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Role of Cyclooxygenase-2 and 5-Lipoxygenase Polymorphisms in Alzheimer's Disease in a Population from Northern Italy: Implication for Pharmacogenomics

Florinda Listì[a], Calogero Caruso[a], Domenico Lio[a], Giuseppina Colonna-Romano[a], Martina Chiappelli[b], Federico Licastro[b] and Giuseppina Candore[a,*]
[a]*Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Italy*
[b]*Dipartimento di Patologia Sperimentale, Università di Bologna, Italy*

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Abstract. Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by cognitive deficit with progressive worsening of memory. Recent data indicate that neurons, as well as other brain cells, can express enzymes such as cyclooxygenases (COXs) and 5-lipoxygenase (5-LO) which are considered important in inflammatory cells. Moreover, it has been demonstrated that COX-2 and 5-LO enzymes play a considerable role in the pathophysiology of AD. In order to assess the possible role of COX-2 and 5-LO single nucleotide polymorphisms (SNPs) in AD, we examined their distribution in 341 AD patients and 190 controls from Northern Italy. A significant difference was observed in the distribution of the −765G COX-2 and −1708A 5-LO alleles between AD cases and controls ($p = 0.03$ for −765G/C COX-2 SNP; and $p = 0.007$ for −1708G/A 5-LO SNP). Hence, COX-2 −765G and 5-LO −1708A alleles were overrepresented in AD patients and underrepresented in controls. Our data suggest that these alleles of COX-2 and 5-LO could be risk factors for AD. These results seem of some importance for a pharmacogenomic approach.

Keywords: Alzheimer's disease, COX-2, 5-LO, pharmacogenomics

INTRODUCTION

Inflammation clearly occurs in the brain of Alzheimer's disease (AD) patients and the classical mediators of inflammation, eicosanoids and cytokines, contribute to the neurodegeneration [1–3]. Recent data indicate that neurons can also express enzymes such as cyclooxygenases (COXs) and 5-lipoxygenase (5-LO) which typically are considered important in inflammatory cells [4,5]. The cyclooxygenases (COX-1, COX-2) are the key enzymes in the conversion of arachidonic acid to the precursors of bioactive lipid mediators, eicosanoids, including prostaglandin (PG), thromboxane, and prostacyclin [6]. COX-2 inducible enzyme is widely expressed in the AD patient brain and its expression correlates with amyloid plaque density and neurofibrillary tangles [4,7–10]. In particular, COX-2 is expressed primarily in neurons, and possibly in oth-

*Corresponding author: Giuseppina Candore, PhD, Gruppo di Studio sull'Immunosenescenza, Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, 90134 Palermo, Italy. E-mail: gcandore@unipa.it.

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er brain cells under certain circumstances during the clinical progression of the disease [11]. One possible mechanism of increased COX-2 gene expression in the brain may involve the inflammation-related transcription factor, NFκB; there is a strong correlation between NFκB DNA binding and COX-2 mRNA expression in AD brain [12]. Moreover, the COX-2 promoter possesses multiple recognition sites specific for NFκB DNA binding [13].

Additionally, several epidemiological studies have suggested that long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDs), which include COX-2 inhibitors, may reduce the risk of developing AD [4,14–17]. On the other hand, a recent report based on a longitudinal study of men and women aged 70 years and older with a family history of AD (ADAPT study) showed that use of naproxen or celecoxib did not improve cognitive function [18]. However, several critical issues have been raised concerning the study results, i.e., it has been claimed that the ADAPT study does not indicate that NSAIDs, if taken during adulthood and for an extended period, cannot prevent or delay the onset of dementia [19].

Non-mutually exclusive hypotheses on the role of COX-2 in AD concern its possible influence on processing of the amyloid-β protein precursor through a PG-E2-mediated stimulation of γ-secretase activity [20]. In addition, COX-2 protein and PG-E2 concentrations were selectively increased $>$ 2-fold in the cerebral cortex of subjects with the presenilin-2 (PS2) familiar AD mutation relative to wild-type PS2 AD cases [21]. Moreover, it has been found that neuronal overexpression of human COX-2 in transgenic mice stimulated excitotoxicity *in vivo* and *in vitro*. In particular, the results indicate that human COX-2 overexpression causes neuronal cell cycle deregulation in the brain [22].

Hence, the use of NSAIDs, which block the synthesis of eicosanoids, might interfere with the mechanisms involved in the pathophysiology of AD, whether or not dependent on inflammatory responses [4,14,15,17,20–22].

A number of single nucleotide polymorphisms (SNPs) have been described in the promoter region of the COX-2 gene that probably regulates its transcription. But only one polymorphism located at position -765, a putative stimulatory protein-1 binding site, has been shown to be functional. It has been shown that the -765 CC genotype resulted in a reduction of approximately 30% in *in vitro* promoter activity and was associated with lower plasma levels of inflammatory markers, such as C-reactive protein [23].

