Journal of
Alzheimer's Disease & Parkinsonism

Romero et al., J Alzheimers Dis Parkinsonism 2018, 8:2
DOI: 10.4172/2161-0460.1000436

Research Article Open Access

Role of the TLR4 rs4986791 Polymorphism in the Development of Late-onset Alzheimer Disease and its Relationship with APOE*4

Romero R, Salazar Alcalá E, Cedeño N and Fernández-Mestre M*

Laboratorio de Fisiopatología, Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela

Abstract

Objective: Based on that the association of the ε4 allele of the APOE gene with the development of late-onset AD is one of the strongest and that TLR4 has been involved in AD pathogenesis. The aim of the present work was to study the role of rs4986791 polymorphism of the TLR4 gene in the development of AD and correlate any such association with the presence of allele ε4 of the APOE gene.

Methods: We included 161 unrelated Venezuelan subjects classified as either AD patients (n=61) or healthy individuals (n=100). Polymorphisms of TLR4 and APOE genes were identified with PCR-SSP and PCR-RFLP, respectively.

Results: The rs4986791 polymorphism does not appear to be related to AD, although the presence of the CC genotype and the C allele apparently confers three times higher risk of developing AD. Finally, positive and negative associations among the combinations TLR4 /APOE genes and AD were observed.

Conclusion: The results suggest the absence of any association between rs4986791 polymorphism of TLR4 gene and susceptibility to AD and the association of the ε4 allele of the APOE gene with the development of this pathology was confirmed.

Keywords: Neurodegenerative disorder; Aged tissues; Chronic-inflammation

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, whose histopathological characteristics are the extracellular accumulation of aggregated amyloid β peptide (Aβ) and the intracellular accumulation of neurofibrillary tangles [1]. Although the mechanisms responsible for the manifestation of the disease have not been elucidated, a consensus recently emerged that neuroinflammation plays an important role. In the central nervous system, the mechanisms of inflammation are mainly carried out by astrocyte and microglial cells activation, resulting in the production of neurotoxic mediators such as reactive oxygen and nitrogen species, proteolytic enzymes, glutamate, complement factors or inflammatory cytokines [2]. Although microglial activation by aggregated amyloid β peptide (Aβ) may possess a neuroprotective role by clearing Aβ via increased phagocytosis and proteolytic degradation, it may also contribute to progressive neurodegeneration [3]. In addition, most aged tissues including the brain are characterized by low-level chronic inflammation. However, aged microglias develop an increased pro-inflammatory phenotype characterized by an exaggerated inflammatory response to external and/or internal stimuli [4]. Many factors have been implicated in microglia activation that drives them to sites of Aβ deposition. Microglial cells use a series of receptors that recognize characteristic motifs common in certain pathogens or molecules associated with cell damage [5].

A specific group of these receptors include the Toll-like receptors (TLRs). Toll like receptors belong to I transmembrane receptor family involved in the initial recognition of pathogens through specific pathogen-associated molecular patterns (PAMP) receptors. The activation of TLRs triggers different signaling pathways, leading to the production of proinflammatory cytokines, reactive oxygen and nitrogen species [6]. Microglias express TLRs 1 to 9 and most of these receptors have been associated with its activation and neurotoxicity [2]. A characteristic in normal aging and in AD patients is an increase in innate immune receptors expression in the brain, such as TLRs. Particularly, high levels of CD14 (co-receptor for TLR4) in parenchymal microglia of the frontal and occipital cortex, hippocampus, and around the senile plaques have been observed in brain of AD patients [7]. Furthermore, monocytes derived from elderly individuals show defective TLR signaling and decreases in relative TLR1 and TLR4 expression [8]. TLR2 and TLR4 have been implicated in AD pathogenesis. A functional deficiency of either receptors results in an increased deposits of Aβ and reduced phagocytic clearance [5]. Specifically, TLR4 has been associated with microglia activation, neurotoxicity and Aβ signaling. The investigations suggest that TLR4 play a dual role in the pathogenesis of AD. On the one hand, the TLRs are neuroprotective due to their contribution in the clearance of amyloid β. On the other hand, the inflammatory responses triggered by TLR4, through the recognition of β-amyloid, can lead to neurotoxic effects [2].

