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Research Article

Association Analysis of CHRNA4 Gene Polymorphisms and Levels of Marker of Oxidative DNA Damage and Oxidative Stress in Migraine Patients

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Summary

Aim: Migraine is a primary headache syndrome which has been a genetic factor in quite complex etiopathogenesis. The mechanisms underlying the migraine have not been clearly enlightened. The aim of our study is to investigate the relationship between polymorphisms of CHRNA4 gene and migraine and determined to oxidative DNA damage and oxidative stress with detection of 8-oxo2dG and AOPP levels in plasma of patients with migraine.
Methods: In our study, DNA was obtained from migraine patients (n=79) and unrelated healthy persons (n=68). Alleles and genotypes of CHRNA4 gene polymorphisms (rs1044394, rs1044393) were determined with PCR and RFLP methods. Also, 8-oxo2dG and AOPP levels were measured in plasma of migraine patients.
Results: As a result, we were found a significant relationship between rs1044394 polymorphism of CHRNA4 gene and migraine patients without aura ($p < 0.05$). Also, it was shown a significant association between rs1044394 polymorphism of CHRNA4 gene and smoker migraine patients ($p < 0.05$). As an interesting, 8-oxo2dG levels in migraine patients were determinate significantly lower than healthy controls ($p < 0.05$).
Conclusions: According to our results, CHRNA4 gene may be important for migraine disease. Also, 8-oxo2dG levels in plasma of patients with migraine who have take medicine treatment might be decreased. This situation may show that drug therapy for migraine may reduce oxidative stress.

Key words: Migraine, CHRNA4, Gene polymorphism, 8-oxo2dG, AOPP

Migren Hastalarında CHRNA4 Gen Polimorfizmilerinin, Oksidatif DNA Hasarı ve Oksidatif Stres Markır Seviyelerinin Analizi

Özet

Amaç: Migren, oldukça karmaşık bir genetik etyopatogenezi olan primer başağrısı sendromudur. Migrenin altında yatan mekanizmalar henüz net olarak aydınlatılamamıştır. Çalışmamızın amacı, CHRNA4 geni polimorfizmleri ile migren arasındaki ilişkinin araştırılması ve migren hastalarının plazmalarında 8-oxo2dG ve AOPP düzeylerinin tespiti ile oksidatif DNA hasarı ve oksidatif stresin belirlenmesidir.
Method: Çalışmamızda, migren hastaları (n = 79) ve ilişkisiz sağlıklı bireylerden (n = 68) DNA elde edildi. CHRNA4 geni rs1044393 ve rs1044394 polimorfizmlerine ait allel ve genotipler PCR ve RFLP yöntemleri ile belirlendi. Ayrıca, migrenli hastaların plazmasında 8-oxo2dG ve AOPP düzeyleri ölçüldü.

Bulgular: Çalışmamızın sonucunda, aurasız migren hastaları ile CHRNA4 geni rs1044394 polimorfizmi arasında anlamlı bir ilişki tespit edildi ($p < 0.05$). Ayrıca, CHRNA4 geni rs1044394 polimorfizmi ile sigara kullanan migren hastaları arasında anlamlı bir ilişki olduğu belirlendi ($p < 0.05$). İlginç bir sonuç olarak, sağlıklı kontrollere oranla migren hastalarında 8-oxo2dG düzeyinin daha düşük olduğu tespit edildi. ($p < 0.05$).
Sonuç: Verilerimize göre, CHRNA4 geni migren hastalığı için önemli olabilir. Ayrıca, ilaç tedavisi alan migrenli hastaların plazmasındaki 8-oxo2dG düzeyinde azalma olabileceği belirlenmiştir Bu durum, migrendeki ilaç tedavisinin oksidatif stresi azaltıyor olabileceğini göstermektedir.

