A Study Investigating the Role of 2 Candidate SNPs in Bax and Bcl-2 Genes in Alzheimer's Disease

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Objective: The proto-oncogene *Bax* (Bcl-2-associated X protein) and related protein *Bcl-2* (B-cell chronic lymphocytic leukemia/lymphoma-2) genes are triggers of apoptosis in Alzheimer's disease (AD). The balance of these proteins has an important role in the death or life of a neuronal cell, and the functional polymorphisms in genes expressing these proteins have been found to promote apoptosis. To investigate the role of *Bax* and *Bcl-2* genes in AD, we examined the presence of the 2 polymorphisms in peripheral blood. To our knowledge, this is the first clinical association study of these 2 functional SNPs using the peripheral blood of patients with AD.

Methods: *Bax* (rs4645878) and *Bcl-2* (rs2279115) in Alzheimer's patients (N = 132) and healthy controls (N = 109), aged 65 to 85 years, were analyzed by qPCR (Quantitative Polymerase Chain Reaction) using TaqMan probe technology. Statistical analyses were done using SPSS, 11.5. The differences between groups were analyzed using an independent-samples t test. The relationships between genotypes and alleles were analyzed using chi-square or likelihood ratio test. The Hardy–Weinberg balance was checked for the patient and control groups. A p-value of less than 0.05 was taken as significant.

Results: Sporadic AD patients and non-demented age-matched control subjects were genotyped in this case-control study. No statistically significant relationship was found between the patients and controls for allele or genotype frequencies (p>0.05).

Conclusion: Our data suggest that these two polymorphisms do not contribute to AD in the population from the Mersin region of the Eastern Mediterranean. Further studies with larger sample sizes must be conducted to ascertain the association between the 2 polymorphisms. [*P R Health Sci J 2020;39:264-269*]

Key words: Apoptosis, Polymorphism, Bax, Bcl-2, Alzheimer's disease

Alzheimer's disease (AD) is a chronic, progressive, neurodegenerative brain disease that prevents the continuation of daily life. By nature, the clinical symptoms of AD are both pathologically and structurally heterogeneous, and the relationship between these two characteristics yet remains poorly understood. Although many studies have been conducted to describe AD, there is still no definitive diagnosis. New advancement strategies focusing on new molecules and pathways are urgently needed. In this regard, *Bax* and *Bcl-2* gene variants are two significant polymorphic markers for AD.

The accumulation of amyloid beta (Aβ), neurofibrillary degeneration, and neuronal and synaptic loss are important pathological hallmarks in AD (1). Neurofilament proteins are major components of myelinated axons and the primer cause of neurological disorders as AD. Many molecular alterations can lead to accumulation of neurofilaments. Neurofilament structure is composed of phosphorylated tau proteins. Binding to microtubules, these tau proteins are involved in their stabilization as well as in the protection of the cytoskeleton and axonal transport (2); when tau is phosphorylated, it forms double-stranded neurofibrillary tangles, as are seen in AD (3). The induction of apoptosis occurs with the accumulation of tau. The formation of amyloid plaques contributes to the mechanisms that bring about apoptotic neurodegeneration (4). In addition to these mechanisms, the Bax and Bcl-2 proteins also have an important role in deciding the fate of the apoptotic cell (5): The *Bax* gene regulates cell death, and the *Bcl-2* gene regulates the survival of the cell (6, 7). *Bcl-2* is known to be one of the

