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Research paper

Angiotensin converting enzyme and methylenetetrahydrofolate reductase gene variations in fibromyalgia syndrome

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ABSTRACT

Objective: Fibromyalgia syndrome (FM) is a common disease characterized by generalized body pain, sensitivity in certain physical areas (sensitive points), lowered pain threshold, sleep disorder, and fatigue. The study aimed to determine the effects *ACE* I/D and *MTHFR* C677T gene polymorphisms in Turkish patients with FM and evaluate if there was an association with clinical features.
Methods: This study included 200 FM patients and 190 healthy controls recruited from the department of Physical Medicine and Rehabilitation at Gaziosmanpasa University in Tokat, Turkey. *ACE* I/D polymorphism genotypes were determined by using polymerase chain reaction (PCR) by specific primers. The *MTHFR* C677T mutation was analyzed by PCR-based restriction fragment length polymorphism (RFLP) methods.
Results: We found a statistically significant relation between *ACE* polymorphism and FM ($p < 0.001$, OR: 1.71, 95% CI: 1.28–2.27). However, this was not the case for *ACE* polymorphism and the clinical characteristics of the disease. There was also no statistically significant relation between *MTHFR* C677T mutation and FMS ($p > 0.05$, OR: 1.20, 95% CI: 0.82–1.78), but dry eye and feeling of stiffness which are among the clinical characteristics of FMS were significantly related with *MTHFR* C677T mutation ($p < 0.05$).
Conclusion: Our findings showed that there are associations of *ACE* I/D polymorphism with susceptibility of a person for development of fibromyalgia syndrome. Also, it is determined an association between *MTHFR* C677T polymorphism and feeling of stiffness and dry eye which are among the clinical characteristics of FM. Our study is the first report of *ACE* I/D and *MTHFR* C677T polymorphisms in fibromyalgia syndrome.

1. Introduction

Fibromyalgia syndrome (FM) is a common disease characterized by generalized body pain, sensitivity in certain physical areas (sensitive points), lowered pain threshold, sleep disorder, and fatigue (Matsuda et al., 2010; Alayli et al., 2011). Some of patients with FM present symptoms of ocular dryness, with ocular discomfort being a common complaint. Despite plenty of data on the overall manifestations of FM, little is known about its ocular complications, especially those concerning corneal sensitivity (Gallar et al., 2009; Price and Venables, 2002). Ocular irritation in dry eye disease (DED) may decrease the patient's quality of life and cause substantial discomfort. Studies investigating the prevalence of dry eye disease in the general population indicate that up to 33% of adults or more experience dry eye symptoms (Bourcier et al., 2005). The etiopathogenesis of FM is still not clearly known. Various viral infections, stress, living conditions, chronic sleep disorders, physical and emotional traumas, major neuro-endocrinal malfunctions as well as genetic factors are considered in the etiopathogenesis of FMS (Grodman et al., 2011). In several studies, a systemic involvement of substance P in FM patients has been mentioned (Yigit et al., 2013; Ortega et al., 2009; Su et al., 2007; Wang et al., 2008). Substance P is closely associated with pain (Ortega et al., 2009).

Angiotensin converting enzyme [ACE; also known as peptidyl dipeptidase A or kininase II, encoded by the *ACE* gene (GenBank NM_000789.2)] is synthesized by vascular endothelial cells and expressed into the plasma membrane as integral ectoenzymes (Baudin et al., 1997). Because the *ACE* gene expression is not known to have a great extent, it is considered to be tissue specific (Butler et al., 1999). It is mentioned from the effect of the brain renin–angiotensin system in regulation of mood. Additionally, ACE is involved in the metabolism of the neuropeptide substance

Abbreviations: FM, fibromyalgia syndrome; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; DED, dry eye disease; ACE, angiotensin converting enzyme; Hcy, homocysteine; ATII, angiotensin II; HPA, hypothalamic–pituitary–adrenal axis; eNO, endothelial nitric oxide; *MTHFR*, 5, 10-methylene tetrahydrofolate reductase; *SNP*, single nucleotide polymorphisms.