Anahtar Kelimeler: Migren, CHRNA4, Gen polimorfizm, 8-oxo2dG, AOPP

INTRODUCTION

Migraine is a chronic disease with frequent attacks, high level of pain and disability during which causes reduced quality of life between attacks[4,20]. Migraine is a common neurological disorder affecting between 10 and 20 % of the population. The clinical presentation is heterogeneous and includes recurrent headache attacks, associated symptoms of vegetative disturbance, and hypersensitivity of various functional systems of the nervous system[14,29]. According to classification criteria of the International Headache Society, migraine is subdivided into two common subtypes, namely migraine with (MWA) and migraine without (MWoA) aura. MWA is categorized by a preceding motor and/or visual disturbance lasting 20 to 30 minutes prior to the onset of a migraine attack[28]. It is widely accepted that the intracranial throbbing pain of migraine is mediated primarily by neuronal activity along the trigeminovascular pathway. However the mechanisms underlying the disease have not been clearly understood. Genetic transition of migraine has drawn attention for years and twin studies have supported this issue[29,30]. Also it has suggested that, 3 different groups of genes have been identified and investigated on migraine diseases' basis. These are genes involved in neurological, vascular, or hormonal pathways. Under the broad category of neurological genes include those involved in expression or control of ion channels and the dopamine and serotonergic pathways[6,9,24]. Therefore we attend to investigated to a gene which one of the subunit of Neuronal nicotinic acetylcholine receptors (nAChRs) that has known as ligand-gated channels.

Alpha nicotinic acetylcholine receptor (CHRNA4) as known one of these subunits of nAChRs which is assigned in the central nervous system and encoded by 20q13.33 gene region. Also it has suggested that CHRNA4 is the most important subunit for nicotine-induced reward, tolerance and sensitization[16,32]. It was investigated that the activation of nicotinic receptors modulates centrally induced peripheral neurogenic vasodilatation[17]. It has been identified as one of the candidate genes with its genetic variants, especially for epilepsy which has known as chanelopathy disease[32]. However nicotinic sensitivity of CHRNA4 might important for especially smoked migraine patients. We determinate the functionally polymorphic regions are known as rs 1044393 and rs 1044394 in exon 5 region of CHRNA4 gene in migraine patients.

It has known that oxidative stress leads to damage on DNA with different mechanisms by causing a set of lesions such as alkaline and sucrose modifications, single and double chain fractures, DNA-Protein cross-linking. Oxidative DNA damages are the modification of DNA alkaline, DNA fractures with single or double chains, the loss of purines, the damages of deoxyribose sucroses, the damage of DNA maintenance systems and DNA-protein cross-linking[19,38]. 8-oxo2dG has been defined as the marker of oxidative

DNA damage in cancer, neurodegenerative diseases and aging[26]. Oxidative stress is discussed to be implicated in the pathophysiology of migraine. However, data are in part controversial and the possible underlying mechanisms remain elusive to date[3,37].

Advanced oxidation protein products (AOPP) are defined as dityrosine containing cross-linked protein products and are reflect oxidized protein damage[11,25]. AOPP has been used extensively as biomarkers of inflammation and oxidative stress[22]. Several studies have been shown that AOPP levels are increased in many pathological conditions such as diabetes mellitus[31], chronic kidney disease[21], coronary artery disease[18], Behçet's disease[36]. However, the best of our knowledge; 8-oxo2dG and AOPP status have not been investigated in migraine with patients.

The fact that migraine is a channelopathy disease and we thought that CHRNA4 gene polymorphism might be connected with migraine. For this aim in our study we investigated that, a possible role of CHRNA4 gene polymorphism in migraine disease. Also it has known that oxidative stress and DNA damage are important for migraine disease, considering the evidence of this association, we measured level of AOPP and 8oxo2dG which have known as an oxidative stress markers in this group.

MATERIAL AND METHODS

Participants

The protocol was approved by the regional ethical committee, and procedures were performed according to the principles of The Helsinki Declaration. Migraine diagnosis was made according to International Classification of Headache Disorders-II criteria[15]. Once enrolled, a neurologist administered questionnaires and blood samplings were drawn at the same visit. In the questionnaire the patients were asked to report the onset time, character, location, duration of the pain, associated symptoms, histories, and medications. We evaluated 79 migraine patients (mean age 36.41±10.35 years) from the database of Bülent Ecevit University Medical Faculty, Department of Neurology. Sixty-eight (35.62±10.24 years) unrelated, age and sex matched, healthy were selected from the same geographic area. The healthy volunteer control subjects were recruited from hospital workers, students of the University. The control subjects without headache were generally normal and received no medication. Exclusion criteria were having known inflammatory disorders, infectious, or immune diseases. Patients having chronic migraine or drug overuse headache were also excluded. Table 1 shows the demographic characteristics of migraine patients and healthy controls. No significant difference in the mean ages and gender was observed between the two groups ($p>0.05$).

