Neuroscience & Medicine, **2011, 2, 226-231**
doi:10.4236/nm.2011.23031 Published Online September 2011 (http://www.SciRP.org/journal/nm)

PKR and PP1C Polymorphisms in Alzheimer's Disease Risk

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Received June 30th, 2011; revised July 30th, 2011; accepted August 26th, 2011.

ABSTRACT

Alzheimer's disease (*AD*) *is a neurodegenerative disease characterized by senile plaques and neurofibrillary tangles. Senile plaques are deposits of amyloid ß-peptide* (*Aß*) *produced by the cleavage of a transmembrane protein termed Amyloid Precursor Protein* (*APP*). *The amyloidogenic cleavage of APP is performed by γ-secretase complex and ß-site APP cleaving enzyme* 1 (*BACE*1), *a key enzyme in AD that can be activated by different noxious stimuli. Interestingly, some viruses could activate double-stranded RNA-activated protein kinase* (*PKR*), *which phosphorylates Eukaryotic Initiation Factor* 2 *alpha* (*eIF*2*α*). *This phosphorylation stops global translation to avoid any synthesis of viral infective proteins, but paradoxically up-regulates BACE1 translation. One of the viral mechanisms to circumvent eIF*2*α phosphorylation is the recruitment of protein phosphatase* 1 (*PP*1), *to fully dephosphorylate eIF*2*α and allow viral protein synthesis. Due to the functional relationship between BACE*1, *PKR, PP1 and AD we have performed a large* (1122 *cases and* 1191 *control individuals*) *case-control genetic analysis using two biallelic polymorphisms rs*2254958 *and rs*7480390, *located within the genes coding for PKR and the catalytic unit A of PP1, respectively. Although a trend to association of the rs*2254958 *TT genotype with AD risk was found, our results show that neither rs*7480390 *nor rs*2254958 *are associated with AD susceptibility.*

Keywords: *Alzheimer's Disease, BACE1, PKR, PP*1, *eIF*2*α*

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by intracellular neurofibrillary tangles [1] and extracellular amyloid *ß*-peptide (A*ß*) aggregates [2-4]. A*ß* is released from the amyloid precursor protein (APP) when it is processed by the beta-site APP cleaving enzyme 1 (BACE1) and gamma-secretase [5-7]. Mutations in APP or presenilins (PS; part of the gamma secretase complex) trigger an inherited form of the disease, known as familiar AD (FAD) at early ages [8-10]. However, FAD accounts for less than 3% of AD cases [11], whereas sporadic AD accounts for most AD cases [12,13]. Its aetiology is unclear but the *APOE-ε*4 allele is considered the major risk factor for sporadic AD [14] and some noxious agents such as the neurotropic Herpes simplex Virus Type-1 (HSV1) have been also involved in sporadic AD onset [15-17].

Double-stranded RNA-activated protein kinase (PKR) activation has been recently associated with AD [17-20]. PKR, encoded by the *EIF*2*AK*2 gene, is an anti-viral mechanism used by the cell to prevent viral protein synthesis. When a virus infects eukaryotic cells, dsRNA originated from the viral genome activates PKR, which in turn phosphorylates Eukaryotic Initiation Factor 2 alpha (eIF2*α*) shutting down global protein synthesis [21], and increasing BACE1 protein synthesis as a side effect [17,22]. A mechanism used by HSV1 to avoid the PKR-defensive shutdown of protein synthesis is the de-phosphorylation of eIF2*α*, which is carried out by the catalytic subunit of protein phosphatase 1 (encoded by the *PPP*1*CA* gene) [23].

We hypothesize that the genetic variability within the PKR/eIF2*α* pathway could have a role in AD susceptibility. Therefore, in the present work we study two genetic markers, rs2254958 and rs7480390 within the *EIF*2*AK*2 and *PPP*1*CA* genes, to assess if they could be associated with AD.

2. Materials and Methods

2.1. AD and Control Individual Selection

Blood samples were collected from consecutive AD patients in medical centers in Barcelona (Fundació ACE-Institut Català de Neurociències Aplicades), Madrid (Hospital Universitario La Paz-Cantoblanco) and Murcia (Unidad de Demencias, Hospital Virgen de la Arrixaca and Fundación Alzheimur). The referral centers' ethics committees and Neocodex have approved this research protocol, which was in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association. Written informed consents were obtained from all individuals included in this study.

