Article

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Study of association between genetic polymorphisms of phospholipase A2 enzymes and Alzheimer's disease

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ABSTRACT

Several genes have been related to late-onset Alzheimer's disease (LOAD). Phospholipases A2 (PLA2) influence the processing and secretion of the amyloid precursor protein, which gives rise to the beta-amyloid peptide, the major component of the amyloid plaque in AD. Hence, in the present study, polymorphisms of three genes encoding PLA2 enzymes group (cytosolic PLA2: *Ban*I *cPLA2* polymorphism; calcium-independent PLA2: *Avr*II *iPLA2* polymorphism; PAFAH: Val279Phe *PAFAH* polymorphism) were analysed in a case-control sample using 58 patients with LOAD and 107 matched healthy controls. There was a genotypic association between the *Ban*I *cPLA2* polymorphism and LOAD (χ^2=6.25, 2df, p=0.04), however there was no allelic association. There were no associations between *Avr*II *iPLA2* and Val279Phe *PAFAH* polymorphisms and LOAD. These data suggest that the *Ban*I *cPLA2* polymorphism may play a role in the susceptibility for LOAD in our Brazilian sample.
Key words: PLA2, dementia, gene, genetics, LOAD.

Associação entre polimorfismos das enzimas fosfolipases A2 e doença de Alzheimer

RESUMO

Vários genes têm sido investigados como fatores de risco para o desenvolvimento da doença de Alzheimer (DA) de início tardio. As fosfolipases A2 (PLA2) influenciam o processamento e secreção da proteína precursora do amilóide, que dá origem ao peptídeo meta-amilóide, o principal componente da placa amilóide na DA. Assim, no presente estudo, foram analisados três polimorfismos genéticos que codificam enzimas do grupo das PLA2 (PLA2 citosólica: polimorfismo *Ban*I *cPLA2*; PLA2 cálcio-independente: polimorfismo *Avr*II *iPLA2*; PAFAH: polimorfismo Val279Phe *PAFAH*) em 58 pacientes com DA de início tardio e 107 controles saudáveis pareados. Houve associação genotípica entre o polimorfismo *Ban*I *cPLA2* e DA de início tardio (χ^2=6,25, 2df, p=0,04); no entanto não foi observada associação alélica. Não houve associação entre os polimorfismos *Avr*II *iPLA2* e Val279Phe *PAFAH* com a doença. Tais dados sugerem que o polimorfismo *Ban*I *cPLA2* pode estar envolvido como fator de susceptibilidade para DA de início tardio em nossa amostra brasileira.
Palavras-chave: PLA2, demência, gene, genética, Alzheimer de início tardio.

Alzheimer's disease (DA) is a progressive neurodegenerative disease, being the most common form of dementia among the elderly[1]. Most AD cases are late-onset AD (LOAD), usually with an age of onset greater than 65 years. LOAD is a complex disease that results from multiple genetic and environmental factors. It is accepted that Apolipoprotein E (ApoE) allele 4 is a major genetic risk factor for LOAD[2-4]. However ApoE allele 4 may only account for about 50% of the susceptibility for LOAD which suggests that there may be additional genetic risk factors for LOAD[5].

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Several candidate genetic polymorphisms related to systems supposedly involved with AD pathophysiology have been implicated in the susceptibility for LOAD, but none has been consistently replicated[6,7]. Brain phospholipids are interesting sites for the interaction between genes and environment because enzymes and other proteins that regulate phospholipid metabolism are clearly genetically determined[8].

