

The Relationship Between Blood Antioxidant Enzyme Levels and Genotype During Migraine Attack and Initial Periods in Migraine Patients

[Atak ve Atak Dışı Dönemdeki Migren Hastalarında Kan Antioksidan Enzim Düzeyleri ve Genotip Arasındaki İlişki]

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ABSTRACT

Objective: In recent years, researchers have been focused on the importance of oxidative stress on migraine pathogenesis. We aimed to investigate the roles of *Superoxide Dismutase* (SOD), *Glutathione Peroxidase* (GSH-Px), Catalase (CAT), antioxidant *Glutathione* (GSH) and *Malondialdehyde* (MDA) of a lipid peroxidation product in migraine pathogenesis. Furthermore, we also aimed to investigate the incidence of the gene polymorphisms of these enzymes in Turkish population.

Material and Methods: GSH-Px, CAT, SOD activities and plasma MDA and blood GSH levels were measured in 40 migraine patients at attack and initial period and 40 healthy subjects. The genotype characteristics were determined by PCR-based RFLP method using DNA extracted from peripheral blood.

Results: Statistically significant decrease in GSH-Px, CAT, SOD activities and GSH levels were found in the migraine patients compared to the control group (GSH, CAT $p < 0.001$; GSH-Px, SOD $p < 0.05$). In the migraine patients and control groups no significant differences were observed for Mn-SOD and CAT genotypes, but there was a significant difference in the GSH-Px3 genotype. No difference was detected in the allele frequencies of MnSOD, GSH-Px3 and CAT gene between the patients and control group.

Conclusion: Consequently, oxidative stress and antioxidant enzymes may play an important role in migraine pathogenesis, and according to our data GSH-Px3 gene polymorphism may cause a tendency to migraine for our regional community.

Conflict of Interest: There is no conflict of interest between authors.

Key Words: Migraine, manganese superoxide dismutase, *glutathione peroxidase-3*, catalase, polymorphism

ÖZET

Amaç: Son yıllarda çoğu hastalıkta olduğu gibi migren patogenezinde de oksidatif stresin önemi araştırılmaktadır. Süperoksit Dismutaz (SOD), Glutatyon Peroksidaz (GSH-Px), Katalaz (CAT), antioksidan Glutatyon (GSH) ve lipid peroksidasyon ürünü Malondialdehit (MDA)'in migren patogenezindeki rollerini araştırmayı amaçladık. Ayrıca çalışmamızda, bu enzimlerin gen polimorfizmlerine bakarak Türk popülasyonunda bu polimorfizmin görülme sıklığını araştırmayı amaçladık.

Yöntem: Atak ve atak dışı dönemde bulunan 40 migrenli hasta ve 40 sağlıklı bireyin, GSH-Px, CAT, SOD aktiviteleri ve plazma MDA ve kan GSH düzeyleri ölçüldü. Genotipik özellikler, periferik kandan ekstrakte edilen DNA kullanılarak PCR'a dayalı RFLP metoduyla tespit edildi.

Bulgular: Migren hastalarıyla kontrol grubu karşılaştırıldığında, GSH-Px, CAT, SOD ve GSH düzeylerinde istatistiksel olarak azalma tespit edildi (GSH, CAT $p < 0.001$; GSH-Px, SOD $p < 0.05$). Migren hastaları ve kontrol grubunda Mn-SOD ve CAT genotipleri için anlamlı bir farklılık gözlenmedi, ama GSH-Px3 genotipinde anlamlı bir farklılık bulundu. MnSOD, GSH-Px3 ve CAT genlerinin allel frekansı açısından hasta ve kontrol grubu arasında farklılık bulunmadı.

Sonuç: Sonuç olarak, migren patogenezinde oksidatif stres ve antioksidan enzimler rol oynayabilir ve verilerimize göre GSH-Px3 geni polimorfizmi toplumumuz için migrene yatkınlığa sebep olabilir.

Tarafsızlık Beyanı: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Anahtar Kelimeler: Migren, mangan süperoksit dismutaz, glutatyon peroksidaz-3, katalaz, polimorfizm.