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proteins that inhibit apoptosis in various cell types. It works to protect neurons from apoptosis, and it has an important role in AD pathogenesis (8). Aβ-induced neurotoxicity has been linked to oxidative stress and changes in intracellular calcium levels, both of which are equally likely to contribute to such toxicity. Bcl-2 acts as an anti-death protein in neurons and can also prevent neuronal apoptosis (9). Therefore, it can be seen that Bcl-2 is the key regulator of programmed cell death in neurons. Any change in the level of this protein may cause increased neuronal sensitivity (10). *Bcl-2* is expressed in the embryonic and adult nervous systems (11). In the adult nervous system, it is expressed in areas where neurogenesis occurs (12). When neurogenesis is largely completed, the expression level decreases but remains high in the neurons in the peripheral nervous system. The overexpression of *Bcl-2* reduces neuronal cell death by increasing the number of neurons in specific regions of the brain (10). *Bax* inhibits *Bcl-2* to accelerate apoptosis (13). *Bax* facilitates apoptosis by translocating into the mitochondrial membrane and mediating the survival response that promotes apoptosis. Moreover, it contributes to enhancing the *Bax* / *Bcl-2* ratio within the cell during the increase of apoptotic cell death (14). These two genes are the bridge between life and death. Programmed cell death occurs throughout life, starting from development and eliminates many neurons during development. These data were obtained by preventing the mechanisms in the formation of proteins synthesized by *Bax* and *Bcl-2* genes in many neuron populations (15).

Although there is new information that illuminates the molecular and genetic bases of AD (16), its clinical diagnosis remains difficult, and more postmortem tissue samples are needed. There are several techniques for reaching a clinical diagnosis of AD. However, new strategies and target genes are needed to slow down or stop the progression of neurodegeneration in AD for an early and accurate diagnosis (17). Genome-wide association studies have revealed a risk for the SNPs of *APP*, *PSEN1*, and *PSEN2* genes and the APOE ε4 allele in late-onset AD (16). SNPs are powerful biomarkers in human disease. Therefore, we examined whether SNPs have a role in characteristic changes in AD patients.

SNPs are important genetic markers that can be used to assess an individual's susceptibility to a disease. Surprisingly, although these two functional SNPs (rs4645878 and rs2279115) have been studied in complex diseases such as cancer, no similar studies have been conducted on the apoptosis pathway genes in AD. These genes were chosen for our study because of the gap in the literature on this matter and the importance of the apoptosis mechanism in AD. The aim of the study was to evaluate the possible association between AD and the two selected functional SNPs (rs4645878, rs2279115) in the *Bax* and *Bcl-2* genes.

Materials and Methods

Study population and Epidemiological data

The study population was recruited at the Neurology Department of the Medical Faculty of Mersin University in Turkey. Participants consisted of 132 Turkish patients with AD (50 males, 82 females) and 109 (52 males, 57 females) healthy volunteers (The number N is different due to technical problems in the recruiting process). The patients with AD were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders-IV criteria (DSM-IV). Using the DSM-IV as our guide, we accepted into the study those individuals who had the following conditions and/or dysfunctions: memory impairment (struggles with learning new information, recalling previously learned information, or both), aphasia, apraxia, agnosia, deficits in executive functioning (planning, organizing, sequencing, and abstracting), and impaired professional and/or social functioning. Importantly, the presence in potential participants of complex comorbid disorders such as hypertension, diabetes, or vascular disease was determined by taking detailed medical histories; the individuals who had such comorbidities were not included in the study group. The control subjects were recruited from the same clinic. They were cognitively intact and had no AD history. The study protocol was approved by the Medical Faculty Ethics Committee. Consent was obtained from all the individuals. Whole blood samples were collected into heparinized tubes coded for each individual. Table 1 shows the gene names, SNP IDs, chromosomal locations, polymorphisms and their position, amino acid changes, and minor allele frequencies of the selected SNPs. The SNPs were located in the promoter, and these SNPs were studied for the first time in terms of their link to AD.

DNA extraction

DNA was extracted from whole blood using a modified salt-precipitation method (18). Eight ml of sterile distilled water (cold) was added to 5 ml of blood sample in a 15 ml polypropylene tube, belonging to each individual, and the tubes were shaken rapidly for 2 to 3 minutes. The mixture was centrifuged at 2000 rpm for 10 min at room temperature. After discarding the supernatant, approximately 8 ml of cold sterile distilled water was added to the tubes. The tubes were centrifuged at 2000 rpm for 10 minutes at room temperature, the supernatant was completely discarded and only the pellet

Table 1. Gene Polymorphisms in AD.

