TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2015) 45: 1082-1086

doi:10.3906/sag-1406-64

Association of *CLU* and *TLR2* gene polymorphisms with late-onset Alzheimer disease in a northwestern Iranian population

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Received: 15.06.2014 • **Accepted/Published Online:** 14.03.2015 • **Printed:** 30.10.2015

Background/aim: A number of genetic variants from different genes have been reported to be related to late-onset Alzheimer disease (LOAD) susceptibility. From these genes, polymorphisms in *CLU* and *TLR2* have been replicated in several studies. In this study we examined the association of rs11136000 in *CLU* and the *TLR2* –196 to –174 del polymorphism with the risk of LOAD in a northwestern Iranian population.

Materials and methods: We conducted a case-control study with a dataset of 160 LOAD patients and 163 healthy controls. To examine polymorphisms of *CLU* and *TLR2* in LOAD we used the PCR/RFLP method and genotype frequencies were statistically determined.

Results: There was no association between CLU polymorphism and the risk of LOAD, but for deletion in *TLR2* we found significant differences between LOAD and the control group ($P > 0.001$, OR = 0.55).

Conclusion: This result suggests that the TLR2 –196 to –174 del polymorphism is an additional risk factor for LOAD. Allelic frequencies of *CLU* may have no effect on risk of LOAD.

Key words: Alzheimer disease, *TLR2*, *CLU*, polymorphism, northwestern Iran

1. Introduction

The commonest cause of dementia in adults is Alzheimer disease (AD), pathologically characterized by senile plaques containing amyloid beta (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein (1). It is a degenerative, incurable, and lethal disease, usually diagnosed in elderly people (2). From a clinical point view, AD is characterized by a progressive loss of memory and cognitive functions in later life (3). Mutations in the amyloid precursor protein (*APP*) gene and the presenilin 1 and 2 genes (*PSEN1* and *PSEN2*, respectively) lead to Mendelian forms of AD. These mutations, however, explain less than 1% of all cases of AD, whereas the vast majority of cases (especially for late-onset forms of the disease) have other more complex genetic determinants (4). At present, apolipoprotein E (*APOE*), which carries 3 alleles, 2, 3, and 4, is the unique and well-established susceptibility gene for late-onset AD (LOAD) (5). While only *APOE* has been clearly identified as a susceptibility gene in the more common form of AD, data from recent genome-wide association studies (GWASs) have implicated several other common risk variants (6–8).

To date, a number of genetic variants from different genes have been reported to be related to LOAD susceptibility by using new large-scale genotyping technologies according to the AlzGene database (www.alzforum.org/res/com/gen/alzgene/largescale.asp). However, none of those genes exhibited replicable results for disease risk association until recently when *CLU* was simultaneously reported by 2 independent research groups, both based on the GWAS approach.

The *CLU* transcriptional unit is located in the chromosomal region 8p21-p12 and comprises 9 exons in the longest transcript that translates in the main CLU protein isoform of 449 amino acid residues (9). The CLU precursor peptide is internally cleaved to produce an a- and b-subunit, held together by disulfide bridges and subsequently secreted from the cell (9).

In the AD brain, *CLU* expression is reported to be increased in affected cortical areas and is present in amyloid

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plaques and in the cerebrospinal fluid of AD cases (10). Similar to *APOE*, clusterin appears to act as a molecular chaperone for Aβ and regulates both the toxicity and its conversion into insoluble forms (11). Furthermore, *APOE* and *CLU* have been shown to cooperate in suppressing Aβ deposition and *APOE* and *CLU* may critically modify Aβ clearance at the blood–brain barrier, suggesting a role for clusterin in the amyloidogenic pathway. *CLU* levels are increased in proportion to *APOE*-ε4 allele dose, suggesting an induction of clusterin in individuals with low *APOE* levels (12).

Toll-like receptor 2 (*TLR2*) represents a reasonable functional and positional candidate gene for AD as it is located under the linkage region of AD on chromosome 4q and is functionally involved in the microglia-mediated inflammatory response and Aβ clearance (13,14). A 22-bp nucleotide deletion at position –196 to –174 of the untranslated 5'-region in the *TLR2* gene is associated with reduced transcriptional activity compared to the wild-type allele in luciferase reporter assays (15). Many experimental and clinical studies have suggested that *TLR2* might play an important role in the pathogenesis of AD (14). TLR2 is a member of pattern recognition receptors in the innate immune system (16). Increased levels of *TLR2* mRNA have been found in microglia isolated from AD patients (14).