In a previous study, this SNP was found to be associated with reduced risk of AD [24]. That study reported a significantly different distribution, in patients and controls, of the -765 SNP whose G allele was underrepresented in control subjects and overrepresented in patients with AD [24]. Recently, a study in the Chinese population has demonstrated a significant association between the polymorphisms of COX-2 and AD. However, they showed that carriers of $+837$ T allele in the exon 10 had a 1.5-fold increase in the risk of AD [25].

An alternative pathway of arachidonic acid generates leukotrienes (LTs), through the action of 5-LO together with the 5-lipoxygenase-activating protein (FLAP). LTs are implicated in a wide variety of inflammatory disorders, including the chronic ones [26]. 5-LO has also been described in neurons and in some glial cells throughout the cerebrum, basal ganglia, and hippocampus [5,27,28]. Compared to controls, a significant increase of LTs was observed in cerebrospinal fluid from AD and mild cognitive impairment patients. So the activation of this enzyme occurs early in the course of AD, before the onset of overt dementia, thereby implicating its metabolites in the pathophysiology of AD [5, 27,28].

Several SNPs of these enzymes have been described [29,30]. In particular, the -1708GA and -1761GA SNPs in promoter region of 5-LO gene and -336GA in promoter region of FLAP have been claimed able to modify 5-LO/FLAP gene transcription or the putative protein derived from translation of 5-LO/FLAP mRNA [29]. Most studies have analyzed these SNPs in association with asthma susceptibility [29,30]. Only a few genetic studies have identified variants of the 5-LO gene and the FLAP gene promoter as risk factors in atherosclerosis and myocardial infarction [31,32]. These studies have shown that 5-LO polymorphisms, involved in a decreased expression of 5-LO, are less represented in patients with myocardial infarction and severe atherosclerosis [31,32].

Accordingly, it has been suggested that the polymorphisms putatively involved in a decreased expression of 5-LO are underrepresented in AD patients. But an overexpressed 5-LO gene could significantly increase the brain's vulnerability to neurodegeneration [27].

Since few studies have investigated the role of these COX-2 and 5-LO polymorphisms in relation to AD [24, 25,27], we have evaluated whether these COX-2 and 5-LO SNPs can be considered risk factors for AD. If responsible for a differential production of the relevant enzymes, these SNPs could be involved in the pathophysiology of AD.

MATERIALS AND METHODS

Subjects

Diagnosis of probable AD was according to standard clinical procedures and followed the NINCDS/ADRDA and DSM-III-R criteria [33,34]. Cognitive performance and alterations were measured according to the Mini-Mental State Evaluation and the global deterioration scale. All AD cases were defined as sporadic because their family history did not mention any first-degree relative with dementia. In addition, 80% of AD patients showed clinical onset of the disease after 65 years of age (late-onset AD, LOAD), and 20% before this age (early-onset AD, EOAD). The population of AD consisted of 341 patients from Northern Italy with clinical diagnosis of probable AD (238 women and 103 men; age range: 53–98 years; mean age: 74.88 ± 8.44). AD patients included in the study did not present major co-morbidity such as cancer, symptomatic (present or previous) cardiovascular diseases, and major inflammatory diseases as autoimmunity and infections. Controls were 190 unrelated individuals (100 women and 90 men; age range: 65–93; mean age 73.21 ± 8.24) randomly selected from a retirement home. These subjects were checked and judged to be in good health based on their clinical history and on blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins, cholesterol, and triglycerides). The controls were collected from the same population as the patient cohort. Patients and controls were assessed to have parents and grandparents born in Northern Italy to ensure ethnicity. Consequently, possible confounding effects, like the inclusion in the study of members of different ethnic groups, have been minimized. Informed consent was obtained from all guardians of patients and controls according to Italian law.

Molecular methods

The salting-out method was used to extract the DNA, following the standard protocol [35]. DNA was extracted and genotyped for 765 G/C COX-2, −1708 G/A and −1761 G/A 5-LO, −336 G/A FLAP SNPs, and ApoE4 polymorphism by using a polymerase chain reaction [32,36].

Statistical information

The data were tested by χ^2 test for the goodness of fit between the observed and expected genotype frequencies according to the Hardy-Weinberg equilibrium (HWE). Differences in allele and genotypic frequencies of the different SNPs among the groups were evaluated by gene count and χ^2 test with Yate's correction. Odd ratio (OR) with confidence interval (CI) was also calculated. It might be argued that a Bonferroni-type adjustment should be performed to correct for the testing of multiple polymorphisms. However, this correction is too stringent and has the potential to ignore important observations [37,38], hence we did not carry out this correction. In patients and controls, a logistic regression analysis was used to investigate the associations of genotypes with AD, after adjustment for ApoE4 allele, gender, and age at onset (LOAD vs. EOAD). The OR (with CI) was calculated as exponential of regression coefficient and its standard error.

