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Peripheral Expression Levels of Selected Oxidative Stress-Related Genes in Alzheimer's Disease

Alzheimer Hastalığında Oksidatif Stres ile İlişkili Seçilmiş Genlerin Periferik Kandaki Anlatım Düzeyi

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ABSTRACT

Objective: The etiology of Alzheimer's disease (AD) is affected via oxidative stress. Antioxidant enzymes are extremely important in preventing reactive oxygen species (ROS) causing damage in the cell. The changes in expression levels of oxidant and antioxidant genes are key factors in cell response to oxidative stress. As a result, this study investigated the change in expression levels of specific oxidative stress related genes (*solute carrier family 7 member 11 (SLC7A11), glutathione peroxidase 4 (GPX4), catalase (CAT)* and *acyl-coa synthetase long chain family member 4 (ACSL4)*) in peripheral blood of AD patients.

Material and Method: Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to assess the expression levels of oxidative stress-related genes in 25 AD patients and 22 controls, and the findings were statistically evaluated.

Results: *SLC7A11, GPX4, CAT,* and *ACSL4* gene expression levels did not vary significantly between AD patients and controls. The results also showed significant negative correlation between age of onset and *ACSL4* expression.

Conclusion: This is the first study that evaluated mRNA expression levels of *SLC7A11, GPX4* and *ACSL4* genes in AD. The results suggested that the peripheral blood expression of above-mentioned genes did not alter in AD. However, due to the small number of subjects, this findings are preliminary and should be validated with a larger number of subjects.

Keywords: Alzheimer's Disease, oxidative stress, *SLC7A11, GPX4, CAT, ACSL4*

ÖZ

Amaç: Oksidatif stres, Alzheimer hastalığının (AH) etiyolojisinde önemli bir rol oynamaktadır. Antioksidan enzimler reaktif oksijen türevlerinin (ROS) hücreye zarar vermesini önlemek için çok önemlidir. Oksidan ve antioksidan enzimleri kodlayan genlerin anlatım seviyelerindeki değişimler hücrenin oksidatif strese karşı verdiği yanıtta anahtar faktördür. Bu nedenle çalışmamızda, AH hastalarının periferik kanlarında spesifik oksidatif stres ile ilişkili genlerin (*solut taşıyıcı aile 7 üye 11 (SLC7A11), glutatyon peroksidaz 4 (GPX4), katalaz (CAT) ve açil-koA sentetaz uzun zincir aile üyesi 4 (ACSL4)*) anlatım düzeylerindeki değişimin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Periferik kan lökositlerinde oksidatif stresle ilgili genlerin ekspresyon düzeylerindeki değişiklikler 25 AH hastası ve 22 kontrolde kantitatif ters transkripsiyon polimeraz zincir reaksiyonu (qRT-PCR) ile belirlenmiş ve sonuçlar istatistiksel olarak değerlendirilmiştir.

Bulgular: *SLC7A11, GPX4, CAT* ve *ACSL4* genlerinin anlatım düzeyleri, AH hastaları ve kontroller arasında istatistiksel olarak bir farklılık göstermemiştir. Ayrıca, sonuçlarımız başlangıç yaşı ile *ACSL4* ekspresyonu arasında anlamlı negatif bir korelasyon olduğunu göstermiştir.

Sonuç: Araştırmamız, AH hastalarının periferik kanlarında *SLC7A11, GPX4* ve *ACSL4* genlerinin anlatım düzeylerini değerlendiren ilk çalışma olup, sonuçlarımız söz konusu genlerin periferik kandaki anlatımlarının AH'de değişmediğini göstermiştir. Bununla birlikte, az sayıda hasta ve kontrol nedeniyle, bu bulgular başlangıç niteliğindedir ve daha geniş çalışma gruplarında doğrulanması gerekmektedir.