Introduction

Migraine headache is a public health problem of enormous scope that has an impact both on the individual sufferer and on the society. Migraine without aura is idiopathic, recurring headache disorder manifesting in attacks lasting 4 to 72 hours. Typical characteristics of such headaches are unilateral location, pulsating type, moderate or severe intensity, aggravation by routine physical activity and association with nausea, photophobia and phonophobia. Migraine with aura is a recurring disorder manifesting with attacks of neurologic symptoms unequivocally gradually developed over 5 to 20 minutes or photophobia usually preceding the onset of unilateral pulsating headache. Tension-type headache is more common in women, with gender ratios ranging from 1.04 to 1.4. Prevalence peaks between the ages of 20 and 50 years. Tension-type headaches often interfere with the activities of daily living. Eighteen percent of tension-type headache sufferers have to discontinue normal activity, while 44% experience some limitation of function [1-2]. Attacks occur with a mean frequency of 2.9 days a month or 35 days a year, most sufferers have less than 1 attack a month and about one-third have 2 or more attacks a month [1-2].

The pathophysiology of migraine is not well known. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are the antioxidant enzymes which are known to have critical importance in antioxidant system. These enzymes affect free radicals in metabolic pathways in different places. They have an important role in clearance of free radicals against tissue defect caused by these radicals [3,4]. The role of antioxidant enzymes in migraine pathophysiology has been reported [5-6].

In this study, we examined antioxidant enzyme activities which play an important role in migraine patients. All primary and secondary headaches are classified and defined by explicit diagnostic criteria in the International Classification of Headache Disorders second edition (ICHD-2) [7]. Migraine is the most common neurological disorder, but the molecular basis is still not completely understood. An impairment of oxidative metabolism might play a role in the pathophysiology.

The goal of this study was to investigate the differences in oxidative stress status with the measurement of SOD, CAT, GSH-Px activities and malondialdehyde (MDA), glutathione (GSH) concentrations in the migraine patients with or without aura and attack. However, with these respects in mind, the present study was carried out to examine the relationship of Mn-SOD, CAT, GSH-Px gene promotor polymorphism for migraine by using restriction fragment length polymorphism (RFLP) in the Turkish population.

Material and Methods

Cases

In our hospital-based case-control study, 40 patients with migraine (10 males and 30 females) and 40 controls (10 males and 30 females) without migraine disease were recruited. The diagnosis of headache was made in accordance with the International Headache Society (IHS) criteria; [7] the migraine with aura patients group consisted of 40; the mean age was 32.45 ± 6.30 years (mean \pm SD). The 40 controls without migraine disease was recruited; the mean age was 30.42 ± 3.75 (mean \pm SD). Written informed consent was obtained from all subjects. The study was carried out in the Neurology clinic of Firat University, Research and Application Hospital. Patients and healthy volunteers were recruited from the Department of Neurology, University of Firat. Furthermore, this study was approved by the Ethics Committee of the Medical Faculty of the same university. Medical, neurologic, psychiatric evaluations were made for all of the patients. Patients having these characteristic were excluded; first-axis psychiatric disorder present or in the past, history of alcohol and cigarette consumption, history of any kind of medicine consumption within the last 2 weeks prior to the study (except simple analgesic), neurologic disorder and /or symptom in clinic and history, history of head trauma, history of cardiovascular, renal and endocrinologic disorder and existing medical disorder. Among all participants, information on demographic characteristics and risk factors was collected using a structured questionnaire.

Measurement of GSH-Px, CAT, SOD activities and MDA, GSH levels

Venous blood samples were taken from the antecubital vein with suitable vacutainers with EDTA as anticoagulant. The basal venous blood was obtained from all the participants in this study in the morning after 12 hours of overnight fasting. In all cases blood samples were taken according to the principles of the Helsinki declaration. The blood samples were drawn from the patients and controls. Whole blood was separated for GSH-Px and GSH assays. Then remaining blood immediately centrifuged at 1500 g for 5 min. The erythrocytes and plasma were collected separately for CAT, SOD and MDA assays, respectively. All samples were kept at -20°C until assays.

Erythrocyte SOD activity was determined according to the method of Sun et al. [8]. Nitroblue tetrazolium salt (NBT), a superoxide radical scavenger, is reduced by superoxide to form blue formazan, whose absorption can be measured at 560 nm. One unit of SOD is defined as the amount of enzyme required to give 50% inhibition of the NBT reduction reaction compared with enzyme control. The enzyme activity in erythrocytes was expressed as units per gr of Hb (U/g Hb).

