Association study of *COX-2 (PTGS2)* –765 G/C promoter polymorphism by pyrosequencing in Sicilian patients with Alzheimer's disease

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Submitted: 18 February 2013
Accepted: 18 June 2013

Arch Med Sci 2014; 10, 6: 1235–1238
DOI: 10.5114/aoms.2014.47832

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Abstract

Introduction: Alzheimer's disease (AD) is characterized by progression of memory problems to a slow global decline of cognitive function. Inflammation when left unregulated becomes a major cofactor in the pathogenesis of AD. PTGS2 is of crucial relevance in the inflammatory response, and it has been shown to play a considerable role in AD pathogenesis.
Material and methods: To assess the possible putative role of a PTGS2 polymorphism (–765 G/C) in AD patients, we examined, by pyrosequencing, its distribution in 84 Sicilian AD patients and in 80 controls.
Results: No significant statistical difference in PTGS2 –765 G/C genotype distribution was found comparing patients with AD and controls. In addition, no significant difference was observed in the distribution of the PTGS2 –765 alleles between AD patients and controls.
Conclusions: These findings suggest that the PTGS2 –765 G/C polymorphism may not be associated with AD in the Sicilian population.

Key words: Alzheimer disease, COX-2 gene, polymorphism, pyrosequencing.

Introduction

Dementia has been estimated to affect about 25 million persons worldwide and it is projected to nearly double every 20 years, reaching about 66 million in 2030 and about 115 million in 2050. The total number of new cases of dementia each year is nearly 7.7 million worldwide, implying one new case every 4 s [1]. About 60–70% of cases of dementia are due to Alzheimer's disease (AD).

The AD is clinically characterized by progression from episodic memory problems to a slow global decline of the cognitive function that leaves patients with end-stage AD bedridden and dependent on custodial care, with death occurring on average 9 years after diagnosis [2]. Neuropathologically, AD is characterized by the presence of extracellular amyloid deposits composed of aggregated β-amyloid (Aβ) peptides and intracellular neurofibrillary tangles containing hyperphosphorylated, aggregated tau protein [2]. To date, the etiological mechanisms underlying the neuropathological changes in AD remain unknown; on the other hand, inflammation is a fundamental protective immunological response, but when

left unregulated, it can become a major cofactor in the pathogenesis of many chronic human diseases, including AD. The role of neuroinflammation in AD has been intensely investigated, in part because of its tremendous clinical implications [2].

Prostaglandin-endoperoxide synthase 2 (PTGS2), also called cyclooxygenase 2 (COX2) (MIM# 600262), has been shown to be expressed mostly in the central nervous system and inflammatory cells [3, 4]. The gene coding for PTGS2 has been mapped to 1q31.1 between two regions to which genetic linkage to AD has been reported [5]. In addition, elevated PTGS2 levels are present in neurons from hippocampal pyramidal regions of the AD brain [6]. A number of single nucleotide polymorphisms (SNPs) have been described in the promoter region of the PTGS2 gene that probably regulates its transcription, but only one polymorphism located at position –765 G/C, a putative stimulatory protein-1 binding site, has been shown to be functional [7]. The C allele of COX2-765 polymorphism has been associated with decreased risk of AD [7]; however, other findings seem to suggest that the distribution of COX2-765G/C polymorphism is similar between patients with AD and controls [8].

Pyrosequencing is a DNA sequencing technology based on the sequencing-by-synthesis principle [9], and the method is based on a real-time bioluminescence technique. Gharizadeh *et al.* analyzed in detail the sequence data of a large number of amplicons, using two techniques (pyrosequencing and Sanger dideoxy sequencing). Their results demonstrate that for short DNA sequences, pyrosequencing excels in reducing sample preparation time, providing ease-of-use and cost and labor savings [10]. Also, the drawbacks of pyrosequencing are rapidly being addressed with the use of Sequenase, and automated software [10]. In the present study, we evaluated the –765 G/C promoter variant of the PTGS2 gene by pyrosequencing technology in Sicilian patients with AD and in normal controls.

Material and methods

Patients

A total of 164 subjects, including 84 AD patients (41 males and 43 females; mean age: 75.9 ±7.8 years; age range: 56–95 years) and 80 normal subjects (37 males and 43 females; mean age: 77.3 ±8.5 years; age range: 56–95) were recruited at the IRCCS Associazione Oasi of Troina (Italy). The diagnosis of probable AD was made according to the recent National Institute of Aging (NIA) and Alzheimer's Association (AA) work group (NIA-AA) revision of the diagnostic criteria that enlarges the window for the detection of the disease to the early stages [11]. Symptoms of cognitive impairment or family history of dementia were exclusion criteria for the controls. Written informed consent was obtained from the participants or from their families. Patients and controls were all born in Sicily and were of European origin.

