Gene 564 (2015) 188–192

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Angiotensin converting enzyme and methylenetetrahydrofolate reductase gene variations in fibromyalgia syndrome

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ARTICLE INFO

Article history:
Received 17 November 2014
Received in revised form 14 March 2015
Accepted 22 March 2015
Available online 28 March 2015

Keywords:
Fibromyalgia syndrome
MTHFR C677T
ACE I/D

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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http://dx.doi.org/10.1016/j.gene.2015.03.051
0378-1119/

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