Gene	Gene ID	Chromosome Location	SNP ID	Position	Variant (M>m)	MAF (%)
Bax (-248 G>A)	581	chr19:48954681	rs4645878	Promoter	G>A	A = 0.0797
Bcl-2 (-938 C>A)	596	chr18:63319604	rs2279115	Promoter	C>A, T	T = 0.3854

M stands for major allele; m, minor allele; MAF, minor allele frequency (reported in the dbSNP database); and SNP, single nucleotide polymorphism.

remained in each tube of the patient and control samples. Three ml of nuclear lysis buffer (10mM Tris-HCl, 400mM NaCl, and 2mM Na2EDTA) was added to each pellet. Ten mg/µg of Proteinase K and 10% SDS (sodium dodecyl sulphate) were added slowly to this mixture, without foaming. Incubated overnight at 37 °C, after incubation, the samples were incubated, again, for 1 hour at 55 °C to inactivate the Proteinase K. Ammonium acetate (6M) was added to this mixture and rapidly quenched ~ 20 times. It was kept at room temperature for 10 min and centrifuged at 3500 rpm for 15 min. The supernatant was transferred to another tube, and pure ethanol was added at room temperature. Tris-EDTA (10mM Tris-HCl, 1mM Na2EDTA; pH = 8) buffer solution was added to the tubes, and then DNA was measured using a spectrophotometer (Multiskan™ GO; Thermo Scientific, MA; ABD).

Real-Time PCR analyses of SNPs

SNP genotyping primers were purchased from Thermo Fisher Scientific (based on TaqMan probe technology). The rs4645878 (C_27848291_10) and rs2279115 (C_3044428_30) promoter polymorphisms' characterizations were accomplished in a final volume of 20µl containing 10ng of DNA, 2X TaqMan Universal PCR Master Mix, 900nM of primer, 200nM of the probe, and nuclease-free water. PCR conditions started with pre-incubation for 1 min at 60 °C and initial denaturation, 10 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 15 sec and annealing at 62 °C for 1 min. Analyses were performed with the ABI PRISM™ 7000 real-time PCR system (Applied Biosystems), and genotyping data were obtained using the SDS software (version 2.0.6) for allelic discrimination (Applied Biosystems).

Statistical analyses

Statistical analyses were performed using SPSS for Windows, version 11.5 (Chicago, IL: SPSS Inc.). Data were expressed as means (± standard deviations) or N (%). An independent-samples t test was used to test whether there was a statistically significant difference between the 2 independent groups, by the average difference. Associations of genotypes and alleles were analyzed using the χ^2 or the likelihood-ratio test. Chi-square tests were used to compare categorical variables. The chi-square data for the alleles not seen in either the control subjects or the patients were calculated using a recessive model (19). Hardy–Weinberg equilibrium was checked for the patient and control groups. Two-tailed p values less than 0.05 were regarded as significant.

Results

The study population consisted of 241 individuals, 132 AD patients and 109 healthy controls. Their genders and ages are presented in Table 2. The genotype and allele distributions of SNPs in the patient and the control groups are displayed in Table 3. A statistically significant association with the risk of AD was not observed with the genotypes for rs4645878 on the *Bax* gene (p = 0.956; OR = 1.022; 95% CI = 0.452–2.117 for AA+AG) or for rs2279115 on the *Bcl-2* gene (p = 0.031; OR = 0.474; 95% CI = 0.240–0.933 for the CA genotype; p = 0.078; OR = 0.506; 95% CI = 0.238–1.078 for the AA genotype according to the wild-type CC genotype). Genotype and allele frequencies were consistent with Hardy–Weinberg Equilibrium (HWE) in all groups for the *Bcl-2* gene. The SNP on the *Bax* gene was not in linkage disequilibrium. Age was a risk factor for AD risk (p<0.001; OR = 0.878–2.451; CI = 0.090–0.674), whereas gender was not associated with AD (p = 0.058). Our

Table 2. Sample description

Variable	Patients (N = 132)	Controls (N = 109)	P-value
Age (mean ± SE)	73.92 ± 7.25	69.60 ± 6.11	p<0.001
Gender (n (%)			
Female	82 (62.1)	57 (52.3)	p>0.05
Male	50 (37.9)	52 (47.7)	

P-values were derived from the χ^2 test (for categorical variables [gender]) and the t test (for continuous variables [age]). N stands for number of individuals.

