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C-338A polymorphism of the endothelin-converting enzyme (ECE-1) gene and the susceptibility to sporadic late-onset Alzheimer's disease and coronary artery disease

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Abstract. The human endothelin-converting enzyme (ECE) is involved in β-amyloid synthesis and regulation of the endothelin-1 (ET-1) vasoconstricting peptide. We investigated the distribution of the C-338A polymorphism of the ECE-1b gene in sporadic late-onset Alzheimer's disease (LOAD) and in coronary artery disease (CAD) to verify its role in the onset of these two complex diseases. Two cohorts of 458 Italian Caucasian LOAD patients and 165 CAD patients were examined for the C-338A polymorphism and compared with respective control samples (260 and 106 subjects, respectively) . The A allele was less present in LOAD patients than in controls, but an at limits statistically significant difference was achieved only in subjects aged less than 80 years, where only the AA genotypes appeared to have a protective role against the onset of the sporadic LOAD. For the overall CAD sample the pattern was similar and significant differences were observed only in subjects non carrying the apolipoprotein E (APOE) e*4 allele, where the A allele carrying genotypes had a protective role against the onset of the disease.

Keywords: Alzheimer's disease, apolipoprotein E, APOE, coronary artery disease, DNA polymorphisms, endothelin-converting enzyme, ECE-1

1. Introduction

Endothelin-converting enzyme (ECE), an enzyme involved in the biosynthesis of *big* endothelin (ET) [7], generates ET-1, a potent vasoconstricting peptide with an important role in the regulation of basal vascular tone [11]. Two homologous proteins have been isolated: ECE-1 and ECE-2. ECE-1, being implicated in ET-1 activation, is believed to contribute to the regulation of vascular tone and cell growth in atherosclerosis [11]. The two proteins are encoded by different genes, ECE-1 and ECE-2, that chiefly act in endothelial and neuronal tissues, respectively [7]. The ECE-1 gene, the more extensively studied of the two, is located on chromosome 1, has 20 exons and four different isoforms (ECE-1a, b, c, d) generated by alternative promoters [15]. They have similar enzyme kinetics but dif-

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ferent tissue expression and subcellular localization [7, 8].

ECE-1 gene variation has been recently investigated [5–7,9,10] and several variants described in particular in the promoter region. One of these variants, C-338A, is polymorphic and the A allele is reportedly associated with higher blood pressure (BP) levels in women [5,7].

Recently, a role of ECE has been hypothesized in the clearance of β-amyloid (Aβ), a peptide present in the abnormal plaques of the brain of Alzheimer's disease (AD) patients. While this role has been ascertained in mice, no direct evidence in man has been found so far. In their study on C-338A polymorphism in relation to the late-onset form of AD (LOAD) in a French Caucasian population, Funalot et al. [6] found that the A Allele homozygotes had a reduced risk for the disease. This result was attributed to the increased promoter activity associated with the A allele and, consequently, with AA homozygotes.

LOAD and coronary artery disease (CAD) are both complex disorders with different pathological hallmarks, yet some convincing links between them exist. It has been reported that cardiovascular disease is associated with AD, as are the factors that cause it [14]. Studies on the association between ECE-1 gene variation and these two diseases are still quite rare. To fill this gap, we investigated C-338A polymorphism in two Italian patient samples affected with these disorders.

2. Materials and methods

A cohort of 458 Italian Caucasian subjects (mean age $\pm$ S.D. $=$ 79.5 $\pm$ 7.07 years; range 62–96; men $=$ 30.1 %, women 69.9%) affected with the sporadic late-onset ($\geqslant$ 60 years) form of Alzheimer's disease was enrolled for the study at the Division of Neurology of Verona Hospital (Northern Italy). Dementia was diagnosed according to the DSM-IV-R criteria [1]. Probable sporadic late-onset Alzheimer's disease was diagnosed according to the NINCDS-ADRDA Work Group guidelines (revised) [12]. The control group, composed of unrelated individuals with no neurodegenerative disorders, included 260 subjects from the same area (78.9 $\pm$ 7.7; 60–99; men 41.2%, women 58.8%). The CAD group comprised 165 patients (70.7 $\pm$ 12.1; 31–98; men 69.1 %, women 30.9 %) recruited at S. Giovanni-Addolorata Hospital, Rome, who came mostly from Central and Southern Italy. The diagnosis of CAD was supported by enzyme and electrocardiographic examinations, as previously described [3]. The control group came from the same area and included 106 subjects (73.7 $\pm$ 11.3; 44–98; men 44.3%, women 55.7%) with no history of infarction or hypertension, and a normal electrocardiogram.