To better understand the genetic aspect of LOAD in the northwestern Iranian population, we followed those susceptible polymorphisms of *CLU* and *TLR2* from GWASs in independent Iranian subjects.

2. Materials and methods

2.1. Sample preparation

The study included 160 AD patients (women and men, mean age 76.06±7.75 years, ranging from 65 to 99) and a healthy control group including 163 healthy individuals of the same ethnicity (women and men, mean age 75.29 ± 6.75 years, ranging from 65 to 89 years), who were randomly selected from a distinguished laboratory. All AD patients were diagnosed by expert clinicians according to Mini-Mental State Examination criteria (17). The age of onset was above 65 years, and the sporadic form of the disease was ensured whereby no affected individuals were present in first-degree relatives of the subjects. All subjects included in the study were Azeri Turks originating from a limited population area in the northwest of Iran.

2.2. DNA preparation and genotyping

Genomic DNA was extracted from whole blood collected in sterile tubes containing EDTA by using the salting-out method.

CLU gene polymorphism identification was determined by PCR/RFLP. Products of 155 bp in length were obtained with a pair of primers (5'-ACGTTGGATGGAATGGCAGGCATTCAGCAC-3' and 5'-ACGTTGGATGTATTGGGTCAAGTGGCAAGG-3'). The primer design was carried out using online Primer 3 programs and the Ensembl Genome Browser for blasting. The PCR reaction was prepared in a total volume of 25 μL, containing 0.1 μg of genomic DNA, 0.01 μg each of the primers, 2.5 μg of 10X PCR buffer (670 mM Tris-HCl, pH 8.8, 160 mM $(NH_4)_2SO_4$, 0.1% Tween-20), dNTP mix (10 mM each), 50 mM $MgCl_2$, and Taq DNA polymerase (5000 U/mL). After denaturation of template DNA at 94 °C for 5 min, 30 cycles of PCR reactions were optimized and performed by denaturation at 94 °C for 1 min, annealing at 68.1 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were digested with restriction endonuclease XapI to identify the rs11136000 polymorphism. The PCR products were digested using 2 U/μL of restriction enzyme in a total volume of 25 μL, containing 5 μL of PCR product in supplied buffer. The mixture was incubated at 50 °C for 12–16 h. The digested PCR product was fractionated on 8% polyacrylamide gel and visualized after staining by $AgNO_3$. The restriction endonucleases were purchased from Roche Applied Science.

Purified PCR products from 21 AD cases and 20 healthy controls were randomly sequenced bidirectionally.

Polymorphism at *TLR2* –196 to –174 del was examined by using the PCR method followed by RFLP. Based on existence of deletion in samples, PCR products of 286 bp or 264 bp in length were obtained with a pair of primers (5'-CACGGAGGCAGCGAGAAA-3' and 5'-CTGGGCCGTGCAAAGAAG-3'). The PCR reaction was prepared in a total volume of 25 μL, containing 0.1 μg of genomic DNA, 0.01 μg each of primers, 2.5 μg of 10X PCR buffer (670 mM Tris-HCl, pH 8.8, 160 mM $(NH_4)_2SO_4$, 0.1% Tween-20), dNTP mix (10 mM each), 50 mM $MgCl_2$, and Taq DNA polymerase (5000 U/mL). After denaturation of template DNA at 94 °C for 5 min, 30 cycles of PCR reactions were optimized and performed by denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. In the next steps, samples were randomly analyzed by bidirectional sequencing.

2.3. Statistical analysis

We analyzed our data statistically using Sigma Stat 2.0 software. Allelic and genotypic frequencies were obtained by direct counting. Hardy–Weinberg equilibrium was tested by using a chi-square goodness-of-fit test. Fisher's exact test was used for differences in genotypes and haplotypes between the groups. Statistical significance was set at $P < 0.05$. The odds ratio (OR) was calculated at the 95% confidence interval (CI).

3. Results

A total of 323 individuals were examined in the present study to evaluate the association of the rs11136000 polymorphism in the *CLU* gene and *TLR2* –196 to –174 del polymorphism with AD using PCR/RFLP and PCR procedures, respectively. The patient and control groups were matched by age and sex.