Table 3. Allele and Genotype distributions of Two SNPs in Patients and Controls.

Gene Name (Patient/Control)	Recessive Model	Patient		Control		P-Value	Genotype	Gender	Patient		Control		P-Value
		N	F (%)	N	F (%)				N	F (%)	N	F (%)	
Bax rs4645878	AA + AG	15	0.52	15	0.50	>0.05	AA	Female	1	0.01	0	-	>0.05
								Male	0				
(108/106)	GG	14	0.48	15	0.50		AG	Female	9	0.13	15	0.14	
								Male	5				
							GG	Female	45	0.86	91	0.86	
								Male	40				
Bcl-2 rs2279115	C	38	0.88	18	0.25	>0.05	CC	Female	20	0.31	18	0.18	>0.05
								Male	18				
(122/100)	A	5	0.12	53	0.75		CA	Female	30	0.44	53	0.53	
								Male	23				
							AA	Female	16	0.25	29	0.29	
								Male	15				

N stands for number of individuals; F, frequency of genotype and alleles; a χ^2 test was used for categorical variables; p-values were derived from the χ^2 test for categorical variables.

results show that no statistically significant association with the risk of AD was observed for the genotype and allele frequencies of rs4645878 on the *Bax* gene or rs2279115 on the *Bcl-2* gene (Table 3). The SNPs explored in the present study were not statistically associated with AD ($p>0.05$).

Discussion

Apoptosis as a general cell death pathway in neurodegenerative diseases (20). Neurodegeneration is the most important neuropathological hallmark of AD, and in vitro studies have shown that apoptotic mechanisms are affected by Aβ neurotoxicity, which is a major cause of AD (21, 22). Aβ is a key molecule in the neurodegenerative pathways of AD. This molecule directly induces neuronal apoptosis. However, the mechanisms of neuronal apoptosis induced by Aβ have not been fully elucidated.

Aβ deposition, neurofibrillary degeneration, and the loss of neurons and synapses are important pathological evidence of the presence of AD in samples obtained from the postmortem brain tissue of patients with AD (23). *Bax* and *Bcl-2* genes have important roles in apoptotic cell death and cell survival mechanisms, respectively. Some of the studies in neuronal cell cultures showed that Aβ promoted neuronal apoptosis (24). The reflection of neuronal apoptosis in AD can be explained by the presence of features such as chromatin condensation and nuclear fragmentation in the brain tissues of these patients. Immunohistochemistry studies with hippocampal samples from patients with AD showed that Bax concentration was high in senile plaques. The Aβ-positive plaques that may lead towards AD pathology can be said to be formed by an increase (by the apoptotic mechanism) in the amount of Bax protein in these areas (25). The rs4645878 and rs2279115 polymorphisms may cause losses in *Bax* gene expression, which then leads to neuronal cell death. Moreover, excessive expression in the *Bcl-2* gene may result in vulnerability to apoptotic mechanisms in AD. The overexpression of the anti-apoptotic protein Bcl-2 was established in transgenic animal models (26). In this animal study, the overexpression of *Bcl-2* inhibited the activation of caspase-9, and decreases the caspase cleavage of tau. These findings suggest that the activation of apoptotic mechanisms can be an untimely event in AD, and such activation can trigger disease mechanisms underlying AD. In a study using a rat model of AD, the test animals were treated with curcumin, the thinking being that it would alter the expression of *Bax* and *Bcl-2* genes. It was found that *Bcl-2* expression increased while *Bax* expression decreased. At the same time, gene expression has been observed to change significantly in the learning and memory functions of rats (27).

SNPs have notable importance in the genetic bases of diseases (28). In addition to the *APP*, *PSEN1*, *PSEN2*, and *ApoE* genes, which are already known to be responsible for AD, new genetic markers are needed if we are to diagnose AD (11). Several studies have shown that the *Bax* and *Bcl-2* genes have important roles in several ailments, such as different types of cancer, diabetes, Crohn's disease, Huntington's disease, and AD (29–33).

