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MTHFR gene C677T mutation and *ACE* gene I/D polymorphism in Turkish patients with osteoarthritis

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Abstract. Osteoarthritis is a degenerative joint disorder resulting in destruction of articular cartilage, osteophyte formation, and subchondral bone sclerosis. In recent years, numerous genetic factors have been identified and implicated in osteoarthritis. The aim of the current study was to examine the influence of methylenetetrahydrofolate reductase (*MTHFR*) gene C677T mutation and angiotensin converting enzyme (*ACE*) gene insertion/deletion (I/D) variations on the risk of osteoarthritis.
Genomic DNA is obtained from 421 persons (221 patients with osteoarthritis and 200 healthy controls). *ACE* gene I/D polymorphism genotypes were determined using polymerase chain reaction using I and D allele-specific primers. The *MTHFR* C677T mutation was analyzed by polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) methods. We found significant difference between the groups with respect to both *ACE* and *MTHFR* genotype distributions ($p < 0.001$, $p < 0.001$ respectively). Our study suggests that *ACE* gene DD genotype and *MTHFR* gene CC genotype could be used as genetic markers in osteoarthritis in Turkish study populations.

Keywords: Angiotensin converting enzyme (*ACE*) gene, methylenetetrahydrofolate reductase (*MTHFR*) gene, osteoarthritis

1. Introduction

Osteoarthritis is a common disease of the synovial joint characterized by articular cartilage thinning and loss, which is often accompanied by alteration in the normal function of other tissues of the joint [1]. Despite the increase in molecular knowledge accrued during the past years, the exact pathogenesis of the destructive process remains unknown. Osteoarthritis is caused by genetic and environmental factors and is considered a multifactorial disease in which age, body mass index, hormonal and local biomechanical factors together with genetic predisposition play a role [2]. The genetics of OA is complex and is not completely understood, and to assess the validity of reported genetic associations, the best strategy is to reproduce those associations in independent cohorts and analysis of a relatively large number of candidate genes [1]. Genetic contribution to OA has been suggested in several epidemiologic studies [3,4]. Twin studies, segregation analyses, linkage analyses, and candidate gene association studies have generated important information about inheritance patterns and the genome location of potentially causative mutations [5]. Classic twin studies have shown that the influence of genetic factors is between 39% and 65% in radiographic OA of the hand and knee in women, about 60% in OA of the hip, and about 70% in OA of the spine. Taken together, these estimates suggest a

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heritability of OA of 50% or more, indicating that half the variation in susceptibility to disease in the population is explained by genetic factors [3]. Genes act through a complex web of mechanisms involving injury and its avoidance; response to injury; body weight; muscle mass; and bone structure and bone turnover or cartilage structure and cartilage turnover or, synergistically, the two together [3–6]. Better and larger association studies are needed that are adequately powered and use greater numbers of genetic markers, such as either anonymous single nucleotide polymorphisms (SNPs) or specified candidates. Clinical and genetic programs incorporating study of both OA and osteoporosis will also provide extra power to find the genes for both disorders as well as new pathways [3–6]. There are limited studies about OA and ACE gene I/D polymmorphism [7–9]. In Bayram et al.'s study, DD genotype of ACE gene I/D polymorphism is associated in a Turkish study population with osteoarthritis, however in Shehab and Hong's studies *ACE* gene were not found asssociated with OA [7–9].

One of the candidate gene for the development of osteoarthritis is methylenetetrahydrofolate reductase (*MTHFR*) which is a regulatory enzyme of the homocysteine metabolism. The gene is also involved in the metabolism of tetrahydrofolate, synthesis of purine and nucleic asids [10]. The *MTHFR* gene has been mapped to the chromosomal region 1p36.3. A common C to T transition at nucleotide 677 (C677T) of the *MTHFR* gene causes substitution of alanine by valine in the protein structure. This genetic polymorphism is located in exon 4 of the *MTHFR* gene corresponding to the folate binding site of the protein [10]. The presence of this mutation was shown to correlate with increased *MTHFR* thermolability and reduced specific activity [11]. This mutation is considered the most common genetic cause of elevated homocysteine levels [12–14]. *MTHFR* is a good candidate gene gene for OA because in previous studies this polymorphism was found to be associated with inflammation process. We hypothesized to examine *MTHFR* gene C677T variation in osteoarthritis patients. To our knowledge, this is the first study regarding *MTHFR* gene C677T variation in osteoarthritis patients.