Genotypic Analysis

After written, informed consent was obtained, venous blood samples were collected into vacutainer plastic tubes containing sodium/ potassium EDTA. Blood samples were centrifuged at 1000 ×g for 10 min. Plasma samples were separated and immediately stored at -20 0C. DNA was extracted from whole blood by salting out procedure[27]. Genotyping of the samples were examined in Muğla Sıtkı Koçman University, School of Health Sciences Laboratory. The CHRNA4 genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, using published PCR primers (shown at table 2). The amplification was followed by restriction digestion with the FokI (c.555C>T; rs1044393), CfoI (c.594C>T; rs1044394). Digested PCR products were directly analyzed by electrophoresis either on 2 % agarose gels and each allele were identified according to its size.

To determine levels of 8-oxo2dG in plasma of blood

To determine 8-oxo2dG (Enzo Life Sciences, USA) levels in plasma use with ELISA method. Results were expressed with ng/mL.

To determine levels of AOPP in plasma of blood

Plasma AOPP levels were measured by spectrophotometric method described by Hanasand et al.[13]. In summary, 40 µl and 160 µl of plasma on the citric acid added to 10 µl KI and 10 ml of the KI and 190 ml of vortexed samples prepared with citric acid was measured by spectrophotometric absorbance at 340 nm against blank. 0-100 mol/L chloramine T standards, such as the sample studied. Results calculated standard curve mol/L was given as the equivalent of chloramine. Results calculated standard curve mol/L was given as the equivalent of chloramine.

Statistical Analysis

Data are presented as mean ± standard error for continuous data or frequencies and percentages for categorical data. Chi-square or Fisher exact tests were used to compare proportions of patients for categorical data among groups. Analysis of variance or t-tests were used to compare the continuous variables among groups. Linkage disequilibrium test, haplotype analysis was evaluated for the two polymorphic regions. Frequencies of values which are smaller than 0:03 were ignored. SPSS 11.5 for Windows program was used for statistical analysis.

Table 1: Describes the distribution of the cases, and the controls by sex, age and smoking status as appropriate and clinical manifestations of migraine

Total	Migraines n (%)	Controls n (%)	p
Age, years MeanSD	36.41±10.35	35.62±10.24	>0.05
Sex			
Female	70 (88.6)	51 (75.1)	>0.05
Male	9 (11.4)	17 (24.9)	
Smoking Status			
Never	57 (72.2)	45 (66.2)	
Ever	22 (27.8)	23 (33.8)	
Treatment			
Triptan	30 (38.1)		
Beta blockers	21 (25.4)		
Antidepressant	28 (36.5)		
Aura			
MwoA	40 (50.6)		
MwA	39 (49.4)		

Table 2: Primers and Restriction Enzymes (RE) used for SNP1044393, SNP 1044394 polymorphisms of CHRNA4 gene.

Gene	Polymorphism	RE		Primers Sequence 5'-3'	Ann. Temp (°C)	Fragment Size (bp)
CHRNA4	SNP1044393	*Fok*I	Sense	GGCGAGTGGGTCATCGTGG	62	TT; 17bp, 25bp, 39bp, 78bp, 132bp TC; 25bp, 39bp, 78 bp, 95bp, 132bp
			Anti sense	GATGACCAGTGAGGTGGACG		CC; 25bp, 39bp, 95bp, 132bp
	SNP 1044394	*Cfo*I	Sense	AACACCAGGAAGTACGAGTGC	62	TT; 255 bp TC; 255 bp, 233 bp, 22 bp
			Anti sense	GATGACCAGTGAGGTGGACG		CC; 233 bp, 22 bp

RESULTS

A power calculation analyses was performed using the Power for Genetic Association Analyses (PGA) software. Given the sample size of this study, we obtained a statistical power of 70 %. According to this base, 79 migraine patients (mean age 36.41±10.35 years) and 69 (35.62±10.24 years) unrelated, age and sex matched, healthy were compared.