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P which is supposed to play a role in depression. The polymorphisms of the various genes including ACE gene is effective in the development of the migraine, fibromyalgia, cardiovascular disorders and psychiatric conditions (Bondy, 2003a). The *ACE* gene is localized on the chromosome 17 and polymorphism occurs by repetition of a 287 base region in the intron 16 of the gene (Butler et al., 1999; Prasad et al., 2000). Over 70 polymorphisms have been detected in *ACE* gene; insertion/deletion (I/D) of 287 base region is the most studied one (Jeunemaitre, 2008). *ACE* contains polymorphisms based on the presence (insertion, I) or absence (deletion, D) within intron 16, of a 287 base pair ALU repeat sequence; resulting in 3 genotypes: DD and II homozygous and ID heterozygous. Plasma ACE levels vary with polymorphism: Individual homozygous for the D allele has the highest levels of enzyme while those homozygous for the insertion allele and heterozygous subjects have the lowest and intermediate levels, respectively (Inanır et al., 2012).

Methylenetetrahydrofolate reductase (*MTHFR*) gene mutations affect homocysteine (Hcy) metabolism, and provoke hyperhomocysteinemia (HHcy). This increases the homocysteine levels, which in turn causes activity in cytokine, lipid peroxidation accompanied by vascular endothelial damage, prothrombotic process, aterothrombogenesis, thromboembolizm, and systemic vascular occlusive diseases. The increases in the homocysteine levels have been reported as a risk factor in vascular stiffness, hypercoagulability, and for thrombotic complications (Brustolin et al., 2010; Friso et al., 2005). Also, MTHFR polymorphisms influence folic acid-B12-B6 metabolism and are implicated in vascular disease associated with aging (Schmechel and Edwards, 2012). Genetic studies about FM have not been able to establish a clear genetic association. Since gene polymorphisms are of great importance in understanding the basis of multifactorial diseases, we aimed to evaluate the effects the *ACE* (I/D) and *MTHFR* (C677T) gene polymorphisms in FM patients.

2. Material and methods

2.1. Study population

This study is a case–control study and included 200 FM patients and 190 healthy controls. After all patients and controls are examined on the clinics of Physical Medicine and Rehabilitation at Gaziosmanpasa University in Tokat, Turkey, they are included to this study. Informed consent is in accordance with the study protocol, approved by the ethics committee of Medical Faculty (12-BADK-044). All patients signed a written consent form after being informed about the details of the study. A complete clinical evaluation was done for all patients. The controls were selected by excluding the diagnosis of FM. All the individuals in the control group were healthy. Data collection sheet included information such as age, sleep disturbances, fatigue, and feeling of stiffness, irritable bowel syndrome, and dryness of eye. Individual features of patients with FM were summarized in Table 1.

Table 1
Baseline clinical and demographic features of the 200 study patients with FM.

Characteristic	Study group, n (%)
Gender, male/female	3/197 (1.5/98.5)
Age, mean ± SD, years	43.24 ± 10,779
Height, mean ± SD, years	161.67 ± 6110
Weight, mean ± SD, years	74.30 ± 10,671
BMI, mean ± SD, years	28.47 ± 4193
Sleep disturbances	124 (62.0)
Fatigue	128 (64.0)
Difficulty concentrating	79 (39.5)
Headache	142 (71.0)
Paresthesia	45 (22.5)
Feeling of stiffness	105 (52.5)
Feeling of swelling in soft tissues	108 (54.0)
Morning fatigue	146 (73.0)
Irritable bowel syndrome	80 (40.0)
Dysmenorrhea	50/197 (25.4)
Dryness of eye	67 (33.5)
Raynaud's syndrome	11 (5.5)
Dysuria	18 (9.0)
Restless legs	97 (48.5)
Dryness of mouth	115 (57.5)

BMI: Body mass index.