This analysis comprised 1122 unrelated sporadic AD patients (mean age at diagnosis ± SD: 77.55 ± 7.73 years old) and 1191 unrelated controls (52.14 ± 11.95). Control subjects were recruited from the general population. All AD patients fulfilled DSM-IV criteria for dementia and were diagnosed according to the NINCDS-ADRDA criteria for possible and probable AD [24]. All patients received a thorough clinical and neurological examination and a comprehensive neuropsychological evaluation including tests for general cognition, memory, language, perceptual and constructional abilities and executive functions. Complete blood analysis and neuroimaging studies were performed in all subjects to exclude other potential causes of dementia. Following the guidelines for the diagnosis of AD from the Study group on Behavioral Neurology and Dementia of the Spanish Neurological Society, AD patients were consecutively recruited at the four participating centers. To avoid population stratification problems, all individuals enrolled in this study were white Mediterranean with registered Spanish ancestors (two generations), as recorded by clinical researchers.

2.2. DNA Extraction Procedures

We obtained 5 mL of peripheral blood from all individuals to isolate genomic DNA from leukocytes. DNA extraction was performed in a MagNa Pure LC Instrument (Roche, Basel, Switzerland), using MagNa Pure LC DNA Isolation kit (Roche) in accordance with the manufacturer's instructions.

2.3. Genetic Association Study

Genotypes for *EIF*2*AK*2 rs2254958 polymorphism, located at the 5'-UTR region within a putative exonic splicing enhancer, were obtained using Real-time PCR coupled to Fluorescense Resonance Energy Transfer (FRET) probes. Primers and probes employed for this genotyping protocol are summarized in **Table 1**. The technique was performed in the LightCycler® 480 System (Roche Diagnostics). Briefly, PCR was carried out in a final volume of 20 µL using 10 ng of genomic DNA, 0.1 µM of forward amplification primer, 0.25 µM of reverse amplification primer, 0.1 µM each detection probe, and 4 µL of LC480 Genotyping Master (5X, Roche Diagnostics, Germany). We used an initial denaturation step of 95˚C for 5 min, followed by 50 cycles of 95˚C for 30 sec, 62˚C for 30 sec, and 72˚C for 30 sec. For melting curve analyses, after an initial denaturation at 95˚C for 2 minutes at a ramp rate of 4.4˚C/second, the samples were incubated at 64˚C for 30 sec and 40˚C for 30 sec at a ramp rate of 2.2˚C/sec, and finally taken to 80˚C with one acquisition per ˚C. Genotypes for *PPP1CA* rs7480390 polymorphism, located in a putative promoter region, were obtained using Real-time PCR coupled to FRET probes. Briefly, PCR was carried out in a final volume of 20 µL using 10 ng of genomic DNA, 0.1 µM of forward amplification primer, 0.25 µM of reverse amplification primer, 0.1 µM each detection probe, and 4 µL of LC480 Genotyping Master (5X, Roche Diagnostics, Germany). We used an initial denaturation step of 95˚C for 5 min, followed by 50 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. For melting curve analyses, after an initial denaturation at 95˚C for 2 minutes at a ramp rate of 4.4˚C/second, the samples were incubated at 40˚C for 30 sec at a ramp rate of 2.2˚C/sec, and finally taken to 80˚C with one acquisition per ˚C.

Table 1. Amplification primers and FRET probes employed in the genotyping protocols.

rs2554958
Primers
Forward 5'-CCACAGGCACGACAAGCATA-3'
Reverse 5'-CGAGTGATACCAGCGAAGACTAAG-3'
FRET probes
Sensor 5'-GGCATCGAGGTCCATCCC[Flc]-3'
Anchor5'-[Cy5]TCAGGAGACCCTGGCTATCATAG[Phos]-3'
rs7480390
Primers
Forward 5'-CGGAAGGACTGTGGAAGTTG-3'
Reverse 5'-GTTAACCCTCAGTGCCTAGC-3'
FRET probes
Sensor 5'-AAAGCCCAACAGACTTTCC[Flc]-3'
Anchor 5'-[Cy5]CCCCTTCCCTGGGTGGAAGGTG[Phos]-3'

Cy5:Cy5 fluorochrome; Flc: Fluorescein fluorochrome; Phos: Phosphothionate.

*APOE-ε*4 genotyping protocol has been previously described [25].