According to our results, a meaningful difference was found between the values of MWoA and the control group (*p=0.03). CC genotype was detected to be higher for MWoA than the control individuals. As a result, it was determined that CC genotype may be a risk factor for MWoA in proportion to other genotypes (shown at table 3). CHRNA4 rs1044393 polymorphism were not significant risk factors for migraine disease (p>0.05 for genotypes and alleles). We also analyzed smoker migraine and smoker control and we found a significant relationship between rs1044394 polymorphism of CHRNA4 and smoker migraine (*p=0.017). Based on this conclusion, CC genotype might be a risk for smoker migraine (shown at table 4). Also, we have not found any significant relationship between alleles of rs1044393, rs1044394 and migraine disease (p>0.05)

When haplotypes for CHRNA4 rs1044393 and rs1044394 polymorphism were determined CT haplotype frequency was slightly higher in cases than in controls but the difference did not reach statistical significance (p>0.05, shown at table 5).

Levels of the oxidative DNA damage in the form of 8-oxo2dG and AOPP were examined in peripheral blood lymphocytes of migraine patients and of control group individuals (Figure 1). 8-oxo2dG level (ng/ml) in plasma of migraine patients were significantly lower than control subjects (p<0.05). Figure 2 shows that 8-oxo2dG level in plasma that we have found significant difference between smoker migraine patients with smoker controls (p<0.05). But mean AOPP level of migraine subjects was not different from controls (p > 0.05).

We have not shown any association between 8-oxo2dG incision in individuals with CHRNA4 rs1044393 and rs1044394 polymorphism and the amounts of 8-oxo2dG and AOPP. Also, we analyzed the levels of 8-oxodG and AOPP in relation to genotypes in migraine patients and controls. We did not find any statistically significant difference in 8-oxo2dG and AOPP levels between populations of patients and controls with these polymorphism (p>0.05).

Table 3: Genotypes for CHRNA4 rs1044393, CHRNA4 rs1044394 polymorphism and the risk of developing for MWA and MWoA.

Genotypes		Control n (%)	MWA / MWoA n (%)	X^2 p Value	OR (95% CI)
CHRNA4 rs1044393	**CC**	48 (70.6)	29 (74.4)		Reference
			30 (78.9)	0.08	Reference
	CT	20 (29.4)	8 (20.5)		0.6 (0.2-1.6)
			7 (18.4)		0.56 (0.21-1.48)
	TT	0 (0)	2 (5.1)	0.174	*
			1 (2.6)		*
CHRNA4 rs1044394	**CC**	38 (55.9)	23 (59.0)		Reference
			32 (80.0)	0.92	Reference
	CT	27 (39.7)	14 (34.1)		0.85 (0.37-1.96)
			7 (17.5)		0.30 (0.11-0.80)
	TT	3 (4.4)	2 (5.1)	**0.03***	1.1 (0.17-7.09)
			1 (2.5)		0.39 (0.03-3.9)

‡ ORs (odds ratio); CI (confidence interval) from conditional logistic regression.

* Carriers of at least one intact allele are used as reference.

Table 4: Genotypes of rs1044393 rs1044394 polymorphism of CHRNA4 gene and risk of developing in smoker and non-smoker migraine patients.

Genotypes		Control Smoker / Control Nonsmoker n (%)	Migraine Smoker / Migraine Nonsmoker n (%)	X^2 p Value	OR (95% CI)
CHRNA4 rs1044393	**CC**	18 (78.3)	20 (90.9)		Reference
		30 (66.7)	39 (70.9)	0.414	Reference
	CT	5 (21.7)	2 (9.1)		0.36 (0.06-2.09)
		15 (33.3)	13 (23.6)		0.6 (0.2-1.6)
	TT	0	0	0.107	**
		0 (0)	3 (5.5)		**
CHRNA4 rs1044394	**CC**	13 (56.5)	18 (81.8)		Reference
		25 (55.6)	37 (64.9)	**0.017***	Reference
	CT	9 (39.1)	1 (4.5)		0.08 (0.009-0.17)
		18 (40.0)	20 (35.1)		0.75 (0.33-1.6)
	TT	1 (4.3)	3 (13.6)	0.150	2.16 (0.20-23.2)
		2 (4.4)	0 (0)		**

‡ ORs (odds ratio); CI (confidence interval) from conditional logistic regression.