2.1.1. Genotype determination of MTHFR

Genomic DNA was extracted from ethylendiamine-tetraacetate (EDTA)-treated whole venous blood samples using a commercial DNA isolation kit (Sigma-Aldrich, Taufkirchen, Germany). The *MTHFR C677T* mutation was analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) methods as described previously (Frosst et al., 1995). The PCR protocol was consisted of an initial melting step of 5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 61 °C, and 30 s at 72 °C; and a final elongation step of 5 min at 72 °C. PCR primers (5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′) were used to amplify a portion of the *MTHFR* gene from 100 ng of genomic DNA in a 25 μl reaction containing 2.5 μl of 10× PCR buffer, 200 μM of dNTP, 10 pM of each primer, and one unit of Taq DNA polymerase. After amplification, the 198 bp of PCR product was digested with Hinf I in a 15 μl reaction solution containing 10 μl of PCR product, 1.5 μl of 10× buffer, and two units of Hinf I at 37 °C overnight. The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. Wild type (CC) individuals were identified by only a 198 bp fragment, heterozygotes (CT) by both the 175/23 bp, and homozygote variants (TT) by the 175 bp.

2.1.2. Genotype determination of ACE (rs1799752)

DNA was extracted from 2 mL of venous blood according to kit procedure (Sigma, USA) and stored at −20 °C. *ACE* genotypes were determined by polymerase chain reaction (PCR). Reactions were performed with 10 pmol of each primer: sense oligo: 5′CTG GAGACCACT CCCATC CTT TCT 3′ and antisense oligo: 5′GAT GTG GCC ATC ACATTC GTC AGAT 3′ in a final volume of 50 μl, containing 3 mM of $MgCl_2$, 50 mM of KCl, 10 mM of Tris–HCl pH 8.4, 0.1 mg/ml of gelatin, 0.5 mM of each dNTP (Geneun), and 2.5 μl of Taq DNA polymerase (Fermentas). DNA was amplified for 30 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min 45 s, and extension at 72 °C for 1 min 30 s using a thermal cycler (Techne, USA). PCR products were analyzed on 2% agarose gels after staining with ethidium bromide. In the absence of the 287 bp in intron 16 of the *ACE* gene, this PCR method resulted in a 190 bp product (D allele) and in the presence of 287 bp, produced a 490 bp product (I allele). In heterozygous samples, 2 bands (490 and 190 bp) were detected. In order to validate the accuracy and reproducibility of this method, each PCR reaction included internal controls for each genotype. Second PCR was performed to confirm samples whose results were not clear. Also, to confirm the accuracy of the genotyping, repeated analysis was performed on all selected samples. No discrepancies were found.

2.2. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 13.0) and the OpenEpi Info software package version 2.2 (www.openepi.com). Results were given as mean ± standard deviation (S.D.). Chi-square ($\chi 2$) test was used to evaluate the Hardy–Weinberg equilibrium (HWE) for the distribution of the genotypes of the patients and the controls. Additionally, the frequencies of genotypes in patients and controls were compared with multiple conditional logistic regression. In this analysis, elimination methods were Backward and Forward. Chi-square test and Fisher's exact test were used to compare categorical variables appropriately, and odds ratio

(OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All p values were 2-tailed, and confidence intervals (CIs) were set at 95%. p values less than 0.05 were considered as significant.

3. Results

Demographic variables and baseline characteristics of patients were given in Table 1. The mean age ± standard deviation (SD) was 43,24 ± 10,77 years in patients and 36,39 ± 11,79 years in control group. There were 197 (98.5%) females and 3 (1.5%) males and 183 (96.3%) females and 7 (3.7%) males in patient and control groups, respectively. Although statistically significant relation was found between *ACE* polymorphism and FM ($p < 0.0002$, OR: 1.71, 95% CI: 1.28–2.27), this was not the case for *ACE* polymorphism and the clinical characteristics of the disease ($p > 0.05$). In the multiple conditional logistic regression analysis, significant relation was found between *ACE* polymorphism DD genotype and FM ($p < 0.001$, OR: 2.65) (Table 2). On the other hand, although there was no statistically significant relation between *MTHFR* C677T polymorphism and FM ($p > 0.05$, OR: 1.20, 95% CI: 0.82–1.78), dry eye which is among the clinical characteristics of FM, was significantly related with *MTHFR C677T* mutation ($p = 0.03$) (Table 3).