2.4. Statistics

We explored the polymorphisms association with AD phenotype using different tests adapted from Sasieni based on chi-squared test (http://ihg2.helmholtz-muenchen. de/). SPSS statistical package was used to develop binary logistic regression models adjusting for age, gender and *APOE* dosage.

3. Results

3.1. *EIF*2*AK*2 rs2254958 Polymorphism and AD Susceptibility

A bi-allelic polymorphism (rs2254958) located in a highly conserved SRp55 binding enhancer element of the *EIF*2*AK*2 gene (which encodes PKR protein) has been previously associated with age of AD onset and susceptibility to AD [20]. We carried out an independent case-control genetic association study, and we found that rs2254958 allele and genotype frequencies were not statistically different between AD patients and controls (**Table 2**). In order to address whether the *APOE-ε*4 allele could be a confounding factor in our analyses, we used the Odds ratio homogeneity test but, in contrast to Bullido *et al.* 2008 [20], we did not detect any interaction between *APOE-ε*4 and rs2254958 (p = 0.580). However, we observed a trend towards association of the rs2254958 TT genotype (recessive model) with AD susceptibility when we carried out a binary logistic regression analysis adjusting for gender and *APOE-ε*4 allele (**Table 3**). Finally, we performed a Cox regression analysis to test the effect of the rs2254958 TT genotype on the age of AD onset, adjusting for *APOE-ε*4 allele and gender, but no statistically significant results were obtained (data not shown).

Table 2. Genotype frequency distribution.

rs2254958					
	MAF	CC	CT	TT	HWE (p value)
Controls	0.46	335 (0.30)	550 (0.49)	237 (0.21)	0.31
AD cases	0.45	356 (0.30)	606 (0.51)	229 (0.19)	0.69
rs7480390					
	MAF	CC	CG	GG	HWE (p value)
Controls	0.08	698 (0.86)	109 (0.13)	7 (0.01)	0.24
AD cases	0.08	786 (0.86)	122 (0.13)	7 (0.01)	0.34

Genotype frequencies are indicated in parenthesis. MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium.

Table 3. Binary logistic regression analyses adjusting for gender and *APOE-ε*4 allele.

rs2254958			
	OR	95% CI	p value
gender	1.93	1.64 - 2.34	<0.001
*APOE-ε*4	3.21	2.65 - 3.88	<0.001
(CC + CT vs TT)	1.24	1.00 - 1.53	0.05
rs7480390			
	OR	95% CI	p value
gender	3.55	2.83 - 4.47	<0.001
*APOE-ε*4	2.45	2.00 - 3.02	<0.001
(CC+CG vs GG)	1.03	0.77 - 1.38	0.85

3.2. *PPP*1*CA* rs7480390 Polymorphism and AD Susceptibility

We found that the allele and genotype frequencies of rs7480390 were not statistically different between AD patients and controls (**Table 2**). In order to address whether the *APOE-ε*4 allele could be a confusing factor in our analyses, we used the Odds ratio homogeneity test. We did not detect any interaction between *APOE-ε*4 and rs7480390 (p = 0.785) even when a binary logistic regression analysis adjusting by gender and *APOE-ε*4 allele was calculated (**Table 3**).

4. Discussion

The causes of sporadic AD are still unknown. Several polymorphism in different genes have been reported to increase AD risk such as *APOE-ε*4 allele [26-28] or neprilysin [29], a protein that degrades A*β*. However the relationship of these proteins with the onset of the disease is unclear. In the present work we focused in the genetic study of two intracellular proteins (PKR and PP1) that can influence the expression levels of BACE1, the rate-limiting enzyme in the production of A*β*. PKR is activated by double stranded RNA from viral origin and phosphorylates eIF2*α* in serine 51. The phosphorylation of this factor allows BACE1 translation from its basally dormant mRNA [17]. On the other hand, PP1 is recruited by the HSV1 protein ICP34.5 to de-phosphorylate eIF2*α* in an attempt to circumvent the PKR/eIF2*α* defensive mechanism and allow global protein synthesis (together with viral protein synthesis) to go on in the host cell.

Paradoxically, under these circumstances BACE1 translation from its mRNA remains repressed as in basal conditions.