* Carriers of at least one intact allele are used as reference.

** Odds ratio cannot be calculated.

Table 5. Haplotype for CHRNA4 rs1044393 and rs1044394 polymorphism and the risk of developing Migraine Disease

Haplotype	Migraine (N=79) n (%)	Controls (N=68) n (%)	OR ‡	95% CI
CC	115 (74.7)	91 (66.9)	1 (reference)	
CT	17 (11.0)	25 (18.4)	1.8	0.94-3.64
TC	12 (7.8)	12 (8.8)	1.2	0.54-2.94
TT	10 (6.5)	8 (5.9)	1.0	0.38-2.66

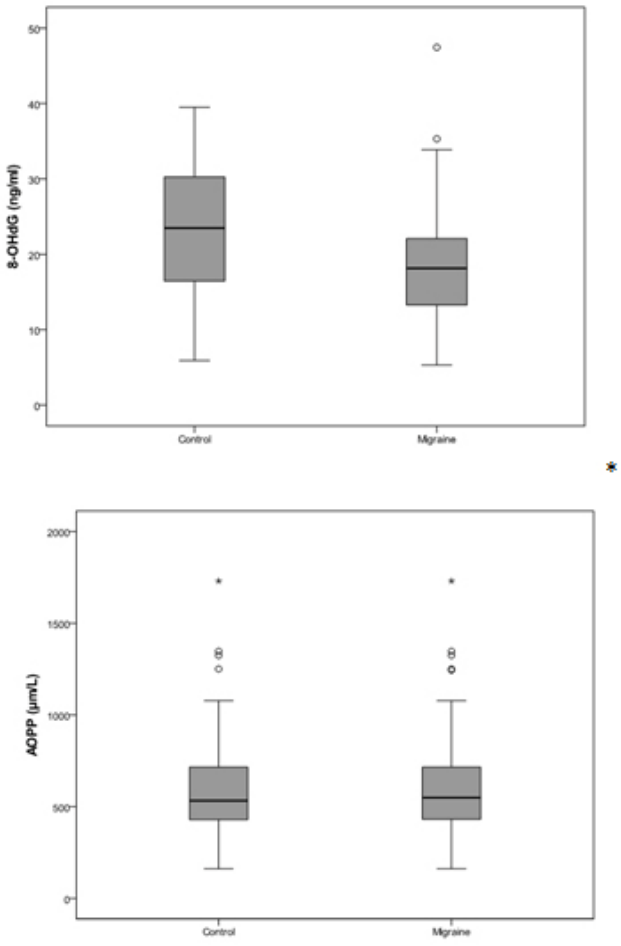

Figure 1: *Levels of 8-oxo2dG (p<0.001) and AOPP (p=0.796) on plasma in patients with migraine and in control group*