The other candidate genes for the development of OA is angiotensin converting enzyme (ACE) which plays an important role in inflammatory and immune related disorders [7]. ACE (also known as peptidyl dipeptidase A or kininase II) is encoded by the *ACE* gene (Gene Bank accession number: NM 000789.2) located on the long arm of chromosome 17 and can be expressed in multiple tissues. *ACE* gene contains a polymorphism based on the presence (insertion, I) or absence (deletion, D) of a 287 base pair ALU repeat swquence within intron 16, resulting in 3 different genotypes: DD and II homozygous and ID heterozygous. Plasma ACE levels vary with polymorphism; individuals homozygous for the D allele have the highest levels of enzyme, those homozygous for the insertion allele have the lowest and heterozygous subjects have an intermediate level [8–15]. This polymorphism has been particularly related to rheumatic and autoimmune diseases. Based on these findings we hypothesized that the genotype of *ACE* gene I/D polymorphism and *MTHFR* gene C677T mutation in osteoarthritis patients may be a determining factor in pathogenesis of osteoarthritis.

2. Materials and methods

2.1. Study population

This study included 221 osteoarthritis patients and 200 controls recruited from the Department of Physical Medicine and Rehabilitation at Gazi Osmanpaşa University (Tokat, Turkey). Informed consent is in accordance with the study protocol, approved by the ethics committee of Medical Faculty. Inclusion criteria were; 1) any symptom and/or sign of OA, 2) radiographic abnormalities according to Kellgren-Lawrence grading [definition KL>2], 3) no evidence for any other form of arthritis, 4) informed consent obtained. In addition, demographic characteristics (age, gender), body mass index (BMI), clinical features on disease severity were observed by way of Kellgren-Lawrence grade [9–16] and Lequesnes' functional index [9–17]. Kellgren-Lawrence grade represents disease severity reflected on radiographs whereas Lequesne's functional index represents fuctional or symptomatic status of patients. Radiographic findings of OA were classified into Kellgren-Lawrence grade 1, 2, 3, or 4. Functional or symptomatic statuses of patients were classified into mild (Lequesne's functional index IB10) or severe (Lequesne's functional index IC10). Pain was evaluated by VAS (Visual Analogue Scale) is a pain measurement scale used for pain scoring. 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 osteoarthritis. All the individuals in the control group were healthy and OA patients also healthy other than having OA. Data col-

Table 1
Demographic variables and clinical findings of the study group

Individual characteristics	Patients ($n = 221$) (%) /(mean ± SD) [min-max]	Controls ($n = 200$) (%) /(mean ± SD) [min-max]
Sex		
Female n(%)	161 (72.85%)	135 (67.5%)
Male n(%)	60 (27.14%)	65 (32.5%)
Mean age, years	58.04 ± 10.87 [25–97]	53.03 ± 12.88 [18–85]
Height	162.88 ± 7.071 [161–164]	–
Weight	77.16 ± 7.104 [62–104]	–
Disease duation	3.85 ± 3.94	–
Visual analog scale (VAS)	6.68 ± 1.55	
Articular involvement		–
Knee	86 (24.41)	–
Hand	55 (25)	
Hip	59 (26.50)	
		–
Grades		
Grade 1	45 (20.36)	–
Grade 2	117 (52.94)	–
Grade 3	50 (22.62)	–
Grade 4	8 (3.61) [2–10]	

lection sheet included information such as age, weight, height, disease duration and VAS. Individual features of patients with OA and controls were summarized in Table 1.