GSH-Px activity was measured by the method of Beutler [9] in which cumene hydroperoxide was used as a substrate. Oxidized glutathione (GSSG) produced by the ac-

tion of erythrocyte GSH-Px and cumene hydroperoxide was reduced by glutathione reductase and NADPH. The decrease of the NADPH concentration was measured at 340 nm. The enzyme activity in whole blood was expressed as U/g Hb.

The level of reduced glutathione was assayed by Beutler et al. [10] method and expressed as $\mu\text{mol/g Hb}$. The method is based on the capacity of sulfhydryl groups present in whole blood to react with 5, 5'-dithiobis-(2-nitrobenzoic acid) (Ellmann's reagent) and form a yellow dye with maximum absorbance at 412 nm.

The end-product of polyunsaturated fatty acid peroxidation, MDA, which reacts with thiobarbituric acid in plasma samples, was determined by the method of Placer et al. [11]. Reaction of MDA with thiobarbituric acid (TBA) has been applied widely to assess lipid peroxidation in biological material. The reaction yields a pink MDA-TBA adduct. The colored complex can be quantified spectrophotometrically from its visible absorbance (max 532 nm). The values of MDA reactive material was expressed as MDA quantities for plasma volume (nmol/ml plasma).

Erythrocyte CAT activity were determined according to the method of Aebi [12] and expressed as k/gHb. The decomposition of H_2O_2 can be directly followed by the decrease of absorbance at 240 nm. The difference in absorbance at 240 nm per time unit allows determining the CAT activity.

Haemoglobin (Hb) concentration was determined according to cyanmethaemoglobin method [13].

DNA extraction

Genomic DNA was extracted from whole blood by proteinase K using a method described elsewhere [14]. The DNA samples were then stored at 4 °C until used as a template DNA in polymerase chain reaction (PCR).

Determination of Mn-SOD, CAT, GSH-Px3 genotype

Mn-SOD, CAT, GSH-Px3 genotypes were determined by using a polymerase chain reaction (PCR)-restriction fragment length polymorphism, the PCR primers were designed based on described previously [15-17]. Sequences of primers, restriction enzyme and sizes of resultant PCR products specific for Mn-SOD, CAT and GSH-Px were summarized in Table 1. PCR reactions were carried out in a total volume of 25 μL , using approximately 100 ng DNA, 2.5 mmol/L MgCl_2 , 200 mol/L dNTPs, 12.5 ng of each primer, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI) in the PCR buffer provided by the manufacturer (10 mmol/L Tris-HCl, pH 9.0, and 50 mmol/L KCl). The PCR procedure was as follows: an initial denaturation step at 95°C for 5 min, and then amplification for 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min using the Gene Amp PCR

System 9700 thermocycler (PE Applied BioSystems).

The Mn-SOD RFLP (Ala-9 Val) gene; F: 5'-AC-CAGCAGGCAGCTGGCGCCGG-3', and the R: 5'-GCGTTGATGTGAGGTTCCAG-3' amplified a 107-bp PCR product [15]. The GSH-Px3 RFLP F: 5'-GAAATCCCAGCCGCCTA-3' and the R: 5'-CACT-CACCTTCGACTTCTCTTGCT-3' amplified a 260-bp PCR product [16]. The CAT RFLP sense primer 5'-TA-AGAGCTGAGAAAGCATAGCT-3' antisense primer: 5'-AGAGCCTCGCCCCGCCGGACCG-3' amplified a 340-bp PCR product [17]. Ten percent of PCR products were digested with MroN I (New England BioLabs) at 37°C for 16 h, with Bsa I (New England BioLabs) at 50°C for 4 h, with Sma I (New England BioLabs) at 37°C for 16 h. Digests were separated on a 3 % agarose gel with ethidium bromide. The electrophoresis was run for 2.5 h at 150 V. DNA fragments were stained with ethidium bromide. The ethidium bromide staining fragments were analyzed on UV source using the image analysis system Kodak EDAS 120.

Statistical analysis

Kruskal Wallis variant analysis was used in comparing groups statistically. For significant values, Mann-Whitney U-test was applied in comparing groups in couples; $p < 0.05$ was considered to be significant.

Results

GSH-Px, CAT, SOD activities and GSH concentrations in migraine patients were significantly lower than in control groups (GSH, CAT $p < 0.001$; GSH-Px, SOD $p < 0.05$). MDA value difference was not statistically significant ($p > 0.05$) (Table 2).