Power estimates were calculated with PS software, v. 2.1.31. Briefly, power was estimated using the number of cases and controls and the prevalence of the putative susceptibility allele or genotype in the control population [39]. The average power across individual SNPs was 0.45, which is higher than most AD association studies [1,40].

RESULTS

The frequencies of the genotypes of all SNPs under investigation, in AD patients and age-related controls from Northern Italy, were in HWE. Table 1 shows the genotype and allele frequencies of the different SNPs of COX-2, 5-LO, and FLAP genes in AD patients and controls.

The distribution of COX-2 genotypes between AD patients and controls was significantly different. Subjects with the −765GG genotype of COX-2 were overrepresented among AD patients and underrepresented in age-related controls. According to genotype, a significant difference in the G allele frequency between AD patients and controls was observed. The G allele frequency was found to be higher in patients than in controls.

Concerning the genotype and allele frequencies of 5-LO and FLAP polymorphisms, there was a significant difference between AD patients and age-related controls for 5-LO −1078 SNP. In fact, the 1708AA genotype was overrepresented among AD patients and

Table 1
Genotype and allele frequencies of different polymorphisms of COX-2, 5-LO, and FLAP genes in 190 controls and 341 AD patients from Northern Italy

Locus	Genotype/allele	AD (N=341)	Controls (N=190)	P-value
	GG	237 (69.5%)	115 (60.5%)	0.032*
−765 G/C	GC	94 (27.5%)	62 (32.6%)	
(COX-2)	CC	10 (3%)	13 (6.9%)	
	G	568 (83.3%)	292 (76.8%)	0.03**
	C	114 (16.7%)	88 (23.2%)	
	GG	256 (75%)	159 (83.7%)	0.018*
−1708G/A	GA	77 (22.5%)	31 (16.3%)	
(5-LO)	AA	8 (2.5%)	0 (0)	
	G	589 (86.4%)	349 (91.8%)	0.007**
	A	93 (13.6%)	31 (8.2%)	
	GG	223 (65.4%)	137 (72%)	0.07*
−1761G/A	GA	112 (32.8%)	53 (28%)	
(5-LO)	AA	6 (1.8%)	0 (0)	
	G	558 (82%)	327 (86%)	0.07**
	A	124 (18%)	53 (14%)	
	GG	272 (80%)	146 (76.8%)	0.06*
−336 G/A	GA	62 (18%)	44 (23.2%)	
(FLAP)	AA	7 (2%)	0 (0)	
	G	606 (88.8%)	336 (88.4%)	0.08**
	A	76 (11.2%)	44 (11.6%)	

*The significance of the different genotype distribution between patients and controls was calculated by chi-square test (3×2 table).
**The significance of the different allele distribution between patients and controls was calculated by chi-square test (2×2 table).

underrepresented in age-related controls. According to genotype, a significant difference in the A allele frequency between AD patients and controls was observed. The A allele frequency was found to be higher in patients than in controls. There were no significant differences for genotype and allele frequencies in the −1761G/A 5-LO and −336G/A FLAP SNPs between patients and controls.

Gender analysis demonstrated that the significant differences were not present by separately analyzing male and female patients and controls (data not shown). Comparing the genotypes and the alleles of −765G/C COX-2 SNP and −1708G/A 5-LO SNP between the two groups, the ORs for these genotypes and alleles were statistically significant (Table 2).

In addition, we performed a logistic regression analysis to test the association of genotypes of −765G/C COX-2 and −1708G/A 5-LO SNPs with AD after adjustment for the presence of ApoE4 allele, gender, and age at onset. This analysis demonstrated that a significant difference in genotype frequency of −765GG COX-2 persisted between AD patients and controls ($p < 0.0001$). In addition, using the same logistic regression analysis, the difference in genotype frequency of −1708AA 5-LO polymorphism persisted between AD patients and controls ($p < 0.0001$). These results indicate that the genotypes under study are independent risk factors for developing AD in this population from Northern Italy.

DISCUSSION

Inflammatory processes play a crucial role in the pathophysiology of AD. Many inflammatory mediators have been detected in regions of the brain of patients with AD and activation of astrocytes and microglial cells causes expression of pro-inflammatory cytokines, complement and acute phase proteins [2,3,41]. In particular, it has been demonstrated by recent findings that COX-2 and 5-LO genes play a considerable role in AD pathophysiology. In fact, COX-2 is largely expressed in the AD patient brain; its expression correlates with amyloid plaque density and neurofibrillary tangles [4, 7–10]. However, it has been suggested that COX-2 and PG can play a role in AD pathophysiology that is not dependent on an inflammatory pathway [20–22]. On the other hand, epidemiological studies have suggested that NSAIDs decrease the risk of developing AD [4, 14–17].