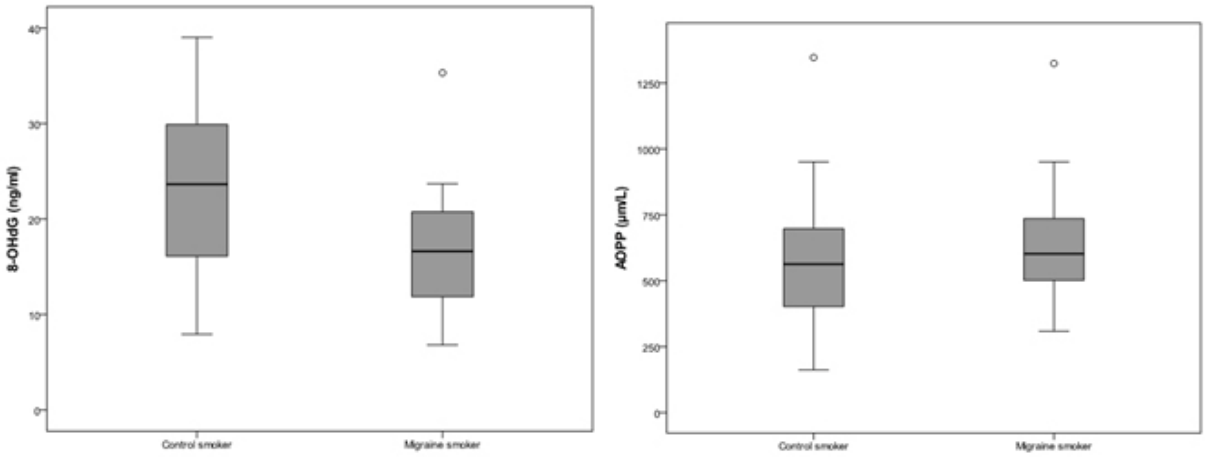

Figure 2: *Levels of 8-oxo2dG (p=0.002) and AOPP (p=0.897)on plasma in patients with smoker migraine and in smoker control group.*

DISCUSSION

In our project which is planned to research a part of the puzzle pertaining to migraine disease that is one of the multigene diseases. To our knowledge, this is the first study to show that functional CHRNA4 gene polymorphisms and levels of 8-oxo2dG and AOPP in patients with migraine. The pathology of the diseases such as migraine and epilepsy which are neurological channelopathies has not been clearly enlightened yet. Ion channels give clues about these diseases[23,32]. In this study we used four-group case-control (MWA, MWoA smoked, nonsmoked) designed. We analyzed to association of CHRNA4 gene polymorphism and levels of 8-oxo2dG and AOPP in migraine patients. Migraine patients group were take regular medicine treatment. As a result of this study we have found an association between rs1044394 polymorphism of CHRNA4 gene in MWoA and smoked migraine patients.

Recently, polymorphisms in CHRNA4 gene that provide protection against nicotine addiction have been described. It was suggested that CHRNA4 gene, one of the subunits of neuronal nicotinic acetylcholine receptors (nAChR), may be connected with idiopathic partial epilepsy, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), idiopathic generalized epilepsy syndrome (IGE) and juvenile myoclonic epilepsy (JME) diseases and febrile convulsions[5]. Also, in another study showed that CHRNA4 gene polymorphisms may be associated with Alzheimer's disease[7]. Our study supported that CHRNA4 gene may important for migraine disease.

8-oxo2dG is known as the oxidative DNA damage marker which increases especially in neurodegenerative diseases[8]. In an investigation suggested that, MDA and NO levels in platelets were surveyed from the oxidative stress markers of migraine patients and it has been determined that there was a meaningful increase[37]. It was confirmed that oxidative stress increased especially throughout migraine attacks[12].We showed that 8-oxo2dG level in migraine patients were significantly lower than control. In our study, the group of migraine patients consists of individuals who received regular treatment and who were not in the attack period. The fact that the patients were not in the attack period and their treatment might affect the decrease of 8-oxo2dG level. Medications containing triptan, beta-bloker and antidepressant are generally used for the

patients. The effects of these medications on the oxidative stress haven't been determined yet. The effect of this medicine treatment of migraine patients to oxidative stress were not shown yet.

Recently, it has suggested that oxidative stress may represent a key event in the pathophysiology of migraine and a suitable therapeutic target[2]. Also, it was determinate that AOPP is a marker of oxidative stress. It was determinate that patients with epileptic encephalopathy have increased levels of oxidative stress markers[10,35]. However, in our study we have not found a meaningful difference between AOPP level in migraine patients and controls.

In conclusion, the fact that 8-oxo2dG, one of the DNA damage markers, is slightly available in migraine patients cannot be explained fully in our study. However, the procedure of our study which has involved taking blood from the patients who are in treatment process (medications like zolmitriptan, eletriptan, beta blocker and antidepressant) during the period when they do not experience attacks may have affected the result in this direction. Studying the effect of medications used for migraine treatment on oxidative damage will contribute to the definition of migraine pathology. In further studies it might be investigation migraine treatment effect of oxidative stress. Furthermore, researching different polymorphisms belonging to CHRNA4 gene in more patients and control individuals and determining gene expression will contribute to the study of connection with migraine disease.

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