No statistically significant difference was found for GSH-Px, CAT, SOD activities and MDA, GSH concentrations between the attack period and initial period in migraine patients ($p > 0.05$) (Table 3).

Identification of the 2 alleles at each polymorphic site was performed by incubating PCR product with a restriction enzyme chosen to cut 1 of the 2 alleles (Table 1), followed by electrophoresis on agarose gels (3%). MnSOD genotypes were named according to the presence or absence of the enzyme restriction sites, i.e. *MroN* I VV, VA and AA are homozygous for absence of the site (107 bp), heterozygous for the presence of the site (107/98/18 bp) and homozygous for the presence of the site (89/18 bp), respectively (Figure 1).

GSH-Px3 genotypes were named according to the presence or absence of the enzyme restriction sites, i.e. *Bsa* I TT, TC, and CC are homozygous for the presence of the site (224/36 bp), heterozygous for the presence of the site (260/224/36bp), and homozygous for absence of the site (260 bp), respectively (Figure 2).

Similarly, the CAT genotypes were named according to the presence or absence of the enzyme restriction sites, i.e. *Sma* I TT, TC, and CC are homozygous for the pre-

Table 1. Primer sequences and reaction conditions for genotyping Mn-SOD, CAT, GSH-Px3 genotype polymorphisms

Polymorphism	Primer sequence	No. of bases	Annealing temperature (°C)	Restriction enzyme	Product size (bp)	Genotype
Mn-SOD	F: 5'ACCAGCAGGCAGCTGGCGCCGG3'	22	60	<i>MroNI</i> (<i>Ngo-MIV</i>)	107	VV
	R: 5'-GCGTTGATGTGAGGTTCCAG-3'	20			107,89,18	VA
					89,18	AA
GSH-Px3	F: 5'-GAAATCCCAGCCGCCTA-3'	17	58	Eco31I (<i>Bsa</i> 1)	224,36	TT
	R: 5'-CACTCACCT-TCGACTTCTCTTGCT-3'	24			260,224,36	TC
					260	CC
CAT	F: 5'-TAAGAGCTGAGAAAGCATAGCT-3'	22	59	<i>Sma</i> 1	340	TT
	R: 5'-AGAGCCTCGCCCGCCGGACCG-3'	22			340,185,155	TC
					185,155	CC

Table 2. GSH-Px, CAT, SOD activities and MDA, GSH concentrations in migraine patients and control groups

	Patients (n: 40)	Control (n: 40)	P
MDA (nmol/ml)	4.89 ± 1.21	5.20 ± 1.90	-
GSH (µmol/g Hb)	0.56 ± 0.33	0.90 ± 0.41	**
GSH-Px (U/g Hb)	41.56 ± 5.17	44.60 ± 5.96	*
CAT (k/g Hb)	22.14 ± 7.07	31.98 ± 13.87	**
SOD (U/g Hb)	39.67 ± 3.39	42.10 ± 3.99	*

Data are expressed as mean ± SD.

- : (p>0.05)

* : Significant difference from control and patients groups (p<0.05)

** : Significant difference from control and patients groups (p<0.001).

Table 3. GSH-Px, CAT, SOD activities and MDA, GSH concentrations in the attack period and initial period in migraine patients

	The attack period	Initial period	P
MDA (nmol/ml)	4.86 ± 0.80	4.89 ± 1.21	-
GSH (µmol/g Hb)	0.57 ± 0.23	0.56 ± 0.33	-
GSH-Px (U/g Hb)	39.87 ± 7.23	41.56 ± 5.17	-
CAT (k/g Hb)	22.76 ± 7.98	22.14 ± 7.07	-
SOD (U/g Hb)	39.33 ± 5.48	39.64 ± 3.39	-

- : (p>0.05)

sence of the site (185/55 bp), heterozygous for the presence of the site (340/185/55 bp) and homozygous for absence of the site (340 bp), respectively (Figure 3).

We analyzed MnSOD, GSH-Px3 and CAT gene polymorphisms in 40 patients with migraine and 40 healthy controls. All the markers of the MnSOD, GSH-Px3 and

CAT gene in our study were in Hardy-Weinberg equilibrium ($p > 0.05$).