The neurotoxicity associated with microglial activation results from the production of neurotoxic mediators, such as reactive oxygen and nitrogen species, proteolytic enzymes, glutamate, complement factors or pro-inflammatory cytokines. These products lead to neuronal death as has been demonstrated in murine models and may contribute to chronic neurodegenerative conditions in AD [9]. Therefore, blocking TLR4 would inhibit the activation of microglia, reducing the production of cytokines, but it would impair the clearance of Aβ by increasing its deposition. Alternatively, the induction of TLR4, with an agonist of lower toxicity than LPS, would increase the clearance of Aβ with a reduced production of cytokines [2]. Several polymorphisms of a single nucleotide have been identified in the TLR4 gene, generating two of them a non-synonymous

***Corresponding author:** Mercedes Fernández-Mestre, Laboratorio de Fisiopatología. Centro de Medicina Experimental. Instituto Venezolano de Investigaciones Científicas. Altos de Pipe, Apartado 21827. Zona Postal 1020-A. Caracas, Venezuela. Tel: 58 212 5041155; Fax: 58 212 5041086; E-mail: mfernandezmestre@gmail.com

Received March 09, 2018; **Accepted** April 05, 2018; **Published** April 14, 2018

Citation: Romero R, Salazar Alcalá E, Cedeño N, Fernández-Mestre M (2018) Role of the TLR4 rs4986791 Polymorphism in the Development of Late-onset Alzheimer Disease and its Relationship with APOE*4. J Alzheimers Dis Parkinsonism 8: 436. doi: 10.4172/2161-0460.1000436

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change in the amino acid sequence: +896A/G (rs4986790) and +1196C/T (rs4986791). These polymorphisms have been linked with susceptibility to various disease associated with age, because the Toll-Like Receptor 4 with these polymorphisms have lower affinity for their ligands [10]. Since the Aβ activates microglia through toll like receptor 4 (TLR4), the purpose of the present work was to assess whether polymorphism *rs4986791*of the TLR4 gene is associated with the development of AD, and correlates with the presence of allele ε4 of the *APOE* gene.

Materials and Methods

Patients

The study was carried out in 61 patients (mean age, 70 ± 10 years), all of whom had been diagnosed with sporadic AD. All patients had received treatment in the Neurology Department at Hospital Clínico Universitario de Caracas (Venezuela) between September 2004 and October 2006. These patients were selected according to the clinical protocol implemented in the Luis Borges Neuropsychology Unit of the Neurology Department at Hospital Clínico Universitario de Caracas. The protocol was framed in accordance with the specifications of the American Psychiatric Association (DSM-IV) and the NINCDS-ADRDA Alzheimer's Criteria (National Institute of Neurological Disorders, Communicative Disorders, and Stroke; Alzheimer's disease and Related Disorders Association). The control group consisted of 100 healthy Venezuelan residents with a mean age of 71 ± 10 years. These subjects completed the Mini-Mental State Examination and laboratory and imaging studies. All participants in the study gave their informed consent; the consent of participants with AD was authorized by their legal guardians. The consent procedure was approved by the bioethics committees at Instituto Venezolano de Investigaciones Científicas and Hospital Clínico Universitario de Caracas.

Genomic DNA extraction

Genomic DNA was extracted from leukocytes and lymphocytes of peripheral blood according to Bunce's method [11]. This method uses chloroform and high salt concentrations to remove proteins.

Genotyping of the *TLR4* gene

The rs4986791 polymorphism was carried out with sequence specific primer-polymerase chain reaction (SSP-PCR), using the initiators and protocol described by Smit et al. [12]. Primers of the blood group ABO gene were used as an internal positive control for PCR amplification [13]. With the SSP-PCR method, two reaction mixtures are prepared, one for the ancestral or wild allele and the other for the mutated or infrequent allele. Each reaction contained 1X PCR Buffer, 0.4 μM each of TLR4-specific primers (ancestral allele or mutated allele and reverse primer), 0.1 μM of each primer ABO, 2.5 mM KCl, 0.4 mM Tris-HCl-NH4, 0.2 mM dNTPs, 1 mM MgCl2, 0.02 U/μL de Taq polymerase Platinum (Invitrogen), 0.02 μg/μL of Genomic DNA and water to a final volume of 10μL. The following conditions were used in the amplification: 96°C for 1 min, 4 cycles of 96°C for 25 s, 70°C for 45 s and 75°C for 25 s; 20 cycles of 96°C for 25 s, 65°C for 1 min and 72°C for 30 s; 3 cycles of 96°C for 30 s, 55°C for 1 min and 75°C for 90 s. At the end, the samples were incubated for 10 min at 22°C. The absence or presence of PCR products was visualized by electrophoresis in 1.5% agarose gels treated with ethidium bromide.