We also examined the risk associated with inheriting the combined genotypes for the two polymorphisms (Table 4). CC-II combined genotype of *MTHFR* C677T-*ACE* I/D were significantly higher in the control group than in the patient group that these combined genotypes seem to have protective effect against FM ($p = 0.00$). Allele and genotype frequencies of SNPs were calculated and tested for departure from Hardy–Weinberg equilibrium using the Chi-square test. The observed and expected frequencies of *MTHFR C677T-ACE I/D* polymorphisms were in Hardy–Weinberg equilibrium in both groups.

4. Discussion

FM is characterized by chronic, diffuse, widespread pain, fatigue, and sleep disturbance and often accompanied by a variety of associated symptoms such as headache and mood disorders (Gallar et al., 2009; Neumann and Buskila, 2003). Among many other signs and symptoms, a considerable portion of patients with FM present concurrent symptoms of oral and ocular dryness. Despite abundant data on the general manifestations of FM, little is known about its ocular complications, in particular those concerning corneal sensitivity (Gallar et al., 2009). As a result the quality of FM patient's life deteriorates. FM has a prevalence that may range between 0.66% and 10.5% for the adult population (Wolfe et al., 1995; Topbas et al., 2005). Angiotensin converting enzyme is a zinc metallopeptidase involved in blood pressure regulation via the angiotensin–renin cascade, generating angiotensin II (ATII) from angiotensin I, and via degradation of the powerful vasodilator bradykinin. Several studies have also demonstrated that ACE might be involved in the hypothalamic–pituitary–adrenal axis (HPA) regulation and catecholamine production and is thus required for sympathoadrenal activation during stress (Bondy, 2003b). The co-localization of angiotensin with dopamine-synthesizing neurons can suggest an involvement of the brain renin–angiotensin system in regulation of mood (Jenkins et al., 1997). Bondy et al. have shown that the combined action of ACE and Gβ3 genotypes accumulates in carriers of the ACE D allele (ID and DD) and Gβ3 TT homozygotes with ID/DD-TT carriers showing a more than five-fold increase in risk for major depression (Bondy et al., 2002). It is suggested that ACE polymorphism is a risk factor for neuropsychiatric disturbances and related diseases. We are thinking that it must

Table 2
Genotype and allele frequencies of MTHFR and ACE gene polymorphisms in FM patient and control groups.

Gene	FM patients n = 200	Healthy controls n = 190	p	OR (CI 95%)
MTHFR (C677T)				
Genotypes				
C/C	133 (66.5%)	137 (72.1%)	0.375	
C/T	65 (32.5%)	50 (26.3%)		
T/T	2 (1.0%)	3 (1.6%)		
Alleles				
C	331 (82.8%)	324 (85.3%)	0.339	1.20 (0.82–1.78)
T	69 (17.2%)	56 (14.7%)		
ACE (I/D)				
Genotypes				
I/I	38 (19.0%)	68 (35.8%)	**0.001**	
I/D	94 (47.0%)	76 (40.0%)		
D/D	68 (34.0%)	46 (24.2%)		
Alleles				
I	170 (42.5%)	212 (55.8%)	**0.0002**	**1.71 (1.28–2.27)**
D	230 (57.5%)	168 (44.2%)		

The results that are statistically significant are typed in bold.

Table 3
Clinical characteristics of FM patients.