Phospholipases A2 (PLA2) are enzymes involved in several physiological processes, such as phospholipids metabolism, remodeling of cell membranes, and intracellular signaling. PLA2 has been related to the cleavage of membrane phosphatidylcoline and releasing of choline, the principal precursor for the synthesis of acethylcoline. Problematic functioning of the neuronal unsaturatted fatty acid metabolism by PLA2 may provoke alterations in the neuronal membrane structure and fluidity, modify intracellular signaling and neurotransmission[9]. Investigations with 31P-spectroscopy studies performed with AD patients have evidenced decreased membrane phospholipids turnover in temporoparietal cerebral areas[10], being these data in accordance with the findings of decreased PLA2 metabolites in the parietal cortex of postmortem AD brains[11]. Brain and platelet PLA2 activity reduced has also been described in patients with AD[12,13]. Decreased activity of PLA2 in the frontal and parietal cortex was associated to earlier onset of dementia, earlier age at death, and higher counts of neurofibrillary tangles and senile plaques[13]. *In vitro* study has shown that PLA2 are involved in the regulation of amyloid precursor protein[14]. Thus findings from experimental and clinical investigations have supported the hypothesis that alterations in membrane phospholipids metabolism, related to reduced PLA2 activity, may be involved in the pathogenesis of AD[15].

In face of the evidences for the involvement of the PLA2 system in the pathophysiology of LOAD, the genes encoding PLA2 enzymes could be considered as plausible candidate in the susceptibility for the disease. Thus, in the present study, we examined, in a case-control sample, polymorphisms in the genes codifying the following PLA_2 isoforms: the cytosolic phospholipase A2 (cPLA2), the calcium-independent phospholipase A2 (iPLA2) and the plasma platelet-activating factor acetylhydrolase (PAFAH)[16].

cPLA2, PLA2 group 4A, also known as PLA2G4A, is a large molecular weight protein, activated by calcium in the cytosol, and catalyzes the release of arachidonic acid from membrane phospholipids. cPLA2 gene (*cPLA2*) is located on chromosome 1q25. The *Ban*I *cPLA2* polymorphism (A/G polymorphism: A1 versus A2 allele), located near the first intron of *cPLA2*, will be investigated[17].

iPLA2, PLA2G6, catalyze hydrolysis of the sn-2 acyl-ester bonds in phospholipids, leading to the release of arachidonic acid and other fatty acids. PLA2G6 is a calcium-independent PLA2. iPLA2 gene (*iPLA2*) was mapped to chromosome 22q13.1 and contains 19 exons[18]. The *iPLA2 Avr*II polymorphism will also be investigated as a possible risk factor for LOAD.

PLA2 group 7, plasma platelet-activating factor acetylhydrolase, PLA2G7, is a secreted enzyme that catalyzes the degradation of platelet-activating factor to inactive products by hydrolysis of the acetyl group at the sn-2 position, producing the biologically inactive products LYSO-PAF and acetate. Stafforini et al.[19] mapped the PLA2G7 gene (*PAFAH*) to chromosome 6p21.1-p12 and found that it contains 12 exons. Deficiency of plasma platelet-activating factor resulting from a missense mutation (Val279-Phe) in exon 9 of the gene has been described.

Val279Phe *PAFAH* polymorphism will be investigated in the present study[19].

METHOD

Sample

Fifty-eight (n=58) unrelated Brazilian patients with LOAD, diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Alzheimer's criteria[20], were recruited from the Institute of Psychiatry of São Paulo University Medical School (31.03% males and 68.96% females; with mean age of 73.56 years 6.60 SD±). A total of 107 matched control subjects (31.77% males and 68.22% females; with mean age of 72.18 years 5.03 SD±) were recruited from the general population in São Paulo. The study was approved by the Ethical Committee of São Paulo University Medical School. The patients or their relatives signed an informed consent form and venous blood samples were collected.

Genotyping

Genomic DNA samples were extracted from lymphocytes. The genetic variants studied are described below:

***cPLA2 – Ban*I polymorphic site**

PCR amplification for this dimorphic site, described by Peet et al. (1998)[21], was performed using the primers: 5'- AAGGGATATTTGTAGAGGACT-3' and 5'-TAGATGATTCGATTTTATGACT-3' and the following conditions: 35 cycles with an annealing temperature of 50.5ºC. The 788 bp product was digested with *BanI* and the alleles separated in 1% agarose gel. The A1 allele was not digested with the enzyme, and A2 allele was digested in two fragments of 452 and 336 base pairs (bp).