The frequencies of the VV, VA and AA genotypes of MnSOD were 27.5%, 70% and 2.5% in cases and were 40%, 60% and 0% in controls, respectively. The frequencies of the V and A alleles of MnSOD were 0.625%

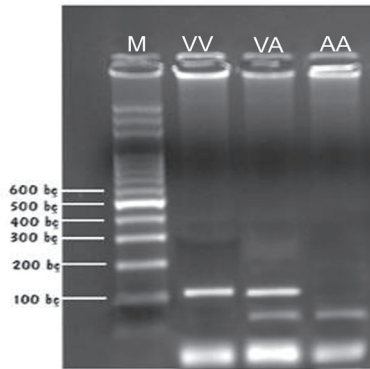


Figure 1. Representative agarose gel electrophoresis illustrating PCR products for the Mn SOD promoter polymorphisms. Lane 1, M was loaded with appropriate molecular markers; lane 2, homozygous VV subject, V allele does not cut with *MroN I* (*NgoMIV*); lane 3, heterozygous subject; lane 4, homozygous AA subject, A allele cuts with *MroN I* (*NgoMIV*) to generate 89/18-bp fragments.

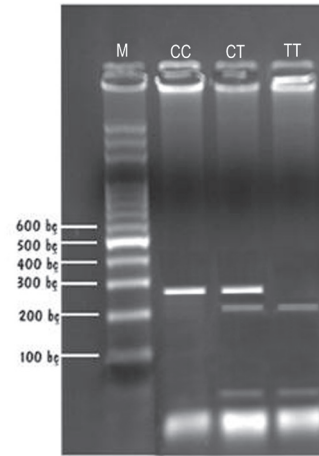


Figure 2. Representative agarose gel electrophoresis illustrating PCR products for the GSH-Px3 promoter polymorphisms. Lane 1, M was loaded with appropriate molecular markers; lane 2, homozygous CC subject, C allele does not cut with *Eco3II* (*Bsa I*). The PCR product has 260 bp; lane 3, heterozygous subject; lane 4, homozygous TT subject, T allele cuts with *Bsa I* to generate 224- and 36-bp fragments.

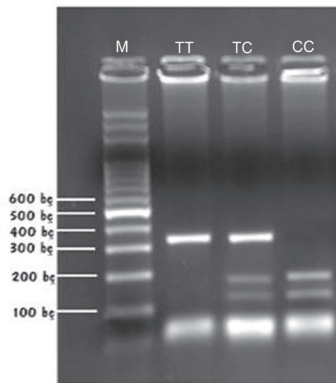


Figure 3. Representative agarose gel electrophoresis illustrating PCR products for the CAT promoter polymorphisms. Lane 1, M was loaded with appropriate molecular markers; lane 2, homozygous TT subject, T allele does not cut with *Sma I*. The PCR product has 340 bp; lane 3, heterozygous subject, T allele cut with *SmaI* generating 185- and 155-bp fragments; lane 4, homozygous CC subject.

and 0% in cases and were 0.7% and 0.6% in controls, respectively. The frequencies of the TT, TC and CC genotypes of GSH-Px3 were 30%, 40% and 30% in cases and were 7.5%, 45% and 47.5% in controls, respectively. The frequencies of the T and C alleles of GSH-Px3 were 0.5% and 0.5% in cases and were 0.7% and 0.3% in controls, respectively. The frequencies of the TT, TC and CC genotypes of CAT were 45%, 25% and 30% in cases and were 50%, 15% and 35% in controls, respectively. The frequencies of the T and C alleles of CAT were 0.4% and 0.6% in cases and were 0.4% and 0.6% in controls, respectively.

The distribution of the genotype at the Mn-SOD and CAT genes between patients with migraine and controls was not significantly different as shown in Table 4 ($p = 0.327$; $p=0.533$, respectively). The distribution of the genotype at the GSH-Px3 gene was found significantly

different between migraine patients and controls ($p = 0.029$).

No significant difference in the alleles at each MnSOD, GSH-Px3 and CAT gene were found as shown in Table 5 ($p > 0.05$).

Discussion

The oxidative stress which occurs in conditions where free radical formation exceed antioxidant capacity, headingly the lipids, proteins and nucleic acids in biological systems are cause harmful effects on all oxidable cellular elements and membrane damaged part is membran lipids. The lipid peroxidation products, in activation of lipoxygenase and inhibition of prostacyclin platelet and leucocyte in the way of prostacyclin/tromboxan cause imbalance. Thus by stimulating the leucotrienes dolorous inflammatual reactions begin [18]. Consequently, the change in lipid peroxidation's thrombocyte functions is thought to be effective in migraine pathogenesis by reduction of cerebral blood fluid.