2.2. Genotype determination

DNA was extracted from 2 mL 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 100 ng of genomic DNA, 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 $MgCl_2$, 50 mM KCl, 10 mMTris-HCl pH 8.4, 0.1 mg/ml gelatin, 0.5 mM of each dNTP (Geneun), 2.5 U 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 sec, and extension at 72°C for 1 min 30 sec using a thermal cycler (Techne, USA). PCR products were analyzed on 2% agarose gels after staining with ethidium bromide. The *MTHFR* C677T mutation was analyzed by PCR based RFLP methods. The PCR protocol was consisted of an initial melting step of 5 minutes at 94°C; followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 61°C, and 30 seconds at 72°C; and a final elongation step of 5 minutes 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 10X PCR buffer, 200 μM dNTP, 10 pM each primers, and one unit of Taq DNA polymerase. After amplification, the 198 bp PCR product was digested with Hinf I in a 15 μl reaction solution containing 10 μl of PCR product, 1.5 μl of 10X buffer, and two units of Hinf I at 37°C.

2.3. Statistical analysis

Analysis of the data was performed using the computer software SPSS 15.0 (SPSS, Chicago, IL, USA) and OpenEpi Info software package program [18]. Continuous data were given as mean ± SD (standart deviation) and (min-max). The frequencies of the alleles and genotypes in patients and controls were compared with X^2 analysis. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated. P value smaller than 0.05 (two-tailed) was regarded as statistically significant. Bonferroni correction was also performed. Power analysis was done by using Minitab 15.0 package program.

3. Results

Demographic variables and baseline characteristics of patients are given in Table 1. The mean age ± standard deviation (SD) was 58.04 ± 10.87 in patients, and 53.03 ± 12.88 in control group respectively. There were 161 (72.9%) female and 60 (27.1%) male patients, while in controls, there were 135 (67.5%) females and 65 (32.5%) males, respectively. In patients group ra-

tio of female higher than male. In power analysis, the power of *ACE* gene DD genotype and *MTHFR* gene CC genotype comparison between patients and controls was over 90% in 95% confidence interval. Tables 2 and 3 presents the distribution of *ACE* gene I/D genotypes and *MTHFR* C677T mutation profiles in patients and control groups. All p values were 2-tailed, and CIs were set at 95%. A p value less than 0.05 was considered significant. A significant difference was found in the frequencies of *ACE* I/D alleles between patients and controls, with OA patients having a higher presence of D allele (0.591 vs 0.418) and lower presence of theI allele (0.409 vs 0.582). compared to controls (Table 2). *MTHFR* gene C allele frequency was observed as 0.674 in the patients and T allele was 0.326, it was 0.842 and 0.158 respectively in the control group (Table 2). After Bonferroni correction, p values were still significant ($<$ 0.001). A significant difference was found in the frequencies of *ACE* I/D alleles between patients and controls, with OA patients having a higher presence of D allele (0.591 vs 0.418) and lower presence of theI allele (0.409 vs 0.582). compared to controls (Table 2).*MTHFR* gene C allele frequency was observed as 0.674 in the patients and T allele was 0.326, it was 0.842 and 0.157 respectively in the control group (Table 2).

4. Discussion

The genetic etiology of osteoarthritis is not entirely known. Recent studies have shown that an inflammatory process plays a part in the pathogenesis of osteoarthritis [13,14]. Genetic factors are involved in its onset and progression [1]. A number of gene polymorphisms involved in development of knee osteoarthritis have been identified, such as those localized in or adjacent to the encoding sequences for the vitamin D receptor [14,15], estrogen receptor alpha [19], calcitonin [20], interleukin-6 [21], *SMAD3* [7], and matrix metalloproteinase-1 [22]. In the last decades, many researchers aimed to identify causal genetic variants by means of candidate gene analyses, Genetic research is done to identify underlying causative genes and pathways, and thereby understand more about the biology of the disease with potential implications for development of novel treatments, and to be able to predict risk of disease by genotyping the identified risk alleles [23]. So far, most research is focused on *GDF5* (growth differentiation factor 5) [23,24]. Also SNPs near the *DIO2* (deiodinase, iodothyronine, type II) gene and recently identified *DOT1L* gene was suggested to play a role in OA [7,22].

In previous studies, *ACE* gene was found associated with rheumatic and autoimmune diseases, so that we aimed to investigate the association between *ACE* gene I/D polymophism and also *MTHFR* gene C677T mutation. In this study, the distribution of *ACE* gene I/D and *MTHFR* gene C677T genotypes were analyzed in osteoarthritis patients in a Turkish population to assess its possible role in the pathogenesis of osteoarthritis. We found that the percentage of *ACE* gene polymorphic and MTHFR mutant alleles and the distribution of genotypes are significantly different between patients and controls.