Anahtar Kelimeler: Alzheimer hastalığı, oksidatif stres, *SLC7A11, GPX4, CAT, ACSL4*

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease marked by increasing cognitive dysfunction, as well as accumulated amyloid plaques and neurofibrillary tangles in the brain. (1). The increase in the prevalence of AD is one of the major health problems. Loss of synaptic communication and neuronal death are among the main underlying causes of AD (2). However, the mechanisms that underpin neuronal death in AD are not fully understood and that holds back the improvement of efficient therapeutic approaches. Data from several studies suggests that approximately 80% of AD cases are due to genetic factors, based on twin and family studies (3,4). In addition to genetic factors, environmental factors are also very important in a sporadic form of AD, especially after the age of 65 (5). The oxidative stress is a common condition that has considerable impact on AD etiology. Oxidative stress causes oxidative modifications in nuclear and mitochondrial DNA in AD. Increased reactive oxygen species (ROS) formation, mitochondrial dysfunction, an impaired antioxidant activity, or a combination of these elements are the most common causes (6). ROS can act as a signal molecule or alter gene expression of signal molecules within the cell. Increased ROS residuals may affect the molecular mechanism of synaptic activity and neurotransmission causing cognitive dysfunction (7). Also, oxidative stress is associated with the deposition of β-amyloid (Aβ) plaque (8), while Aβ plaque leads to the formation of free radicals and oxidative stress (9). Against oxidative stress, the body uses its own defense mechanism through antioxidants. Changes in the expressions of antioxidant enzyme genes are important for the cell response to oxidative damage (10). One of these antioxidant enzymes is glutathione peroxidase 4 (GPX4) which needs glutamate, cystine and cysteine for enzyme activation (11). Catalase (CAT), encoded by the *CAT* gene, is another important antioxidant enzyme that basically converts two molecules of hydrogen peroxide into one molecule of oxygen and two molecules of water in a two-step reaction (12). A study performed by Habib et al. showed catalase-amyloid interactions in neurotoxic Aβ peptides stimulated oxidative stress (13). Solute carrier family 7 member 11 (SLC7A11) has an important function in the antioxidant mechanism as it supports the survival and growth of the cell by providing cystine uptake and promoting glutathione synthesis under oxidative stress (14). Ferroptosis, a recently identified mechanism of cell death, has also been shown to play a role in oxidative stress and neurodegenerative disorders in recent years. The catalytic enzyme acyl-coa synthetase long chain family member 4 (ACSL4) is involved in the ferroptosis lipid metabolism pathway. The ACSL4 establishes cell susceptibility to ferroptosis and leads to ferroptotic cell death (15).

The function of antioxidant enzymes is mostly regulated at the transcriptional level. Inability to produce sufficient antioxidant enzyme mRNA against ROS accumulation causes the cell to be unable to defend itself against oxidative stress. Variations in antioxidant gene expression levels can lead to oxidative harm in AD patients' central nervous systems (10). If oxidative stress increases in AD, an increase in the function of antioxidant enzymes and gene expressions can be expected in AD patients. Yet, conflicting results have been obtained in postmortem studies in AD brains on antioxidant gene expressions (16) and there are few studies showing the alteration of antioxidant gene expression levels in AD (10). This study investigated whether *GPX4, CAT, SLC7A11* and *ACSL4* gene expressions, which are known to be associated with oxidative stress, changed in the peripheral blood of AD patients.

MATERIAL AND METHOD

Study Population

The research involved 25 AD patients and 22 healthy controls who had no background of significant neurologic or mental illness. Participants were recruited from Istanbul Faculty of Medicine, Istanbul University in the Behavioral Neurology and Movement Disorders Unit of Neurology Department. The diagnosis of dementia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders (NINCDS-ADRDA) criteria. All participants signed informed consent forms. The Istanbul University's Ethics Committee gave approval to the study's procedures.