Most humans are infected by HSV1 [30,31]. eIF2*α* phosphorylation after HSV1 infection might be an important event in sporadic AD aetiology [32,33], as phosphorylated eIF2*α* enables BACE1 translation promoting amyloidogenic pathway [22] (**Figure 1**). Interestingly PKR could be also activated by other stimuli than dsRNA viruses such as siRNA (reviewed in [34]) or oxidative stress [35]. It would be also possible the existence of a leak in eIF2*α* phosphorylation due to a dysregulation of PKR or PP1 activities. Therefore, uncovering the genetic variability underlying eIF2*α* phosphorylation after PKR activation and eIF2*α* de-phosphorylation after PP1 activation is critical to understand the role of this pathway in the aetiology of sporadic AD pathology. In fact, a genetic association between rs2254958 polymorphism within *EIF2AK2* gene with the risk of AD has been found [20]. In our study we found a trend to association of the same marker with AD susceptibility in a recessive model. However our results show that the at-risk allele (T allele) described herein is the opposite (C allele) to that reported by Bullido and coworkers, and according to the original study, the effect of the rs2254958 occurs even in the absence of *APOE-ε*4 allele whereas we did not detect any interaction among these variants. Contrary to the reported results, we did not observe any influence of the rs2254958 variant over the age of AD onset. These discrepancies between the two studies could be due to differences in sample size, mean age of AD onset in patients and characteristics of the control group.

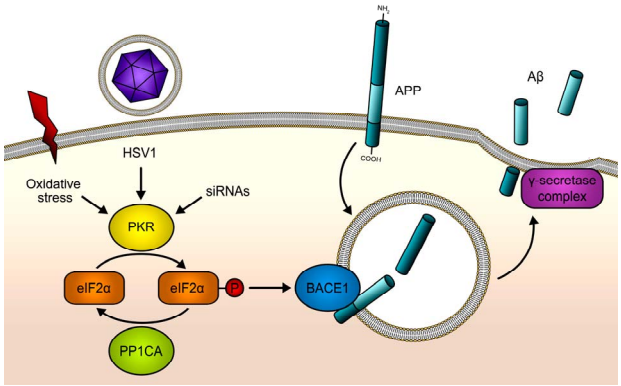

Figure 1. PKR/eIF2*α* pathway in A*ß* production. Double stranded RNA (dsRNA) from HSV1 or other viral origins is sensed by PKR, which becomes activated after the binding of the dsRNA, by oxidative stress action or by the binding of siRNAs. As a consequence eIF2*α* is phosphorylated at Serine 51 by PKR. This phosphorylation will shut down global protein synthesis at the level of translation initiation in a defensive mechanism when PKR is activated by viruses. HSV1 is able to evade this anti-viral PKR pathway through the recruitment of PP1CA, an eIF2*α* phosphatase. BACE1 and other genes with specific features in the 5'UTR of its mature mRNAs respond *inversely* to the phosphorylation of eIF2*α*: their translation is *activated* when eIF2*α* is phosphorylated at Serine 51. Therefore, the activation of the PKR/eIF2*α* pathway will lead to BACE1 up-regulation, promoting the *ß*-cleavage of APP, which followed by the *γ*-cleavage will increase Aß production.

We did not detect any association of the *PPP1CA* rs7480390 polymorphism with AD, therefore we discarded a relevant role of this polymorphism in AD pathology.

In spite of these results, we cannot discard that other polymorphisms within *EIF2AK2*, *PPP1CA* or other genes involved in the PKR/eIF2*α* pathway could be related to AD ethiology. Further studies would be necessary to elucidate the real relevance of this pathway and its interaction with other environmental factors in AD susceptibility.

5. Acknowledgements

This work was supported by the Spanish Ministerio de Ciencia y Tecnología (FIS: PI07/0593 and PI10/00587; ISCIII-RETIC RED HERACLES RD06/0009/002-FEDER). The authors wish to thank to all the individuals and technicians who participated in this work. The authors thank I. Hernández, A. Mauleon, M. Rosende-Roca, P. Martínez-Lage, M. Alegret, S. Ruíz and L. Tárraga (Memory Clinic of Fundació ACE & Institut Català de Neurociències Aplicades; Barcelona, Spain) for their technical support; to the personnel of Unidad de Demencias, Hospital Universitario Virgen de la Arrixaca (Murcia, Spain); and Memory Unit of University Hospital La Paz-Cantoblanco (Madrid, Spain) for their continued effort to support this project.

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