Genotyping of the *APOE* gene

The rs429358 and rs7412 polymorphisms were genotyped using PCR-RFLP (Restriction Fragment Length Polymorphism) with the initiators described by Emi et al. [14]. Amplicons were digested with the enzyme Hha I using conditions described by Hixson and Vernier [15] and the manufacturer's protocol (GIBCO, BRL). The fragments were visualized by electrophoresis in 4% agarose gels treated with ethidium bromide.

Statistical analysis

Genotypic and allelic frequencies were calculated. The Hardy-Weinberg equilibrium was calculated with a chi-squared test. The statistical significance of intergroup differences in frequency (for alleles, genotypes, and genotypic combinations) was estimated using the Mantel-Haenszel chi-square statistic and 2 × 2 contingency tables; p values were corrected (pc, as the acronym of corrected p value, in Spanish) multiplying by the number of comparisons made (Bonferroni correction), and were considered significant when $p<0.05$ [16]. Relative risk with corresponding 95% confidence intervals (95% CI) were calculated as odds ratios (OR) according to Woolf's formula [17].

Results

Allelic and genotypic frequencies of the TLR4 gene in healthy individuals and patients with Alzheimer disease

The allelic and genotypic frequencies of the rs4986791 polymorphism showed Hardy-Weinberg equilibrium in the control cohort. In both groups, the CC genotype had the highest frequency, followed by CT. No difference in the distribution of the genotype and allele frequencies among controls and AD patients was found. However, the presence of the CC genotype (OR: 3.56, 95% CI: 0.7631-16.6627) and the C allele (OR: 3.42, 95% CI: 0.7451-15.6931) apparently confers three times higher risk of developing AD (Table 1). The results contrast

	AD (n=61)	Control (n=100)	OR 95% CI	*P*
Genotype				
CC	97 (59)	89.2 (89)	3.56 (0.7631-16.6627)	ns
CT	3 (2)	10.8 (11)	0.28 (0.06-1.3103)	ns
Alelles				
C	98.4 (120)	94.6 (193)	3.42 (0.7451-15.6931)	ns
T	1.6 (2)	5.4 (11)	0.29 (0.0637-1.3419)	ns

AD: Alzheimer Disease; 95% CI: Confidence Interval; ns: Not Significant; OR: Odds Ratio
Values shown in parentheses indicate the number of repeats of the allele or the number of individuals bearing the genotype for the polymorphic site under study. Frequencies are expressed as percentages

Table 1: Allelic and genotypic frequency of the rs4986791polymorphism of the *TLR4* gene in AD patients and controls.

with those previously reported in the literature. A first study showed that the 299Gly allele of the TLR4 gene may be associated with a decreased risk of late-onset AD in an Italian population sample, and this effect was independent of the APOE e4 status [18]. Other studies showed a strong association between TLR4 polymorphisms and late-onset AD risk, especially among ApoE e4 non-carriers [19,20]. However, the mechanisms by which the polymorphisms in the TLR4 gene could affect susceptibility to late-onset AD remain to be determined. In consequence future investigations should be focused on understanding the role of TLR4 genetic variants in neurodegenerative processes.

Allelic and genotypic frequencies of the APOE gene in healthy individuals and patients with Alzheimer disease

The distribution of the APOE allele and genotype frequencies in controls) and AD patients shows three common alleles (*APOE*2*, *APOE*3* and *APOE*4*). The *APOE*3* allele was the most frequent allele in both groups studied. However, the *APOE*4* allele frequency was significantly higher in patients with AD than in healthy individuals (37.7 % vs. 14.5% respectively, OR: 3.57, 95% CI: 2.0849-6.1093; *p*: 0.000001, *pc*: 0.000003) (Table 2). In addition, five APOE genotypes were identified in patients and four in controls. A comparison of the frequencies of genotypes between AD and controls showed a significantly increased frequency of the ε3/ε4 (40.98% versus 21%, OR: 2.61; 95% CI: 1.2955-5.2677; *p*: 0.003, *pc*: 0.015) and ε4/ε4 (16.4% vs. 4%, OR: 4.7; 95% CI: 1.4058-15.7522; *p*: 0.008, *pc:* 0.04) genotypes in AD patients versus healthy individuals. Likewise, a significantly lower frequency of the ε2/ε3 (1.64% vs. 15%, OR: 0.094, 95% CI: 0.0121-0.7343, *p:* 0.003, *pc:* 0.012) and ε3/ε3 (39.34% vs. 60%, OR: 0.43, CI 95%: 0.2255-0.8292, *p:* 0.005, *pc:* 0.02) genotypes were recorded in patients versus controls (Table 2). Numerous studies have confirmed that the ε4 allele is the strongest genetic risk factor for sporadic AD. As compared to individuals with no ε4 alleles, the increased risk for AD is approximately is 2-3 fold in people with one ε4 allele and about 12-fold in those with two ε4 alleles [20,21]. There are a variety of mechanisms by which apoE isoform may influence risk for AD. Evidence indicates that the apoE isoforms differentially affect Aβ aggregation and clearance in the brain. However, other mechanisms likely to play a role in the ability of apoE to influence CNS (Central Nervous System) function