Collection of the biological material for scientific study was approved by the institutional ethics committees. Informed consent was obtained for all individuals. Venous blood was drawn in $EDTANa_2$ as anticoagulant from all subjects after overnight fasting. Genomic DNA was extracted according to the salting out procedure described by Miller et al. [13]. ECE-1 C-338A polymorphism was investigated according to the technique described by Funalot et al. [5]. The PCR product was digested overnight by *Tsp509*I and then visualized on NuSieve agarose gel under UV. The Apolipoprotein E (APOE) polymorphism had been previously typed by the technique described by Wenham et al. [17].

Allelic frequencies were determined by the gene-counting method. Agreement between the genotype distributions and those expected according to the Hardy-Weinberg equilibrium was verified by a X^2 test. For LOAD and CAD groups, the differences in allele and genotype frequencies between patients and controls were analyzed by a X^2 test. In the case of small values the Fisher's exact test was used. Since some stratifications of the samples were performed, we used the Bonferroni's correction for multiple comparisons. Consequently the 5% level of statistical significance was changed according with the number of post-hoc tests. The risk of developing AD or CAD associated with ECE-1 A allele carrying genotypes was estimated by the odds ratios (O.R.) adjusted for APOE alleles and age or sex, obtained by logistic regression analysis.

3. Results

Table 1 lists the ECE-1 C-338A genotype and allele frequencies in LOAD patients and healthy subjects for the whole sample. The observed genotype frequencies fitted the ones expected according to the Hardy-Weinberg equilibrium. Across the entire sample, the A allele carrying genotypes were less frequent in patients than in controls, but the difference was not statistically significant (crude O.R. $=$ 0.77; 95% C.I. $=$ 0.57–1.05; $p = 0.09$). Frequencies in CAD patients and respective controls are also reported in Table 1. Here too, in the overall sample, the A allele carrying genotypes in the whole sample were less frequent in patients than in

Table 1
ECE-1 C-338A genotype and allele frequencies in the overall LOAD and CAD samples

		Genotypes						Alleles	
		CC	AC	AA	AA+AC	n	p	*C	*A
LOAD									
Patients	obs.	286	151	21	172	458	0.85	0.789	0.211
	%	62.4	33.0	4.6	37.6				
Controls	obs.	146	100	14	114	260	0.56	0.754	0.246
	%	56.2	38.5	5.4	43.9				
Crude OR		1.0	0.77	0.77	0.77				
(95% CI)			0.56–1.06	0.38–1.55	0.57–1.05				
p			0.11	0.46	0.09				
Adjusted OR		1.0	0.79	0.75	0.78				
(95% CI)			0.56–1.10	0.36–1.56	0.57–1.08				
p			0.16	0.45	0.14				
CAD									
Patients	obs.	105	49	11	60	165	0.12	0.785	0.215
	%	0.636	0.297	0.067	0.364				
Controls	obs.	57	42	7	49	106	0.84	0.736	0.264
	%	0.538	0.396	0.066	0.462				
Crude OR		1.0	0.63	0.85	0.67				
(95% CI)			0.38–1.07	0.31–2.32	0.41–1.09				
p			0.09	0.76	0.11				
Adjusted OR		1.0	0.65	0.83	0.67				
(95% CI)			0.38–1.11	0.29–2.34	0.40–1.11				
p			0.12	0.72	0.12				

Table 2
ECE-1 C-338A genotype frequencies stratified by age in LOAD and by APOE allele in CAD

Sample (Variable)	Cases	Controls	Crude OR	(95% CI)	P	Adjusted OR	(95% CI)	P
LOAD (Age)	AA/CC+AC							
($<$ 80 years)	6/218	8/89	0.31	(0.10–0.91)	0.036	0.28	(0.09–0.90)	0.03
($\geqslant$ 80 years)	15/219	6/157	1.79	(0.68–4.72)	0.26	1.96	(0.70–5.48)	0.20
CAD (APOE)	AA+AC/CC							
e*4 allele	14/13	3/12	4.31	(0.99–18.8)	0.06	2.45	(0.45–13.23)	0.30
Non-e*4 allele	46/92	46/45	0.48	(0.28–0.84)	0.013	0.51	(0.29–0.89)	0.018

controls, but the difference was not significant (crude O.R. $=$ 0. 67; 95% CI $=$ 0.40–1.11; $p = 0.11$).

