LACK OF ASSOCIATIONS BETWEEN CLU AND PICALM GENE POLYMORPHISMS AND ALZHEIMER'S DISEASE IN A TURKISH POPULATION

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NINCS ÖSSZEFÜGGÉS A CLU, VALAMINT A PICALM GÉNPOLIMORFIZMUSOK ÉS AZ ALZHEIMER-BETEGSÉG KÖZÖTT EGY TÖRÖK BETEGCSOPORTBAN

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Background and purpose – To investigate the association between the rs11136000 single nucleotide polymorphism (SNP) of the clusterin (CLU) gene, the rs541458 and rs3851179 SNPs of the phosphatidylinositol-binding clathrin assembly protein (PICALM) gene and Alzheimer's disease (AD) in a Turkish population, and to determine whether there are any relationships between the CLU and the PICALM genotypes and behavioral and psychological symptoms of dementia (BPSD) in the Turkish population.
Methods – One-hundred and twelve AD patients and 106 controls were included in this study. BPSD were evaluated by the Behavioral Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD). SNPs in the CLU and the PICALM gene were genotyped by Real-Time PCR. Genotype distributions were assessed for the groups of patients and controls, for the patient groups with and without each BPSD, and "No BPSD" and "BPSD".
Results – The CLU and the PICALM genotypes were similar in the AD and control subjects, and the groups with and without each BPSD. There were also no significant differences between the "No BPSD" and the "BPSD" groups for the PICALM genotypes, but even without a statistical significance, it is notable that none of the "No BPSD" patients had

Háttér és célkitűzés – Vizsgáltuk az összefüggést a clusterin (CLU) gén rs11136000 egyesnukleotid-polimorfizmusa (SNP), a foszfatidilinozitol-kötő clathrin összeszerelő fehérje (PICALM) génjének rs541458 és rs3851179 SNP-i és az Alzheimer-betegség (AD) között török populációban, és meghatároztuk, fennáll-e összefüggés a CLU- és PICALM-genotípusok, valamint a dementia viselkedési és pszichés tünetei (BPSD) között a török populációban.
Módszerek – A vizsgálatba 120 AD-beteget és 106 kontrollt vontunk be. A BPSD-t a Behavioral Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD) segítségével értékeltük. A CLU és a PICALM gének SNP-it valós idejű PCR-rel genotipizáltuk. A betegek és a kontrollok csoportjában értékeltük a genotípusok eloszlását az egyes BPSD szerint, illetve a BPSD-t mutató (BPSD-) és attól mentes (No-BPSD-) betegcsoportokban.
Eredmények – A CLU- és a PICALM-genotípusok hasonlóak voltak az AD- és a kontrollcsoportban, illetve a BPSD-t mutatók és nem mutatók csoportjában. Nem volt szignifikáns a különbség a No-BPSD- és a BPSD-csoportok között a PICALM-genotípusokban, de szignifikáns különbség nélkül is érdemes megemlíteni, hogy a No-BPDS-csoportban egyik esetben sem volt jelen a CLU-rs11136000-TT genotípus, és

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genotype pattern CLU-rs11136000-TT, and the female subjects with genotype pattern CLU-rs11136000-TT had higher mean score of BEHAVE-AD.
Conclusion – This study claims that investigated SNPs are not genetic risk factors for AD in a Turkish population. In addition, the rs541458 and rs3851179 of PICALM SNPs are not related to development of BPSD, but the rs11136000 of CLU SNP might be related to development of BPSD in AD female Turkish subpopulation.

Keywords: *Alzheimer's disease, behavioral and psychological symptoms of dementia, clusterin, phosphatidylinositol-binding clathrin assembly protein, single-nucleotide polymorphism*

a CLU-rs11136000-TT genotípusú nőbetegek magasabb átlagpontszámot értek el a BEHAVE-AD-n.
Következtetés – A vizsgálat eredményei alapján a vizsgált SNP-k nem jelentik az AD kockázati tényezőit ebben a török populációban. Továbbá a PICALM rs541458 és rs3851179 SNP-i nem állnak összefüggésben a BPSD kialakulásával, viszont a CLU rs11136000 SNP-je összefüggésben állhat a BPSD kialakulásával a török AD-s betegek női alcsoportjában.