It has been reported that free radicals have relationship with many diseases such as Alzheimer's disease, Parkinson's disease, schizophrenia, depression, ischemia, epilepsy. It is known that there are enzymes which clean free radicals in order to protect cells from cytotoxic free radicals in all of the animals and plants. SOD, GSH-Px, CAT are free radical cleaning enzymes and have important duties in cleaning these radicals against tissue defect [19].

Migraine pathophysiology is still in discussion, and analysis of responsible etiopathogenesis factors is very important [20,21]. It is true that migraine predisposes to stroke [19]. The aim of this study was to investigate the differences in oxidative stress status with the measure-

Table 4. Frequencies of genotypes of the Mn-SOD, GSH-Px3 and CAT gene promoter in patients with migraine and in controls.

Polymorphisms	Genotype counts frequency			Genotype		
				<i>df</i>	<i>X</i> ²	<i>P value</i>
Mn-SOD	VV	VA	AA			
Migraine patients 40	167(40)	24(60)	0(0.0)	2	1.04	0.327
Control 40	11(27.5)	28(70)	1(2.5)			
GSH-Px3	TT	TC	CC			
Migraine patients 40	72(42.8)	16(40)	12(30)	2	0.74	0.029
Control 40	12(30)	64(37.42)	29(16.95)			
CAT	TT	TC	CC			
Migraine patients 40	18(45)	10(25)	12(30)	2	6.20	0.533
Control 40	20(50)	6(15)	14(35)			

Table 5. Frequencies of the alleles of the Mn-SOD, GSH-Px3 and CAT gene promoter in patients with migraine and in controls.

Polymorphisms	Allele counts frequency		Allele		
			<i>df</i>	<i>X</i> ²	<i>P value</i>
Mn-SOD	V	A			
Migraine patients 40	0.625	0.000	1	0.3	<i>p</i> >0.05
Control 40	0.7	0.6			
GSH-Px-3	C	T			
Migraine patients 40	0.5	0.5	1	0.74	<i>p</i> >0.05
Control 40	0.3	0.7			
CAT	C	T			
Migraine patients 40	0.6	0.4	1	4.44	<i>p</i> >0.05
Control 40	0.6	0.4			

ment of erythrocyte SOD, CAT, GSH-Px, GSH and plasma MDA levels in the migraine patients with or without aura and attack. However, with these respects in mind, the present study was carried out to examine the relationship of Mn-SOD, CAT, GSH-Px gene promoter polymorphism for migraine by using restriction fragment length polymorphism (RFLP) in the Turkish population. It is reported that decreased mitochondrial energy reserve in migraine and habitual deficiency caused migraine attacks by impairing metabolic homeostasis, hemostasis and causing biochemical disorder through activating the trigeminovascular system together with probable genetic and environmental factors [22]. The enzymes which have a material duty in the brain against free radicals are SOD and GSH-Px. Catalase, which exists in some organs at a significant level but in brain at a very low level, could be found in subcellular particles which are called peroxisomes. Under physiologic conditions, it has been

reported that the role of catalase against free radicals is not important [19].

In previous studies, plasma MDA level and thiobarbituric acid reactive agents (TBARS) level were found high in migraine patients according to the control group [20, 23-25]. Furthermore SOD and GSH-Px enzyme activities were found significantly lower in migraine patients than in control group [19-26].

In our study, antioxidant enzyme activities within erythrocytes were examined by using a different method. SOD, CAT, GSH-Px enzyme activities and GSH concentrations were lower in the migraine group than control group. It could be stated that these enzyme activities do not play an important role in patients suffering from tension type headache and healthy people. To reveal if there is an addition of antioxidant enzymes in migraine etiopathogenesis we concluded that it must be researched in the perspective of polymorphic conditions. We came

to conclusion that GSH-Px3 has genotype difference between patient and control group, this gene polymorphism have relation with migraine, but there is no relation between Mn-SOD, CAT gene polymorphism and migraine.

In conclusion oxidative stress and antioxidant enzymes may have an important role in migraine pathogenesis according to our data, GSH-Px3 gene polymorphism may play an important role in the etiology of migraine.

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