***iPLA2 – Avr*II polymorphic site**

PCR amplification for this single nucleotide polymorphism (SNP) C/T that alters the restriction site of *Avr*II on the iPLA2 gene[22], was performed using the primers: 5´ GGGGTTTATTTTGCTGGGTT 3´ (forward) and 5´

CAAGGGTGAT GGGGAGATC 3´(backward). The conditions for thermal cycling were: an initial cycle 94ºC for 5 min followed by 24 cycles each of 93ºC for 30s, 60ºC for 30s and 72ºC for 30s and a final step of 72ºC for 5 min. The 380-bp products were digested with *Avr II* with the T allele uncut, and the C allele cut into 182 bp and 198 bp fragments.

***PAFAH* – Val279Phe variant**

PCR amplification for this variant, described by Stafforini et al. (1996)[19], was performed with primers sequences 5'- CTATAAATTTATATCATGCTT-3' 5'- TTTACTATTCTCTTGCTTTAC-3'. The conditions for thermal cycling consisted of denaturation at 94ºC for 5 min followed by 5 cycles at 94ºC (1 min), 56ºC (1 min), 72ºC (1 mim) and 25 cycles at 94ºC (30 sec), 52ºC (30 sec), 72ºC (30 sec) before a final extension step at 72ºC for 7 min. The 177-bp product was digested with *Mae*II to yield 95 bp and 82 bp fragments. The alleles were separated on 10% polyacrylamide gels.

Statistical analysis

Chi-squared test was performed to compare overall allele and genotype frequencies of the polymorphism between cases and controls using the statistical software EpiInfo 2000, version 1.1.2. The Hardy-Weinberg equilibrium test was performed by the HWE program.

RESULTS

cPLA2

The genotypic and allelic distribution of the dimorphic site for the restriction enzyme *Ban*I are shown on the Table. There was a statistic significant difference in genotypic frequency between LOAD patients and control subjects (χ^2=6.25, 2df, p=0.04). However there was no allelic association (χ^2=2.62, 1df, p=0.10). There was no homozigosity association as well (χ^2=2.48, 1df, p=0.11, OR=1.68, 0.84<OR<3.37). Allele frequencies and genotypic distribution of the polymorphisms were in Hardy-Weinberg equilibrium (patients: p=0.06; controls: p=0.40).

iPLA2

The genotypic and allelic distribution of the dimorphic site of the restriction enzyme *Avr*II in the iPLA2 gene are shown on the Table. There was no significant difference in genotypic (χ^2=3.74, 2df, p=0.15) and allelic (χ^2=1.03, 1df, p=0.30) frequencies between LOAD patients and controls. There was no association with homozigosity (χ^2=2.79, 1df, p=0.09). Allele frequencies and genotypic distribution of the polymorphisms were in Hardy-Weinberg equilibrium (patients: p=0.10; controls: p=0.52).

PAFAH

The Phe mutation in the *PAFAH* occurred in a low frequency in our sample. Only one heterozygous individual was detected. The homozygous genotype (Phe/Phe) was not present in our sample. Therefore the present polymorphism is not likely to be associated with LOAD. Allele frequencies and genotypic distribution of the polymorphisms were in Hardy-Weinberg equilibrium (patients: p=0.94; controls: p=1.00)

DISCUSSION

LOAD etiology is likely to be complex, involving multiple genes in addition to environmental effects and their interactions. Trying to bring light on the findings of an abnormal PLA2 activity in the AD, we examined the association of three genetic polymorphisms encoding PLA2 isoforms and LOAD. We are not aware of any previous report of an association study between these polymorphisms and LOAD to date.

Table. Distribution of genotypes and alleles of cPLA2, iPLA2 and PAFAH genes variants among patients with LOAD and healthy controls.