The finding of several polymorphisms in the promoter region of COX-2 gene has suggested that the reduction of risk by COX inhibitors against AD may be specific for particular genotypes [2], analogous to the

Table 2
Odds ratio (OR) and confidence interval (CI) with P-value comparing 190 controls and 341 AD patients from Northern Italy

	Controls	P-value
AD (−765 GG COX-2)	OR=1.48, 95% CI=1.025–2.15	0.044
AD (−765 G COX-2)	OR=1.50, 95% CI=1.09–2.05	0.011
AD (−1708 AA 5-LO)	OR=9.71, 95% CI=0.55–169.3	0.055
AD (−1708 A 5-LO)	OR=1.77, 95% CI=1.15–2.72	0.007

observed protection against colorectal adenoma [42]. 5-LO has been observed in neurons and in some glial cells throughout the cerebrum, basal ganglia, and hippocampus [5,27,28]. Compared to controls, a significant increase of LTs was observed in cerebrospinal fluid from AD and mild cognitive impairment patients. So the activation of this enzyme occurs early in the course of AD [5,27,28].

The aim of our study was to evaluate whether the COX-2 and 5-LO SNPs likely involved in different enzyme production can be considered risk factors for AD. We found that the −765G of COX-2 and −1708A of 5-LO alleles were significantly higher in AD patients and lower in age-related controls. This interpretation is correct, i.e., these findings are related to an overrepresentation of reported alleles in AD but not to their underrepresentation in the controls, as demonstrated by the fact that these frequencies are not different from those obtained in another Italian cohort and lower than those obtained in Italian centenarians, i.e., a typical example of successful ageing [43,44].

In addition, the stratification for ApoE4 allele, gender, and age at onset clearly demonstrated that −765 G/A and −1708 G/A polymorphisms are risk factors for AD independently of ApoE4, gender, and age at onset in Northern Italy population. Our findings confirm and extend the data of a recent study which demonstrated that −765 COX-2 allele increases the risk for AD independently of ApoE4 [24]. This is the first study where the −1708G/A 5-LO SNP has been analyzed, so it needs to be validated by further studies. A further method of validation should be a meta-analysis that provides a means to quantitatively synthesize association data across studies of the same genetic variant. The use of meta-analyses has recently become an important part of genetic research mainly to reconcile previously conducted studies that gave inconsistent results, but an adequate number of studies is necessary [1,45].

In the present report, we have followed an approach known as candidate gene association, i.e., we have selected the genes to be investigated according to their known or postulated biology. Another approach, genome-wide association (GWA), entails the screening of the whole genome for associations. It investigates hundreds of thousands of SNPs across the genome, without any previous hypotheses about potential mechanisms or candidates. This kind of study has greatly accelerated the rate of detection of genetic associations. Testing so many potential genes simultaneously carries the risk of finding many spurious associations. On the other hand, the statistical correction performed to avoid this type of error may be responsible for missing rare alleles with a low OR. Individual GWAs are underpowered to detect all but the largest effects, and the susceptibility variants identified so far are probably only a subset of the loci that would be detectable using this approach if power was increased [46,47]. Accordingly, in 7 out 9 WGA studies performed until now, as reported on the Alzforum website, the only reproducible featured gene was APOE [48]. In any case, polymorphisms involved in AD are fairly common in the general population, so there is a strong likelihood that any given individual will inherit one or more of the high-risk alleles: the occurrence of the disease is likely to depend on interaction between different high-risk alleles, exposure to pathogens, environmental factors, and lifestyle choices [2,3].

Finally, the differences between patients and controls are significant but relatively small with unimpressive ORs. However, since AD is a multifactorial disease, any single mutation will only provide a small or modest contribution to risk, also depending on interaction with other genes and/or a particular environment [49].

These results might offer an approach to defining individual risk profiles that can be applied to healthy subjects to predict intrinsic risk of AD. Such risk profiles, when better established, can be used to trigger further diagnostic procedures and early therapeutic interventions aimed at preventing or significantly delaying the clinical manifestations of AD. A customized risk profile can also provide useful information for personalized therapeutics, i.e., for a pharmacogenomic approach [50]. The working hypothesis is that these polymorphisms might be a means to determine which AD patients to treat with the inhibitors, and therefore the possibility of preventive treatment with a specific inhibitor of eicosanoids or their enzymes [2,51].

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