In this study, the relationship between these two SNPs and AD risk was investigated. The functional SNPs in the coding regions of the candidate genes have a direct impact on the structure and function of affected proteins. Moreover, SNPs may occur in their specific binding sites for transcription factors, and these SNPs can affect gene expression. The abnormal expression of these genes could lead to instability in several metabolic pathways or biological mechanisms. The investigation of the promoter SNPs of genes can provide important data on the etiology of such complex diseases as AD. *Bcl-2* is one of the several proteins that inhibit apoptosis in various cell types (34). Apoptosis plays an important role in the pathogenesis of AD. In order to better understand the role of these proteins in programmed cell death and survival (neuro-protection), there is a need to investigate additional markers in AD. Therefore, the other SNPs in the *Bax* and *Bcl-2* genes and in other potential genes that are known to be functional must be investigated at the molecular level so that researchers can better assess the complex nature of the disease. Our new findings will contribute to determining the strength of the relationship between AD and apoptosis.

We examined the effects of *Bax* (death) and *Bcl-2* (anti-death) gene polymorphisms on AD. Although, Aβ has an important role in AD, how it induces neuronal cell death has not been clearly elucidated. Bax and Bcl-2 proteins play an important role in neuronal cell death and survival. The expression levels of these proteins are associated with AD pathology, and differences in equilibrium in the expression of the two proteins may also affect AD.

The overexpression of Bcl-2 protein in glial cells surrounding senile plaques suggests that *Bcl-2* may play a role in the survival of glia. On the other hand, the overexpression of *Bax* in senile plaques suggests that *Bax* is associated with neurite degeneration in these plaques. The investigation of other potential SNPs that may be effective in the function of proteins that are the products of the *Bax* and *Bcl-2* genes may be helpful in revealing the relationship between these genes and AD. The limitations of this study are the lack of research on the expression of relevant genes, the large sample size, and the lack of studies in the database, which studies would allow us to compare ethnic differences.

Conclusion

These data provide new information for genetic investigations of AD, focusing particularly on whole blood for new molecular diagnostics for AD. To the best of our knowledge, the present study is the first report in the literature to look at *Bax* and *Bcl-2* candidate gene SNP variations and how they relate to AD susceptibility/risk. Our results prove that the accurate characterization of these SNPs in large-scale studies of different

ethnic groups is essential. The data obtained can provide a new basis for encouraging future discussions on and genetic research of AD by focusing on new biological markers (especially those that might be found in the easily available peripheral tissues) for new molecular diagnoses of AD.

Resumen

Objetivo: El protooncogén *Bax* (*la proteína X asociada con Bcl-2*) y los genes de la proteína *Bcl-2* (*B-cell lymphoma 2*) relacionados son desencadenantes de la apoptosis en la enfermedad de Alzheimer (EA). El balance de estas proteínas tiene un papel importante en la muerte o en la vida de células neuronales y se ha encontrado que los polimorfismos funcionales en los genes que expresan estas proteínas promueven la apoptosis. Para investigar el papel de *Bax* y *Bcl-2* en la EA, examinamos los dos polimorfismos en la sangre periférica. Según nuestro conocimiento, este es el primer estudio clínico de asociación sobre los SNP relacionados con pacientes con la EA que utilizan en sangre periférica. Métodos: Se analizaron *Bax* (rs4645878) y *Bcl-2* (rs2279115) mediante PCR en tiempo real en EA pacientes (N = 132) y controles sanos (N = 109) de 65-85 años con el método de qPCR con sonda TaqMan. Los análisis estadísticos se realizaron utilizando el SPSS-11.5. El valor de p inferior a 0.05 se tomó como significativo. Resultados: Los pacientes con la EA esporádica y los sujetos de control emparejados por edad no demenciales fueron genotipados en este estudio de casos y controles. No se encontró una relación estadísticamente significativa entre los pacientes y los controles para las frecuencias de alelos y genotipos ($p > 0.05$). Conclusión: Nuestros datos sugieren que estos dos polimorfismos no contribuyen a la EA en la población turca. Se deben realizar estudios adicionales de mayor tamaño de muestra para en fin determinar alguna relación definitiva entre los polimorfismos de apoptosis en pacientes con la EA y la asociación definitiva entre los dos polimorfismos.

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