Genotypes APOE	AD (n=61)	Control (n=100)	OR 95% CI	p
ε2/ε3	1.64 (1)	15 (15)	0.094 (0.0121-0.7343)	0.003
ε2/ε4	1.64 (1)	0	4.98 (0.1998-124.2621)	ns
ε3/ε3	39.34 (24)	60 (60)	0.43 (0.2255-0.8292)	0.005
ε3/ε4	40.98 (25)	21 (21)	2.61 (1.2955-5.2677)	0.003
ε4/ε4	16.4 (10)	4 (4)	4.7 (1.4058-15.7522)	0.008
Alleles				
ε2	1.64 (2)	7.5 (15)	0.20 (0.0461-0.9148)	0.016
ε3	60.66 (74)	78 (156)	0.43 (0.2653-0.7124)	0.0004
ε4	37.70 (46)	14.5 (29)	3.57 (2.0849-6.1093)	0.000001

AD: Alzheimer Disease; 95% CI: Confidence Interval; ns: Not Significant; OR: Odds Ratio
Values shown in parentheses indicate the number of repeats of the allele or the number of individuals bearing the genotype for the polymorphic site under study. Frequencies are expressed as percentages

Table 2: Allelic and genotypic frequency of the rs429358 and rs7412 polymorphisms of the *APOE* gene in patients and controls.

Genotypes combination TLR4/APOE	AD (n=61)	Control (n=100)	OR 95% CI	*p*	Association
CC/ε2ε3	1.64 (1)	13 (13)	0.11 (0.0142-0.8753)	0.009	Negative
CC/ ε2ε4	1.64 (1)	0	4.98 (0.1998-124.2621)	ns	-
CC/ε3ε3	37.71 (23)	52 (52)	0.55 (0.2917-1.0697)	0.039	Negative
CC/ε3ε4	39.34 (24)	20 (20)	2.59 (1.2757-5.2766)	0.003	Positive
CC/ε4ε4	16.39 (10)	4 (4)	4.7 (1.4058-15.7522)	0.008	Positive
CT/ε2ε3	0	2 (2)	0.32 (0.01516-7830)	ns	-
CT/ε3ε3	1.64 (1)	8 (8)	0.19 (0.0233-1.5714)	ns	-
CT/ε3ε4	1.64 (1)	1 (1)	1.65 (0.1013-26.8671)	ns	-

AD: Alzheimer Disease; 95% CI: Confidence Interval; ns: Not Significant; OR: Odds Ratio
Values in parentheses show the number of individuals bearing the genotype for the polymorphic site under study. Frequencies are expressed as percentages

Table 3: Frequencies of genotype combinations of the *TLR4/APOE* genes in patients with AD and controls.

as well as AD. These mechanisms include effects on synaptic plasticity, cell signaling, lipid transport and metabolism, and neuroinflammation [22].

Study of the combined effect of polymorphism in TLR4 and APOE genes

There of the 12 possible combinations of *TLR4/APOE*, seven were observed in both groups. The combinations CC/ε3ε4 (OR: 2.6; 95% CI: 1.2757-5.2766; *p:* 0.003; *pc*: 0.021) and *CC/ε4ε4* (OR: 4.7; 95% CI: 1.4058-15.7522; *p*: 0.008, *pc*: 0.05) were significantly more common in AD patients than in controls (associations positive). We must point out that some genotype combinations were more common in the control group: *CC/ε2ε3* (OR: 0.11, 95% CI: 0.0142-0.8753, *p*: 0.009, *pc*: not significant) and *CC/ε3ε3* (OR: 0.55; 95% CI: 0.2917-1.0697; *p*: 0.039, *pc*: not significant) (associations negative) (Table 3).