Kulcsszavak: *Alzheimer-betegség, a dementia viselkedési és pszichés tünetei, clusterin, foszfatidilinozitol-kötő clathrin összeszerelő fehérje, egyesnukleotid-polimorfizmus*

Alzheimer's disease (AD) is a neurodegenerative disorder characterized neuropathologically by extracellular deposition of the amiloid-β (Aβ) peptide in the senile plaques, intracellular deposition of the hyperphosphorylated tau protein in the neurofibrillary tangles, and cortical neuronal loss[1]. It is responsible for the majority of dementia in the elderly and is highly heritable but genetically complex[2]. Several studies have suggested that an array of low-penetrance, common risk alleles influence to late-onset AD (LOAD) (age>65), which is the most common form of AD. Some of these alleles probably affect a variety of pathways involved in the production, aggregation and removal of Aβ[1, 3], and strong evidence suggests that Aβ plays a central role in the pathogenesis of AD[4].

Apolipoprotein E (ApoE) is the main brain cholesterol transport lipoprotein[5], and the APOE ε4 allele (ε4 APOE) is the most important genetic risk factor for LOAD[6, 7]. The Genome-wide association studies (GWASs) conducted by several large consortia have pinpointed other novel AD risk genes outside the APOE region, such as clusterin (CLU, also known as APOJ) and phosphatidylinositol-binding clathrin assembly protein (PICALM)[8–11]. Findings with CLU and PICALM have been replicated in several data sets[12–15], but it has been suggested that, to determine the generalizability of the contribution of each gene to AD risk and the possibility of population-specific causative variants, confirmation in other populations is required[16].

Behavioral and psychological symptoms of dementia (BPSD) are commonly observed over the course of AD[17, 18]. These symptoms are associated with considerable morbidity to patients, increased caregiver burden and earlier institutionalization[20, 21]. A genetic component to BPSD development in AD has been demonstrated[22]. It has been asserted that the genes that increase the risk for AD may also determine the presence of BPSD[23], and suggested that one or more of the APOE actions could be involved in the development of BPSD[24]. CLU has been shown that it binds to and promotes the clearance of Aβ from the brain, a function potentially shared with APOE[25]. It is also involved in immune response[26], membrane recycling and apoptosis[27]. It has been suggested that CLU expression levels are associated with disease status, age at onset, disease duration, and/or Clinical Dementia Rating Scale (CDR) of AD[26]. PICALM also has a role in internalisation and transport of lipids such as that mediated by lipoprotein particles containing apoE and CLU[28]. It has been speculated that the association between AD risk and PICALM might be linked to the production of Aβ via clathrin-mediated endocytosis[10], essential in dendritic outgrowth in neurons[29]. In several genetic studies, significant associations has been reported between APOE genotype and various BPSD[23, 30, 31], but some other studies have reported controversial results[31–33]. The association of BPSD with CLU and PICALM genotypes has been unexplored.

Under the influence of all these suggestions, we aimed to evaluate the rs11136000 single nucleotide polymorphisms (SNPs) of the CLU gene, the rs541458 and rs3851179 SNPs of the PICALM gene as genetic risk factors for AD in a Turkish sample and to determine whether there are any relationships between the CLU and PICALM genotypes and BPSD in AD in a Turkish sample.

Methods

SUBJECTS AND DIAGNOSIS

Of the 120 consecutive Turkish patients with sporadic LOAD, collected prospectively from our Division of Dementia Outpatient Clinic, seven subjects' blood samples and a subjects' clinical data

were missing. These patients were excluded from the study. A total of 112 sporadic LOAD subjects and 106 non-demented age-, sex-, and educational level-matched controls were recruited in this study. The rs11136000 SNP of the CLU gene, the rs541458 and rs3851179 SNPs of the PICALM gene were examined blinded to the diagnosis of patients affected by sporadic LOAD with or without BPSD and controls.