Genetic Analysis

Total RNA was collected from leukocytes in the peripheral blood using trizol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The iScript™ cDNA synthesis kit (Bio-Rad, CA, USA) was used to synthesize cDNA from 1µg of total RNA according to the manufacturer's protocol. The SYBR Green I Assay (Thermo Fisher Scientific, MA, USA) was used in a qRT-PCR on the Lightcycler 480 Real-Time PCR system (Roche, Basel, Switzerland). The data was normalized using *GAPDH* as the housekeeping gene, and the relative expression levels of *SLC7A11, GPX4, CAT,* and *ACSL4* genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Data Analysis

Expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* among groups were compared by the non-parametric two-tailed Mann–Whitney U test. Spearman's rho test was performed to investigate correlations between *SLC7A11, GPX4, CAT* and *ACSL4* mRNA expressions and age, age at onset, and mini mental state examination (MMSE) scores. The categorical variables were compared using Fisher's exact test. A p value was considered statistically significant for less than 0.05. All statistical analysis was performed using SPSS Statistics 23.0 software (IBM Corp., USA).

RESULTS

Table 1 summarizes the descriptive characteristics of the study population. There was a significant age gap between AD patients and controls, and the overall MMSE score in patients was significantly lower than in controls (Table 1).

The expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* genes

Table 1. Descriptive characteristics of study population.

	AD (n=25) (mean±SD)	Control (n=22) (mean±SD)	p-value
Age, years, (range)	77.20±5.058, (67-89)	73.90±6.265, (66-91)	**0.035**
Age of onset, years, (range)	72.20±5.848, (65-84)	-	
Gender, % (n)			0.452
Male	48% (13)	59.1% (13)	
Female	52% (12)	40.9% (9)	
MMSE score (range)	18.39±5.255, (10-26)	27.72±3.232, (17-30)	**< 0.0001**

Mann Whitney-U test was used to compare means and Fisher's exact test for percentages, p values in bold shows statistical significance.
AD: Alzheimer's disease, MMSE: Mini Mental State Examination, SD: standart deviation, n: number of individuals

in leukocyte did not vary significantly between AD patients and control subjects. As shown in Figure 1a, the *SLC7A11* expression level in AD (mean±SEM; 1.21±0.18) was similar to controls (1.12±0.16, p=0.819). Although *GPX4* expression seems to be increased in AD (1.89±0.17) as compared to controls (1.56±0.20), no significant difference was found (p=0.207, Figure 1b). In addition, lower *CAT* expression was observed in AD patients (0.89±0.10) as compared to controls (1.09±0.09) without any significance (p=0.72, Figure 1c). Finally, the *ACSL4* expression appears to be higher in AD patients (2.42± 0.25) as compared to controls (2.19±0.16, Figure 1d) without statistical significance (p=0.889).

In AD patients, a correlation analysis was used to show the relationship between age, age of onset, MMSE score, and the expression levels of *SLC7A11, GPX4, ACSL4* and *CAT* genes. The *ACSL4* expression and age at onset had a significant negative correlation, according to our findings (r=-0.458, p=0.028; Figure 2). Other gene expressions were found to have no significant correlation with age, age of onset, or MMSE score in AD patients (Supplementary Figure 1 and 2).