Discussion

The AD brain is characterized by extensive extracellular deposits of β-amyloid (Aβ) that form senile plaques. In addition, the AD brain exhibits intracellular neurofibrillary tangles and a strong inflammatory response with increased levels of inflammatory cytokines, chemokines, immune cell surface proteins, acute phase proteins, complement proteins, and oxidative damage within the brain [7]. In AD, microglia cells play an important role in disease progression by clearing Aβ deposits, initiating phagocytic activity, and releasing cytotoxic mediators [2]. Nevertheless, the role of microglia in the course of AD remains somewhat controversial, but the involvement of several receptors in this process is evident. Microglia cells express several receptors that cooperate in the recognition, internalization, and clearance of Aβ and in cell activation, such as Toll-like receptors (TLRs) [2]. Among the TLRs, TLR-4 is involved in these processes and experimental evidences have suggesting different roles for TLR4 signaling, which appear to be associated with both beneficial (clearance of Aβ) and detrimental (neurotoxicity) processes [2]. Polymorphism in the TLR4 gene has been associated with the incidence of AD. In a first study, the association between the rs4986790 polymorphism of TLR4 gene and a reduced prevalence of late onset AD was observed [18], while other studies showed that the variant rs1927907 [19] and rs7045953 [20] were associated with an increased risk of developing late-onset AD. Since the Aβ activates microglia through of toll like receptor 4 (TLR4) and that the TLR4 with the rs4986791 polymorphism have a lower affinity for their ligands, we studied whether these polymorphism of the TLR4 gene is associated with the development of AD.

Comparative analysis of allele frequencies of the rs4986791 polymorphism did not showed any statistical differences. However, the presence of the CC genotype apparently confers three times higher risk of developing AD. This result suggests that others polymorphisms of TLR4 gene may be conferring susceptibility to develop late-onset AD as previously described in the literature [18-20]. For example the variants rs4986790 and rs4986791 have been found to be co-segregated; forming haplotypes. Thus the association studies must be conducted that take into account different polymorphisms of TLR4. Finally, when establishing comparisons between the groups, negative (protective) and positive (susceptibility) associations were observed between TLR4/APOE genotypes and the development of AD. These results confirmed the association of the ε4 allele of the APOE gene and the development of this pathology, but contrasts with the study of Chen et al. [19], who determined that the rs1927907 variant of TLR4 was associated with an increased risk of developing late-onset AD, especially among ApoE ε4 non-carriers [19]. Additional investigations with a larger number of patients would be useful to confirm the importance of the TLR4 polymorphisms in the development of late-onset AD. Besides, is very important to investigate the distribution of a second functional TLR4 polymorphism (rs4986790) which may also lead to an altered inflammatory response. TLR4 polymorphisms can alter the receptor functionality or truncates signalling pathways, leading to a decreased pro-inflammatory response that is insufficient to eliminate the Aβ deposits, or exacerbates the pro-inflammatory response. This later process has deleterious effects to the host due to the structural change produced in the extracellular domain of TLR4. Thus the innate immune system is a double-edged sword. At Low Aβ concentrations, corresponding to those observed in the brain of early/middle stage AD, CD14 and TLRs may activate microglia promoting phagocytic clearance of Aβ. While higher Aβ concentrations corresponding to those observed at late stage AD, microglial activation through CD14 and the TLR4 results in production of neurotoxins, thereby damaging surrounding neurons and killing these damaged neurons [7]. In consequence, is very important continuing the study of innate immune receptors as TLRs, due to that these receptors participates in microglial activation, cells responsible for cleaning the deposits of Aβ. Therefore, it is important to emphasize that there are other polymorphisms in the TLR4 gene, such as Asp299Gly (+896 A/G, rs4986790), which co-segregates with Thr399Ile (+1196 C/T, rs4986791), the variant rs1927907, among others. For this reason, it is suggested to carry out the determination of a TLR4 polymorphism group and to detect if the genotypic and haplotype combinations of TLR4 (rs4986791, rs4986790, rs1927907, for example) confer susceptibility to the development of late onset AD, independently of the variability of APOE gene. Finally, the recognition of the contribution of polymorphisms of TLRs and their co-receptors in AD pathogenesis suggests that they may be an appropriate target for therapeutic intervention within the disease progression.

Conclusion

In conclusion, the results suggest the absence of any association between rs4986791 polymorphism of TLR4 gene and susceptibility to AD, but confirmed the association of the ε4 allele of the APOE gene and the development of this pathology. Future investigations should be focused on understanding the role of TLR4 genetic variants in the development of late-onset AD.

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