The diagnosis of AD was performed according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) for a diagnosis of probable AD[34]. No familial cases of AD were included in this study. All patients were over 65 years of age at the onset of their illness, and were free of pharmacological treatment for BPSD and AD. Only patients who lived with family at least three years were included and only a person who spend time together with patient at least three hours a day was accepted as a main caregiver to ensure reliability of data. Controls were recruited from the general community or from among medical and care staff volunteers in hospitals. They were cognitively intact.

All subjects underwent physical, neurological, psychological, and mental status examinations, laboratory blood analyses, neurocognitive evaluation, brain magnetic resonance imaging or computed tomography. Cognitive function was examined using the Mini Mental State Examination (MMSE)[35]. Disease staging was assessed using the CDR[36]. BPSD were evaluated by a direct assessment of the patients and a structured interview with the main caregivers using the items of the Behavioral Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD)[37] at the time of AD diagnosis. BPSD needed to be present had to exist for at least one month, intermittently and severe enough to effect negatively to patients' and/or family members' social life. The BEHAVE-AD is a 25-item scale and consists of seven subscales including Paranoid and Delusional Ideation (seven items), Hallucinations (five items), Activity Disturbances (three items), Aggressiveness (three items), Diurnal Rhythm Disturbances (one item), Affective Disturbance (two items), and Anxieties and Phobias (four items). Each item is scored on a four-point scale rating from 0 (absence of a symptom) to 3 (the most severe category) based on a clinical interview with the main caregiver. Both the presence of symptoms and consideration of the magnitude of symptoms were noted in this study. We divided the patients into a series of pairs of groups, based on a positive or negative result for a certain category; groups with/without paranoid and delusional ideation, hallucinations, activity disturbances, aggressiveness, diurnal rhythm disturbances, affective disturbance and anxieties and phobias. In addition, 11 AD patients, whose BEHAVE-AD score was 0, were assigned to the "No BPSD" group, while 101 AD patients, whose BEHAVE-AD score was 1 or above, were assigned to the "BPSD" group. Genotype distributions were assessed for the groups of patients and controls, for the patient groups with and without each BPSD, and for the groups "No BPSD" and "BPSD".

Patients and controls with a history of traumatic brain injury, drug abuse, neurological diseases, psychiatric disorders, mental retardation, abnormalities in serum vitamin B12 and folate, syphilis serology or thyroid hormone levels, significant systemic medical problems such as clinically significant cardiac, pulmonary, hepatic or renal disorders, cancer, poorly controlled hypertension or metabolic disorders and family (in first-degree relatives) history of neuropsychiatric diseases were excluded. In addition, patients with other forms of dementia were excluded.

All subjects and their legal guardians were informed about the study design. The project was approved by the local ethical committee, and written informed consent was received from all participants and legal guardians. The study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects.

MOLECULAR ANALYSIS

DNA extraction and analysis

A blood sample was drawn from each individual. Genomic DNA was extracted from fresh-frozen blood using High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim Germany).

Genotypic analysis

The genotyping of the CLU gene rs11136000 C>T, PICALM gene rs541458 T>C and rs3851179 G>A polymorphisms was performed using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA). The Assays-on-Demand SNP genotyping kit was used for the polymerase chain reaction (Applied Biosystems). Single nucleotide polymorphism amplification assays were performed according to the manufacturer's instructions. In brief, 25 µl of reaction solution containing 30 ng of DNA was mixed with 12.5 µl of 2X

Table 1. *Genotype distribution of the rs11136000 SNP of the CLU gene, the rs541458 and rs3851179 SNPs of the PICALM gene in AD patients and controls*

	AD patients (N=112)		Controls (N=106)		p
	Observed values	Expected values	Observed values	Expected values	
CLU rs 11136000					
CC/CT/TT	53/45/14	50.9/49.21/11.9-	45/52/942.5/	47.56/46.89/11.56	0.353
(N, %)	47.3/ 40.2/ 12.5	-	49.0/ 8.5	-	
χ^2	1.2606		0.8196		
PICALM rs541458					
CC/CT/TT	17/49/46	15.38/52.25/44.38	18/52/36	18.26/51.47/36.26	0.556
(N, %)	15.2/ 43.8/ 41.0	-	17.0/ 49.0/ 34.0	-	
χ^2	0.0111		0.4322		
PICALM rs3851179					
GG/GA/AA	48/49/15	46.93/51.14/13.93	41/44/21	37.44/51.11/17.44	0.437
(N, %)	42.9/ 43.8/ 13.3	-	38.7/ 41.5/ 19.8	-	
χ^2	2.0529		0.1958		

