease only if linked with the **del* allele of rs1799752 [32]. Therefore, it is possible that the true role of the ACE gene in Alzheimer's

disease be related to a variation in the promoter region. Because of different patterns of linkage disequilibrium, in some populations the **del* allele is in the same haplotype, while in others the **ins* allele is located on the same chromosome as in the majority of people. This would explain the major differences between data found in the literature, since rs4291 was not investigated in the majority of studies, and in those studies relating **del* allele with increased risk, differences would therefore be due to its linkage with the **a* allele.

Although possibly limited by a low-power sample and by the multiple comparisons performed, our data indicate that a possible cause of variation between studies involving data on ACE may lie in its interaction with the APOE/APOC1 cluster, as shown in the significant associations observed according to genetic contexts. However, only two studies have previously investigated these genetic interactions. Monastero et al. [33] detected no influence of the ACE gene on Alzheimer's disease among Italians, even when the enzyme was analyzed in combination with the APOE gene. In contrast, Wang et al. [29] detected a synergistic interaction between the **ins* allele of the ACE gene and the *E*4* allele of the APOE gene among Chinese people: the effect of the **ins* allele was almost three times greater in the presence of the *E*4* allele, when compared with a weak, although significant, association found among non-carriers of the *E*4* allele.

There is no doubt that further studies involving the construction of haplotypes and also gene interactions are needed in order to elucidate these apparent contradictions. It is worth noting that association data may change according to genetic contexts in other loci; thus, investigation of these effects may help explain the reasons for the major differences observed between studies of different ethnic groups. These differences may be caused by cultural and environmental factors, which could modulate the results of different association studies, depending on environmental/cultural contexts. This type of approach may enhance the usefulness of these data for detecting people at high risk of Alzheimer's disease. It is therefore clear that more studies are needed to further investigate genetic risk factors in different populations so to widen the range of identified genetic susceptibilities to Alzheimer's, thereby optimizing prevention of the disease.

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