DNA extraction, polymerase chain reaction amplification and pyrosequencing

DNA was isolated from a lymphocyte-enriched fraction of whole blood. The procedures for detecting the single nucleotide polymorphism (SNP rs 20417) of the –765 G/C promoter variant in the PTGS2 gene were based on polymerase chain reaction (PCR) amplification and pyrosequencing technology (PyroMark ID instrument; Biotage, Uppsala, Sweden) as previously described [12]. Biotinylated PCR primer sequences for the amplification of PTGS2 promoter variant sites were selected according to the same previously published study [12]. Five µl of DNA were added to produce 50 µl of PCR solution mixture that contained 0.2 mmol of each dNTP, 1.5 mmol/l $MgCl_2$, 1X PCR buffer, 20 pmol of each primer and 1.5 units of DNA Taq polymerase. The PCR was performed with an initial denaturation for 5 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C and a final incubation for 10 min at 72°C. PCR products were resolved by agarose gel electrophoresis to confirm successful amplification. The biotinylated products were then immobilized to streptavidin-coated beads using a solution from a commercial PSQ TM 96 sample preparation kit (Biotage, Uppsala, Sweden). Beads (3 µl) were diluted in 15 µl of binding buffer with 10 µl of biotinylated PCR products, incubated for 10 min at room temperature and then transferred to a filter probe where the liquid was removed by vacuum filtration. DNA in the denaturation solution was separated, the templates were washed with washing buffer, transferred to a PSQ 96 SNP plate (Biotage, Uppsala, Sweden) and annealed with the sequencing primers described by Skarke *et al.* [12], in annealing buffer at room temperature. Finally, the samples were analyzed using a PyroMark ID System and the SNP reagent kit (both purchased from Biotage, Uppsala, Sweden).

Statistical analysis

Fisher's exact test was used for comparisons in 2 × 2 and 2 × 3 contingency tables. Statistical significance was accepted when the p value was lower than 0.05. The power of the study was calculated by the methods of Cohen [13]. The χ^2 test was use to determine whether there was a significant difference between the number of actual and expected genotypes in Hardy-Weinberg equilibrium.

Table I. Genotype frequencies of PTGS2 –765 G/C polymorphism in patients with Alzheimer's disease (AD) and controls

Genotype	Controls (%)	AD patients (%)	Value of *p*
GG	49 (61.2)	55 (65.5)	0.381
GC	24 (30.0)	26 (30.9)	
CC	7 (8.8)	3 (3.6)	
Total	80 (100)	84 (100)	

Table II. Allele frequencies of PTGS2 –765 G/C polymorphism in patients with Alzheimer's disease (AD) and controls

Allele	Controls (%)	AD patients (%)	Value of *p*
G	122 (76.2)	136 (80.9)	0.346
C	38 (23.8)	32 (19.1)	
Total	160 (100)	168 (100)	

Results

No statistically significant difference between the observed and expected values in Hardy-Weinberg equilibrium was observed ($p > 0.05$).

The distribution of PTGS2 –765 G/C showed no significant difference between patients with AD and controls. PTGS2 –765 G/C genotype distributions in the cases and controls are presented in Table I. In addition, no significant difference in the G and C allele frequency between AD patients and controls was observed ($p > 0.05$) (Table II). The power of the study is 28% calculated by the methods of Cohen [13].

Discussion

This preliminary study in Sicilian patients with AD showed that PTGS2 –765 G/C alleles and –765 C/C genotype seem not to be associated with the risk of developing AD. These data agree with the data obtained by other authors [8]. The role of PTGS2 in neurodegenerative diseases is very controversial, and conflicting data exist in the literature [7, 8, 14]. For instance, although some studies have demonstrated that the increase in PTGS2 activity greatly contributes to the progression of AD [7], other investigators have suggested an entirely opposite effect [8]. In this latter study, the C allele of PTGS2 –765 G/C promoter polymorphism was associated with decreased risk of AD, a finding which further supports the involvement of PTGS2 in AD etiology. Abdullah *et al.* reported that the C allele of COX-2 –765 promoter polymorphism is associated with decreased risk of AD in their sample of 168 AD cases (recruited from the Memory Disorder Clinics in Tampa and in Miami, Florida) [7]. In fact, the rare C allele is associated with approximately 30% less expression of COX-2 compared to the more commonly occurring G allele [15]. A study of Listi *et al.* suggests that the G allele of COX-2 –765 could be a risk factor for AD in a population from Northern Italy [16]. Fehèr *et al.* indicate that the COX-2 G/G genotype is associated with AD and support the involvement of COX-2 in AD etiology [17]. In addition, in the study of Tang *et al.*, no significant difference in the distributions of COX-2 –765G/C polymorphism was observed between AD cases and controls [18].

Our data do not seem to associate this polymorphism with AD, at least in the Sicilian population, but in light of the limited power of the study (28%) our conclusions should be evaluated with caution. In any case, further and broader studies are needed to confirm the role of PTGS2 –765 G/C polymorphism in AD. We plan to extend our preliminary analysis to a larger cohort of Sicilian patients with AD in the near future.

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