A: adenine; AD: Alzheimer's disease; C: cytosine; CLU: clusterin; G: guanine; PICALM: phosphatidylinositol-binding clathrin assembly protein; SNP: single nucleotide polymorphism; T: thymine

TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 µl of predeveloped assay reagent from the SNP genotyping product (C_11227737_10 for the CLU Gene rs11136000, C_2134590_10 for PICALM Gene rs541458 and C_8748810_10 for PICALM Gene rs3851179, Applied Biosystems) containing 900 nm two primers and 200 nm two MGB TaqMan probes. Reaction conditions consisted of preincubation at 60ºC for 1 minute and at 95ºC for 10 minute, followed by 40 cycles at 95ºC for 15 second and at 60ºC for 1 minute. Amplifications and analysis were performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems), using the SDS 2.0.6 software for allelic discrimination (Applied Biosystems).

All procedures were conducted in a manner blind to the case status and other characteristics of the participants. Scoring of gels and data entry were conducted independently by two persons.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS for Windows (Version 15.0; SPSS Inc. Chicago, IL, USA). Data were presented as mean ± standard deviation or as N (%), as applicable. Chi-square tests were used to compare categorical variables. All two-group comparisons of demographic, clinical and behavioural data were made with the t-test or – when normal distribution was lacking– the Mann-Whitney U test. Three-group comparisons were made with the variance analyse or Kruskal-Wallis tests according to suitability of variable to normal distribution. P value correction was performed for multiple comparisons using Fisher exact test. A p value <0.05 was considered statistically significant.

Results

One-hundred and twelve AD subjects and 106 non-demented controls were screened to asses the CLU gene rs11136000, the PICALM gene rs541458 and rs3851179 genotype in this study. The mean age at onset of AD was 73.59±7.59 (range: 65 to 98) years. No significant differences in the mean age, the mean educational level and gender were noted between AD subjects (75.45±7.59 [range: 65 to 100] years, 3.09±4.14 [range: 0 to 21] years, 45 male/67 female) and controls (74.04±5.29, [range: 65 to 86] years; p=0.196, 3.74±3.97 [range: 0 to 15], years; p=0.241, 52 male/54 female; p=0.187). The mean duration of illness at the point of sampling was 2.58±1.88 (range: 0.5 to 10) years. The mean total score of BEHAVE-AD was 13.25±11.04 (range: 0 to 52). The mean MMSE score of patients and controls were 15.17±4.46 (range: 8 to 24) and 27.79±1.08 (range:26 to 30), respectively (p<0.05). Among the 112 AD patients, 23 (20.5%) patients were CDR 1 and 89 (79.5%) patients were CDR 2.

For all analyzed polymorphisms, data obtained for AD patients and controls were in Hardy-Weinberg equilibrium (The level of significance=0.05, the degrees of freedom=1, and the critical threshold value=3.842) **(Table 1.)**.

In this study, no significant differences were demonstrated between the groups of AD patients and controls for the CLU gene rs11136000, the PICALM gene rs541458 and rs3851179 genotype distributions **(Table 1.)**.

Among the 112 AD patients, 101 patients (90.17%) had at least one positive finding among the BEHAVE-AD subcategories. Activity disturbances (70.5%) was the most common symptom in BPSD, followed by paranoid and delusional ideation (69.6%), anxieties and phobias (65.2%), affective disturbance (57.1%), diurnal rhythm disturbances (50.0%), aggressiveness (49.1%) and hallucinations (37.5%). No significant differences were demonstrated between the patient groups with and without each BPSD for the CLU gene rs11136000, the PICALM gene rs541458 and rs3851179 genotype distributions. Genetic results are summarized in **Table 2**.