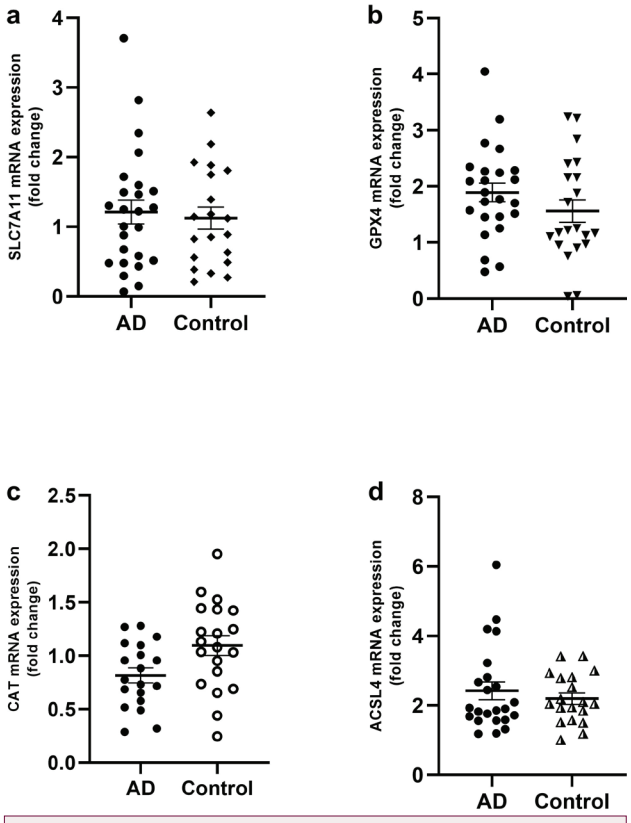

Figure 1. mRNA expression levels in study population **(a)** *SLC7A11* expression in AD and control subjects **(b)** *GPX4* expression in AD and control subjects **(c)** *CAT* expression in AD and control subjects **(d)** *ACSL4* expression in AD and control subjects. Data are presented with mean±SEM, AD Alzheimer disease, SEM standard error of the mean.

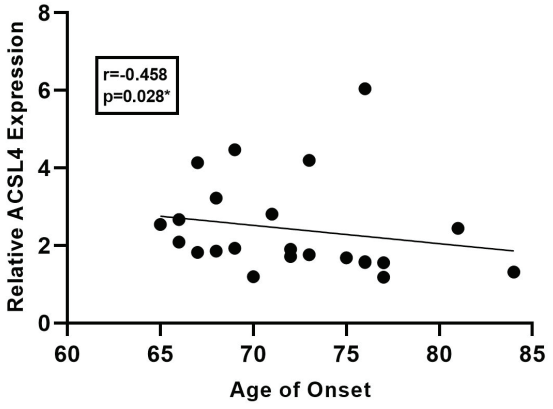

Figure 2. Correlation between *ACSL4* mRNA expression and age of onset in AD patients.

DISCUSSION

Several reports have shown the relationship between oxidative stress and AD etiology (7-9). Oxidative stress tends to increase in the brain with age. Even so, the role of oxidative stress in the etiology of AD is still unknown. The main goal of this research was to investigate whether the expression levels of specific oxidative stress-related genes could be identified in peripheral blood of AD patients. For this purpose, a study was made of the peripheral expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* genes that were known to play a role in oxidative stress.

Cell death by apoptosis is known to occur in response to oxidative stress, and GPX4 has been identified as a key regulator of apoptosis in response to oxidative stress (17). In transgenic mice, increased expression of *GPX4* preserves neurons from oxidative stress and amyloid toxicity (17). Furthermore, in a study by Hambright et al., inactivation of *GPX4* gene in neuronal cells promotes hippocampal neurodegeneration and cognitive dysfunction in a mouse model (18). Studies investigating the relationship between *GPX4* and AD were limited to animal models, so far there has been only one study in humans, and that study explored the effect of *GPX4* polymorphism on episodic memory (19). Therefore, this is the first study to investigate *GPX4* gene expression levels in AD patients. The results showed that *GPX4* expression increased in the peripheral blood of AD patients, although it was not statistically significant. This increase may be the result of neuroprotective effect of *GPX4* in AD.

SLC7A11 plays a crucial role in antioxidant protection by mediating cystine uptake, promoting glutathione synthesis, and sustaining cell viability against oxidative stress conditions (14). In the central nervous system (CNS), glutamate export is particularly important since it is a non-vesicular pathway of release for this excitatory neurotransmitter, which can play a role in neuronal signaling or excitotoxic pathology (20). One study showed that by releasing glutamate through microglia increased Aβ toxicity which suggested that since the *SLC7A11* gene was expressed not only in cultured microglia but also in the reactive microglia inside or around amyloid plaques in transgenic mice, this could be important to Aβ toxicity in AD (21). In a study conducted in the peripheral blood of schizophrenia patients, the significant reduction of *SLC7A11* gene expression supports the hypo-glutamatergic neurotransmission hypothesis in the pathogenesis of schizophrenia and the role of SLC7A11 in neurological diseases (22). However, *SLC7A11* gene expression levels in the peripheral blood of AD patients has not been investigated so far. Although the results showed that the peripheral blood expression of *SLC7A11* did not change in AD, this should be considered preliminary because of the small size and should be further investigated in a larger study.