Locus	Genotypes/alleles	Patients (%)	Controls (%)	p value
CPLA2	A1/A1	5 (8.6)	26 (24.2)	**0.043**
	A1/A2	34 (58.6)	49 (45.7)	
	A2/A2	19 (32.7)	32 (29.9)	
	A1	44 (37.9)	101 (47.1)	0.10
	A2	72 (62.0)	113 (52.8)	
IPLA2	T/T	13 (22.4)	25 (23.3)	0.15
	T/C	35 (60.3)	50 (46.7)	
	C/C	10 (17.2)	32 (29.9)	
	T	61 (52.5)	100 (46.7)	0.30
	C	55 (47.4)	114 (53.2)	

Regarding the *Ban*I polymorphism there was a genotypic association with LOAD. Our findings suggest that the alelle A1 may be a genetic factor of protection to AD in order of the important difference in the distribution of the A1 and A2 alleles between patients and controls and the substantial underrepresentation of the homozygous A1/A1 on AD group.

Functional significance of the *Ban*I *cPLA2* polymorphism has been investigated. Barbosa et al.[8] found that cPLA2 activity in platelets was significantly higher in schizophrenic patients with the A2A2 genotype. Therefore our results with AD patients were in the opposite direction of this functional study of the *Ban*I *cPLA2* polymorphism. Such phenomenon may be related to ethnical population stratification. So it could be premature to assess the validity of the association of LOAD and the investigated polymorphism because it may be in tight linkage with another polymorphism which could influence the risk for the disease. If the *Ban*I *cPLA2* polymorphism is tightly linked with a polymorphism of risk for LOAD, different patterns of linkage disequilibrium may exist between different ethnical samples. Thus a specific allele could be linked to the risk-conferring allele in some populations, but could be linked with the non-risk allele in others[23,24].

Interestingly, *cPLA2* is located on chromosome 1q25, immediately adjacent to the region 1q23 where Blacker et al.[25] reported suggestive linkage with LOAD. This region is also close to the gene encoding nicastrin, which binds presenilin and is required for γ-secretase activity and beta-amyloid generation[26].

The restriction polymorphism by *Avr*II in the $iPLA_2$ gene did not show allelic or genotypic association with LOAD. In addition to the obvious explanation that this polymorphism has no influence on LOAD susceptibility, the study may be limited by the size of the sample. In polygenic and multifactorial model of the etiopathology for LOAD, it has been hypothesized that there may be several additive genes each one of them with low weight for the final product (the phenotype). Thus, the chance of detecting these putative genes may be reduced in relatively small samples and negative findings in this case do not mean necessarily lack of association.

We also investigated if there was association between homozygosity and LOAD. There are some reports showing association of homozygosity of other genetic polymorphisms and some neuropsychiatric disorders. This type of association may represent a heterozygosity advantage, probably because the presence of two different molecular forms of the neurotransmitter receptor, resulting in an increased ability to respond adaptively to variations in the environment[27]. However, in our sample there was not found such association as well.

Finally, we also investigated a point mutation in the exon 9 of PAFAH gene, which alter the enzymatic activity[19]. However, in our Brazilian case-control sample this mutation was very rare, thus we consider unlikely that this variant influences the risk to LOAD in our population.

We are aware of other methodological weakness in our investigation. Population stratification is the most common reason for conflict results in genetic studies[28,29]. Investigations comparing different ethnical populations have shown significant variations in the allelic frequency. Power to detect association may be reduced in ethnic admixture samples[29]. In order to face such a problem we can try to identify the ethnical origin of the sample and try to compound homogenous samples. However, in Brazil, physical characteristics such as skin pigmentation, hair colour and texture, shape of the nose and lips, are poor predictors of genomic ancestry[30]. Moreover the fact that the present sample is in Hardy-Weinberg equilibrium may indicate that our sample may not have important problems of population stratification[29].

In conclusion, in our sample we were able to find an association between LOAD and *Ban*I *cPLA2* polymorphism, indicating that this polymorphism may play a role in the susceptibility for LOAD in our Brazilian sample. However more comprehensive polymorphism coverage within the *cPLA2* and *iPLA2* investigated is warranted in larger samples. Investigations in samples with different ethnic background must be carried out as well.

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