There were no significant differences between the "No BPSD" group and the "BPSD" group for the PICALM gene rs541458 and rs3851179, and On the other hand, even without a statistical significance, it is notable that none of the "No BPSD" patients had TT genotype for the CLU gene rs11136000 (CC: 72.7%, CT: 27.3%, TT: 0% and CC: 44.6%, CT: 41.6%, TT: 13.9% respectively, p=0.056, Fisher Exact test adjusted p=0.187), and the female subjects with TT genotype for the CLU gene rs11136000 had higher mean score of BEHAVE-AD in comparison to CC and CT genotype (p=0.140, **Table 3.**).

Discussion

The results of this study have demonstrated that, there is no significant differences for the CLU gene rs11136000, the PICALM gene rs541458 and rs3851179 genotype distributions between the groups of Turkish AD patients and controls. In addition, our findings did not support the association with BPSD and the rs541458 and rs3851179 SNPs of the PICALM gene. However, there were liminal significant differences between the "No BPSD" group and the "BPSD" group for the CLU gene rs11136000 genotype distributions (p=0.056), but Fisher Exact test adjusted p value as 0.187. We did not find a significant association with BPSD and this locus, nevertheless we thought that this is probably due to the small sample size of the "No BPSD" group. The lack of this association could be also attributed to the small size of the cohort. Consequently genetic variation at the CLU locus in Turkish female subjects might be a genetic factor that influences BPSD.

In a recently published study, it has been suggested that both the PICALM and CLU genetic variants had showed nominal significant association with cognitive decline[38]. Furthermore, a significant association of episodic memory with CLU and PICALM genotypes has been shown[39]. Nevertheless, the association of BPSD with CLU and PICALM genotypes has not been explored before, therefore we could not compare our results with others'.

The association between the rs3851179 SNP of the PICALM and AD was also not replicated in Polish, African American, Caribbean Hispanic, Israeli-Arab and Han Chinese populations by independent studies[12, 16, 40–42]. Moreover, the association between the rs11136000 SNP of the CLU was not replicated in Polish and Caribbean Hispanic populations[41, 43]. In another study, significant association was replicated in the rs11136000 SNP of the CLU based on southern Chinese population, however significant association for the rs3851179 SNP of the PICALM was only identified in the APOE ε4 (-) but not in the APOE ε4 (+) subgroup[44]. It has been suggested that the low replication of AD susceptibility locus in different populations may be caused by different linkage disequilibrium patterns in the human genome in different populations[40].

The prevalence of BPSD in our patients was higher compared to some studies[37, 45], but it has been suggested that prevalence rates of BPSD range from 60% to 90% in the elderly with dementia in some other studies[46–48]. Our hospital is a neuropsychiatry hospital, that we gave a referral center that received many requests to see patients with BPSD. Moreover, all of our patients were free of pharmacological treatment for BPSD and AD.

There are several limitations to the present study. First, the sample size was relatively small, and the No BPSD group consisted of only 11 patients. The relatively small size of the samples might have resulted in inadequate power to detect associations between the CLU and PICALM genotype and BPSD. In addition, longitudinal assessments were not used for measurement of BPSD in this study. They were assessed by the BEHAVE-AD in a cross-sectional study design. Owing to BPSD is observed at any time over the disease course, and would not be expressed at the time of assessment, such genetic predispositions could be missed. Therefore, cross-sectional study design is the second limitation of this study. However, use of pharmacological agents, such as acetylcholinesterase inhibitors or antipsychotics, during follow-up of AD patients can be a confounding factor for the evaluation of BPSD in longitudinal studies. Because, these drugs have been found to be effective for BPSD[49, 50]. In this study, patients receiving pharmacological treatment for BPSD and/or AD were not included. Our cohort was free of pharmacologi-