Since for both these disorders other factors, such as the APOE alleles, sex and age, could infuence the risk associated with the ECE-1 genotypes, the logistic regression analysis was performed and the adjusted O.R. calculated (Table 1). Inserting the interactions between the above mentioned variables and the ECE-1 genotypes revealed that in LOAD a statistically significant interaction was present between ECE-1 AA genotype and age ($p = 0.024$, data not shown). A statistically significant differences was observed when subjects were stratified in two age groups taking 80 years as a cut-off. This age grouping was based on the median value of the patient age distribution, and allowed us to compare very old (octogenarian) with less old subjects. More precisely, in subjects aged less than 80 years the AA genotype was significantly less frequent in patients than in controls (adjusted O.R. $=$ 0.28; 95% C.I. 0.09–0.90; $p = 0.03$) (Table 2). Having stratified by age requested the Bonferroni correction for multiple comparisons to be applied . Consequently the 5% statistical significance was attained at $p = 0.025$ (0.05/2). This made the difference due to age significant at limits ($p =$ 0.03).

The logistic regression analysis in CAD revealed that a statistically significant interaction was present between ECE-1 and APOE alleles ($p = 0.018$, data not shown). After stratification by carrying or not the APOE e*4 allele, the AA + AC genotypes were less frequent in patients with APOE non-e*4 alleles than in controls (adjusted O.R. $=$ 0.51; 95% C.I. 0.29–0.89; p $=$ 0.018) (Table 2). When corrected for multiple comparisons the statistical significance was attained at $P =$ 0.025, hence the difference was yet significant.

4. Discussion

The ECE-1 human gene is involved in both β-amyloid synthesis and regulation of the ET-1 vasocon-

stricting peptide. This prompted us to investigate C-338A polymorphism in LOAD and CAD. Across the entire LOAD patient sample, the frequencies of the A allele carrying genotypes differed from those of the controls, though not significantly, confirming the trend reported by Funalot et al. [6], i.e. a lower AA genotype frequency in patients, and hence a protective effect of this genotype against the disease. After stratifying the sample by age we observed a statistically significant difference only in subjects aged less than 80 years. After correction for multiple comparisons, the AA genotype was found significantly protective though at limits, resembling what observed by Funalot et al. [6] in a sample of similar age composition. It may be supposed that with age $\geqslant 80$ years the pathogenetic factors of AD surpass the protective effect of the ECE-1 A allele.

Also in the CAD patient sample throughout, the A allele was less frequent than in controls, resulting protective though not significantly. This finding cannot be compared with others reported in the literature for westernized populations because of lack of data. The only data that could have a connection with this topic are those of Funke-Kaiser et al. [7] and Funalot et al. [5] who both reported, though at different levels, an association of the A allele with higher blood pressure (BP), chiefly in untreated hypertensive women. Recently Wang et al. [16], in an investigation of this polymorphism in a Chinese CAD sample, reported an association of the A variant with an increased risk for the disease. The opposite trend showed by those data compared with ours could be attributed to the different genetic and environmental make up of the Chinese population with respect to the population we examined [2].

After stratifying our overall sample according with the APOE allele, a significant difference was observed in non-e*4 allele carriers, that after correction for multiple comparisons was yet statistically significant.

While in AD the finding reported in the literature that the A allele, associated with increased ECE-1 promoter activity, is protective agrees with its involvement in β-amyloid clearance, our results for CAD diverge from the data that suggested an association with BP. In this case, the protective role of the A allele contrasts with the higher BP levels reportedly associated with it. In our study we were unable to analyze the relationships between C-338A and BP levels because we had BP data only for medicated CAD patients, making them unsuitable for comparison with subjects without medication.

However, to explain why the ECE-1 A allele is protective only in the absence of the APOE e*4 allele, one needs to consider the pathogenetic mechanism of e*4 in CAD. In CAD the e*4 allele has been reportedly associated with increased levels of LDL cholesterol and total cholesterol, hence to a higher risk of cardiovascular diseases. It can be postulated that the e*4 allele diminishes the protective role of the ECE-1 A allele that can be observed when the other two APOE alleles, e*2 and e*3, are present.

In conclusion, the ECE-1 C-338A frequencies distribution in our sample of patients with sporadic LOAD confirmed the trend previously reported by Funalot et al. [6] showing a protective effect chiefly in less elderly subjects. In CAD, another complex disease in which the effect of e*4 on its onset has been variously reported [4], examined here for the first time in connection with C-338A polymorphism in a westernized population, our results indicate that the protective effect afforded by the A allele seemed to be modulated by the absence of the APOE e*4 allele.

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