Catalase is an antioxidant enzyme which is involved in oxidative stress mechanism (12). It has been suggested that catalase deficiency or degradation is implicated in the pathogenesis of degenerative diseases including diabetes mellitus, cancer, AD, Parkinson's disease and hypertension which increase in frequency with age (23). In the analysis of different brain regions of AD patients, Aksenov et al. found a substantial increase in *CAT* expression in brain regions affected by AD pathology (9). This suggested that this antioxidant enzyme could play an important role in protecting CNS cells from oxidative stress in AD. Also, the same study showed that an increased expression of *CAT* leads to an increased tolerance of cultured neural cells to Aβ toxicity (10). In another study conducted in late-onset AD (LOAD) patients, it was observed that *CAT* gene expression levels in peripheral blood were significantly decreased compared to the controls, but the erythrocyte CAT enzymatic activity was significantly increased in LOAD patients. This suggests that even as the expression of antioxidant genes decreased, their enzymatic activity was not affected (24). Consistent with that study, we observed a decreased mRNA expression of *CAT* in AD patients but without statistical significance. However, since CAT enzyme activity was not measured, a comparison could not be made like the study of González-Mundo et al.

Considering the fact that the brain is the fattiest organ after adipose tissue, ACSL4 is regarded as an important enzyme for neurodegenerative diseases due to its role in lipid metabolism although it has not been fully investigated in AD (25). An *in vitro* study of embryonic stem cells showed that the nerve growth factor, retinoic acid induced neuronal differentiation, and neurite growth were weakened by a knockout of the *ACSL4* gene (26). This study showed a non-significantly decreased expression level of *ACSL4* in AD patients, also a correlation analysis pointed out that the expression of *ACSL4* decreased with increasing age of onset. The fact that AD patients had higher *ACSL4* expression levels may support the assumption that a lipid metabolism pathway is involved in the pathogenesis of oxidative stress in AD. Since our study is the first investigating the *ACSL4* expression levels in AD patients, it is necessary to replicate our findings in larger patient-control groups and to support by further *in vitro* studies.

Aging is a major risk factor for AD, and it is known that the expression of several genes are altered during aging. Many studies have shown that the expression of various genes involved in DNA repair, energy metabolism, oxidative stress response, and synaptic activity changes with aging (27). Although limited to animal studies, *GPX4* and *SLC7A11* gene expressions have been shown to alter with age, while the effect of age on *ACSL4* gene expression is unknown (28,29). Also, a study by Tatone et al. showed that the *CAT* mRNA levels were significantly lower in women over the age of 38 (30). In this study, we did not find any correlation between age and the expression of studied genes. This may suggest that age may not influence the expression of these genes, but it should not be ruled out since this research was conducted with a small study group. Although the age of the patients and controls was similar, we found a statistically significant difference between them. However, no significant effect of age was observed in correlation analysis.

Our study has several limitations. First, the current study was conducted using a small sample size. Second, the activities

of enzymes that are products of studied genes could not be measured. Therefore, the ability to investigate the relationship between extracellular enzymatic activity and mRNA expression levels was hampered. Lastly, considering AD is a CNS disease, the whole blood-based mRNA analysis is a limitation factor. Future research should focus on mRNA expression in brain tissues from Alzheimer's patients and *in vitro* cell cultures. The present study laid the groundwork for future research into oxidative stress related gene expressions in AD.

CONCLUSION

In conclusion, the results showed that peripheral blood expressions of the *GPX4, SLC7A11, CAT* and *ACSL4* genes were not altered in AD. However, further studies investigating the expression of these genes in brain tissue rather than peripheral blood will be more informative to elucidate their roles in AD etiology.

Ethics Committee Approval: The Istanbul University's Ethics Committee gave approval to the study's procedures. All participants signed informed consent forms.

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