Table 2. *Gene polymorphisms for the groups with and without each BPSD*

Items of BEHAVE-AD	CLU rs 11136000 CC/CT/TT (%)	p	PICALM rs541458 CC/CT/TT (%)	p	PICALM rs3851179 GG/GA/AA (%)	p
Paranoid and delusional ideation						
With	66.0/ 66.7/ 92.9	0.130	70.6/ 65.3/ 73.9	0.657	77.1/ 61.2/ 73.3	0.224
Without	34.0/ 33.3/ 57.1		29.4/ 34.7/ 26.1		22.9/ 38.8/ 26.7	
Hallucination						
With	37.7/ 35.6/ 42.9	0.885	35.3/ 30.6/ 45.7	0.312	43.8/ 34.7/ 26.7	0.424
Without	62.3/ 64.4/ 57.1		64.7/ 69.4/ 54.3		56.2/ 65.3/ 73.3	
Activity disturbance						
With	71.7/ 66.7/ 78.6	0.672	64.7/ 71.4/ 71.7	0.848	72.9/ 71.4/ 60.0	0.622
Without	28.3/ 33.3/ 21.4		35.3/ 28.6/ 28.3		27.1/ 28.6/ 40.0	
Agressiveness						
With	45.3/ 46.7/ 71.4	0.201	52.9/ 44.9/ 52.2	0.733	62.5/ 38.8/ 40.0	0.059
Without	54.7/ 53.3/ 28.6		47.1/ 55.1/ 47.8		37.5/ 61.2/ 60.0	
Diurnal rhythm disturbances						
With	47.2/ 48.9/ 64.3	0.513	47.1/ 44.9/ 56.5	0.509	60.4/ 40.8/ 46.7	0.149
Without	52.8/ 51.1/ 35.7		52.9/ 55.1/ 43.5		39.6/ 59.2/ 53.3	
Affective disturbance						
With	49.1/ 64.4/ 64.3	0.261	70.6/ 59.2/ 50.0	0.317	56.3/ 59.2/ 53.3	0.910
Without	50.9/ 35.6/ 35.7		29.4/ 40.8/ 50.0		43.7/ 40.8/ 46.7	
Anxieties and Phobias						
With	60.4/ 66.7/ 78.6	0.430	70.6/ 65.3/ 63.0	0.856	66.7/ 65.3/ 60.0	0.894
Without	39.6/ 33.3/ 21.4		29.4/ 34.7/ 37.0		33.3/ 34.7/ 40.0	

A: adenine; BEHAVE-AD: Behavioral Pathology in Alzheimer's Disease Rating Scale; BPSD: behavioral and psychological symptoms of dementia; C: cytosine; CLU: clusterin; G: guanine; PICALM: phosphatidylinositol-binding clathrin assembly protein; T: thymine

Table 3. *The association of the CLU genotype with mean total BEHAVE-AD score with respect to gender*

Sex	Genotype	N (%)	Total BEHAVE-AD score (mean ± SD)	p
Female	CC	28 (25.0)	12.07±7.76	0.140
	CT	31 (27.6)	10.48±8.77	
	TT	8 (7.2)	20.38±15.86	
Male	CC	25 (22.3)	13.44±13.13	0.302
	CT	14 (12.5)	17.79±13.19	
	TT	6 (5.4)	12.17±9.45	

BEHAVE-AD: Behavioral Pathology in Alzheimer's Disease Rating Scale; C: cytosine; CLU: clusterin; SD: standard deviation; T: thymine

cal treatments and this was the advantage of this study. In addition, this study performed on a well-characterized sample of patients with probable AD, who underwent a careful diagnostic process in our Division of Dementia Outpatient Clinic.

In summary, our study claims that the rs11136000 SNP of the CLU gene, and the rs541458 and rs3851179 SNPs of the PICALM gene are not genetic risk factors for AD in Turkish people. In addition, the rs541458 and rs3851179 SNPs of the PICALM gene are not related to development of BPSD, but the rs11136000 SNP of the CLU gene might be related to the development of BPSD in AD female Turkish subpopulation. To our knowledge, this study was the first to evaluate the investigated SNPs as genetic risk factors for AD in a Turkish sample, and whether there is a relationship between CLU and PICALM gene and BPSD susceptibility. Further studies should verify our data in larger samples.

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