MTHFR C677T					ACE I/D		
		Patients			Patients		
Characteristics	Status	CC	CT+TT	p	II	ID+DD	P
Gender	Male	3	0	>0.05	2	1	>0.05
	Female	130	67		66	131	
Sleep disturbances	Yes	81	43	>0.05	40	84	>0.05
	No	52	24		28	48	
Fatigue	Yes	91	37	>0.05	46	82	>0.05
	No	42	30		22	50	
Difficulty concentrating	Yes	56	23	>0.05	32	47	>0.05
	No	77	44		36	85	
Headache	Yes	96	46	>0.05	52	90	>0.05
	No	37	21		16	42	
Paresthesia	Yes	30	15	>0.05	14	31	>0.05
	No	103	52		54	101	
Feeling of stiffness	Yes	64	41	>0.05	38	67	>0.05
	No	69	26		30	65	
Feeling of swelling in soft tissues	Yes	74	34	>0.05	37	71	>0.05
	No	59	33		31	61	
Morning fatigue	Yes	99	47	>0.05	48	98	>0.05
	No	34	20		20	34	
Irritable bowel syndrome	Yes	51	29	>0.05	25	55	>0.05
	No	82	38		43	77	
Dysmenorrhea	Yes	35	15	>0.05	17	33	>0.05
	No	95	52		49	98	
Dryness of eye	Yes	38	29	**0.03**	25	42	>0.05
	No	95	38		43	90	
Raynaud's syndrome	Yes	6	5	>0.05	5	6	>0.05
	No	127	62		63	126	
Dysuria	Yes	13	5	>0.05	8	10	>0.05
	No	120	62		60	122	
Restless legs	Yes	69	28	>0.05	35	62	>0.05
	No	64	39		33	70	
Dryness of mouth	Yes	75	40	>0.05	38	77	>0.05
	No	58	27		30	55	

The results that are statistically significant are typed in bold.

Table 4
Comparative analysis of combined genotypes of patients and controls.

Genotypes	Patients (n = 200)		Control (n = 190)		P
	n	%	n	%	
MTHFR C677T-ACE I/D					
CC-II	22	11	46	24.2	**0.00**
CT-II	16	8	21	11	0.39
TT-II	0	0	1	0.5	>0.99
CC-ID	60	30	55	28.9	0.81
CT-ID	33	16.5	19	10	0.82
TT-ID	1	0.5	2	1	0.96
CC-DD	52	26	36	18.9	0.09
CT-DD	15	7.5	10	5.2	0.48
TT-DD	1	0.5	0	0	>0.99

The results that are statistically significant are typed in bold.