All analyzed polymorphisms satisfied Hardy–Weinberg equilibrium distribution (P > 0.001). For allele frequency, association of *TLR2* –196 to –174 del polymorphism (P > 0.001, OR = 0.55) was found to enhance LOAD risk, but for rs11136000 polymorphism in the *CLU* gene, no differences between cases and controls were observed (Table 1). Further genotypic analysis of those 2 SNPs showed significantly different distributions between LOAD patients and healthy controls by applying a dominant model for both of them (Table 2). Using the del/del or del/ins genotype for –196 to –174 del polymorphism as a reference, the OR for AD in subjects with the ins/ins genotype was 0.39 (95% CI = 0.24–0.63). The –196 to –174 del allele significantly raised the risk of developing LOAD (OR = 0.55, 95% CI = 0.40–0.76, power = 82.9%).

4. Discussion

We used a PCR/RFLP approach to follow up on the significant association of a common SNP in the *CLU* locus with increased risk for AD in a northwestern Iranian population.

In this study, in agreement with several other reports, we obtained a significant association of genotype frequencies with AD risk. In contrast with previous data we found no allelic association between LOAD cases and nondemented controls (6,7,8,18).

To date, in addition to 2 published GWASs, another 2 case-control studies (19,20) and 1 metaanalysis (21) carried out in parallel on Caucasians successfully demonstrated significant disease associations for *CLU* with compatible genetic effect sizes. A replication study performed by Kamboh et al. based on 2707 Caucasian Americans failed to detect significant LOAD associations for *CLU* (22). We tried to replicate their data in our population. Although a significant genotyping association was successfully replicated in *CLU* based on our dataset, no allelic difference in *CLU* was identified between cases and controls among the Iranian dataset.

Table 1. Allelic distribution of polymorphisms in *CLU* and *TLR2* in LOAD cases and controls.

Allele	AD patients n = 160	Healthy controls n = 163	P	OR (95% CI)
CLU (rs11136000)				
T	177 (55.31)	173 (53.06)	P = 0.58	0.91 (0.67–1.24)
C	143 (44.68)	153 (46.93)		
TLR2 (–196 to –174 del)				
Del	169 (52.8)	218 (66.9)	P > 0.001	0.55 (0.40–0.76)
Ins	151 (47.2)	108 (33.2)		

Table 2. Genotypic association analysis of polymorphisms in *CLU* and *TLR2* with LOAD.

Allele	AD patients n =160	Healthy controls n =163	P
CLU (Rs11136000)			
CC	25 (15.62)	16 (9.81)	
TC	93 (58.12)	121 (74.23)	P = 0.009
TT	42 (26.25)	26 (15.95)	
TLR2 (–196 to –174 del)			
Ins/ins	39 (24.4)	73 (44.8)	
Del/ins	91 (56.9)	72 (44.2)	P > 0.001
Del/del	30 (18.7)	18 (11)	

Compared with the cited previous studies with thousands of subjects, our study was conducted on a much smaller sample size. This may partially explain why no association was observed for allelic association with LOAD in our whole dataset.

We used a PCR approach to follow up on the significant association of a common polymorphism in the *TLR2* locus with increased risk for AD in a northwestern Iranian population.

In this study, in agreement with a Chinese report (23), we obtained a significant association of allelic and genotyping frequencies with AD risk. Many experimental and clinical studies have suggested that *TLR2* might play an important role in the pathogenesis of AD (14). TLR2 is a member of pattern recognition receptors in the innate immune system (16). Although an increasing volume of data favors *TLR2*-mediated neurotoxicity, *TLR2* may also be essential for Aβ clearance and in that way provide neuroprotection in AD (14). The *TLR2* del/del genotype is reported to show decreased transactivation of responsive promoters (15).

Our results suggest a significant association between the –196 to –174 del allele of *TLR2* and the risk of developing LOAD in the northwestern Iranian population.

Our data suggest that the –196 to –174 del/del genotype of *TLR2* may increase the risk of LOAD in the northwestern Iranian population. Additional independent replications and functional genetic analyses are needed to elucidate the potential mechanisms and the epidemiologic relevance of these associations.

Acknowledgments

We are appreciative for the valuable cooperation of the Danesh Laboratory in providing the control samples. We thank Dr Parvin Javadi (Khuban Old People's Home, Tabriz) and Dr Shoukoh Mousavipour (Mehr Old People's Home, Tabriz) for their kind cooperation. The study was funded by a grant from the Deputy for Research, Tabriz University of Medical Sciences.

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