investigate the associations between ACE gene I/D polymorphism and FM. Although Jenkins et al. have also found that in patients with sleep disorders, which are one of the symptoms of fibromyalgia, there were considerable differences in the polymorphism of *ACE* gene (Jenkins et al., 1997; Bondy et al., 2002; Kramer et al., 1998; Gunduz et al., 1999). These patients were not diagnosed as FM. Since FM is associated with depression and sleep disorders, we investigated the relation of FM and *ACE* polymorphism. Although we found a statistically significant relation between *ACE* polymorphism and FM, we did not find any significant relation with *ACE* polymorphism and clinical findings of FM. Although fibromyalgia is not considered an inflammatory disorder, the complex interaction between the biology of pain and inflammation has led to a considerable amount of research aiming to identify alterations in levels of various cytokines in fibromyalgia patients (Ablin et al., 2008; Wallace et al., 2001). Interleukin-1 receptor antibody (IL-1Ra) and IL-6 levels have been shown to be elevated in peripheral macrophages of fibromyalgia patients. Also, inflammatory cytokines such as IL1-beta, IL-6 and tumor necrosis factor alpha (TNFa) have been detected in skin biopsies of fibromyalgia patients, possibly indicating an element of neurogenic inflammation (Wallace et al., 2001; Salemi et al., 2003). *MTHFR* mutations also cause cytokine activation. *MTHFR* is a key enzyme in Hcy metabolism, which plays a major role in regulating endothelial function. It regulates Hcy and methionine metabolism and converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (primary form of folate in circulation). The methyl group serves as donor for methionine production from Hcy. On the other hand, folate could not convert Hcy to methionine. This in turn causes degeneration in conversion of Hcy to methionine and to HHcy. In patients with *MTHFR* mutations, reduced enzyme activity and decreased remethylation of Hcy to methionine lead to elevated total Hcy. Moreover, altered Th1/Th2 balance resulting from inhibition of the remethylation cycle is speculated to cause abnormal cellular immune response in relevant patients (Husemoen et al., 2006). This effect on immune response may cause unresponsiveness to colchicine in *MTHFR* mutation carriers. Homocysteine metabolism is limited to the B12–folate dependent remethylation pathway catalyzed by methionine synthase of the methionine–folate cycle in vascular cells. Thus, smooth muscle cells and vascular cells, particularly endothelial cells may be especially vulnerable to the higher levels of circulating and endogenous Hcy found in patients with HHcy. As Hcy promotes oxidant injury to vascular cells, HHcy may play an important role in oxidative stress (Wallace et al., 2001; Salemi et al., 2003; Husemoen et al., 2006). Folic acid, capable of lowering elevated levels of homocysteine and playing an important role in the remethylation of homocysteine, has a positive role in cardiovascular disease, neurodegenerative disorders, neural tube defects, and cancer. Deficiency or impairment of folate metabolism is associated with HHcy, hypomethylation, DNA damage, impaired cell proliferation, malignancies, and impaired endothelial nitric oxide (eNO) production (Brustolin et al., 2010; Hayden and Tyagi, 2004). The general population of patients with metabolic syndrome, prediabetes and overt type 2 diabetes mellitus in which oxidative stress is involved has a similar frequency of the common polymorphism affecting the folate-dependent, thermolabile gene coding for the 5, 10-methylene tetrahydrofolate reductase (*MTHFR*)(*C677T*, *Ala → Val*). This mutation is associated with a decreased activity of the enzyme resulting to mild to moderate HHcy, which occurs in 10–15% of the general population (Husemoen et al., 2006; Hayden and Tyagi, 2004; Wotherspoon et al., 2008). Also, Schmechel and Edwards stated that *MTHFR* C677T mutation may affect chronic wide-spread pain and FM (Schmechel and Edwards, 2012). The *MTHFR* C677T mutation was also shown to have an association with ocular involvement in Behcet's disease (BD) patients (Ozkul et al., 2005). Furthermore, Cardoso et al. (2007) have reported a patient with Sjögren's syndrome who has livedoid vasculopathy, hypercoagulability and dry eyes, as well as *MTHFR* mutation. These findings may suggest that *C677T* mutation may play a role in the pathogenesis of these diseases. Similarly, it is also possible that this mutation can be involved in the complex mechanism of dry eye, which in turn may be related to FM. Also, there is not much study about genetic factors that causes to fibromyalgia in the literature. So far, 3 studies have attempted to explore the genetic contribution to FM in a genome wide manner (Docampo et al., 2014; Arnold et al., 2013; Smith et al., 2012). The role of the single nucleotide polymorphisms in FM was analyzed with the genome wide association study (GWAS) by Dacompo et al. And, while they did not find any significant association with FM (Docampo et al., 2014), Arnold et al. detected a wide linkage to the chromosome 17p11.2-q11.2 region in the Fibromyalgia Family Study (Arnold et al., 2013). Additionally, they specified that further research with larger and different patient populations was necessary. Nevertheless, our study is the first report of *ACE* I/D and *MTHFR* C677T polymorphisms in fibromyalgia syndrome.

5. Conclusions

Our findings showed that there are associations of *ACE* I/D polymorphism with the susceptibility of a person for development of fibromyalgia syndrome. Also, it is determined an association between *MTHFR* C677T polymorphism and dry eye which are among the clinical characteristics of FM. Our study is the first report of *ACE* I/D and *MTHFR* C677T polymorphisms in fibromyalgia syndrome.

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