There are limited number of studies about *ACE* gene I/D and *MTHFR* gene C677T polymorphism and osteoarthritis in the literature. In a study, Hong et al. reported an association of *ACE* gene I/D polymorphism with primary knee osteoarthritis in a homogeneous Korean population. On the Hang et al.'s data, they suggested that *ACE* I/D gene polymorphism I allele is a risk factor for early onset, severe form of primary knee osteoarthritis [8]. Nevertheless, Shebab et al. have not found a significant association between *ACE* gene I/D polymorphism genotypes in osteoarthritis patients and controls in Kuwaiti Arabs [8]. In a recent study, Bayram et al. examined Turkish osteoarthritis patients to determine the frequency of I/D polymorphism genotypes of *ACE* gene and similarly they reported that there was a statistically significant difference between the groups with respect to genotype distribution (P\0.001). The D allele frequency was indicated as 69% and I allele was as 31% in the patients [8]. There may be ethnic differences in the ability of the ACE polymorphism to influence susceptibility to OA.

To our knowledge, *MTHFR* gene C677T mutation has never been investigated in OA in any ethnic group. Tasbas et al. examined *MTHFR* gene C677T mutation in RA patients and showed that the frequency of *MTHFR* C677T variant was similar in Turkish RA patients and healthy control subjects [25].

Besides *ACE* gene, different gene polymorphisms were found associated with osteoarthritis. Honsawek et al. investigated MMP-3-1612 5A/6A gene polymorphism with knee osteoarthritis in Thai population and indicated that the -1612 5A/6A polymorphism genotypes of MMP-3 gene promoter do not play a role in the development of osteoarthritis in the Thai population [2]. In a recent study Kostopoulo et al. performed a genetic association analysis using a cohort of 1,410 Greek osteoarthritis patients and healthy controls

Table 2
Distribution of *MTHFR* gene C677T mutation profile and allele frequencies between osteoarthritis patients and controls

Genotype	Patients ($n = 21$) (%)	Controls ($n = 200$) (%)	χ^2	**P value**	OR (95%CI)
CC	97 (43.89)	140 (70)	33.12	$p <$ **0.0001**	
TT	20 (9.05)	3 (1.5)			
CT	104 (47.05)	57 (28.5)			
CC+CT:TT	201:20	197:3	11.59	$p =$ **0.0008**	6.511 (1.887–34.750)
CT+TT:CC	124:97	60:140	29.09	$p <$ **0.0001**	2.983 (1.994–4.461)
Allele frequency					
C	298 (67.42)	337 (84.25)	32.508	$p <$ **0.0001**	0.3869 (0.276–0.540)
T	144 (32.58)	63 (15.75)			

The results that are statistically significant are typed in bold.

Table 3
Distribution of *ACE* gene I/D polymorphism and allele frequencies between osteoarthritis patients and controls

Genotype	Patients ($n = 221$) (%)	Controls ($n = 200$) (%)	$\chi 2$	**P value**	OR (95%CI)
II	37 (16.74)	78 (39)	26.92	$p <$ **0.0001**	
DD	77 (34.84)	45 (22.5)			
ID	107 (48.42)	77 (38.5)			
ID+II :DD	144:77	155:45	7.77	$p =$ **0.005**	1.839 (1.196–2.848)
DD+ID:II	184:37	122:78	26.20	$p <$ **0.0001**	0.315 (0.199–0.494)
Allele frequency					
I	181 (40.95)	233 (58.25)	25.14	$p <$ **0.0001**	0.497 (0.377–0.654)
D	261 (59.05)	167 (41.75)			

The results that are statistically significant are typed in bold.

and found significant association between single nucleotide polymorphism (SNP) 1784G>C in *SREBP-2* gene and osteoarthritis development [24]. Using Dutch Caucasian osteoarthritis cases in their discovery sample, with strong signals then genotyped in cohorts from Europe and North America, Kerkhof and colleagues identified a locus on chromosome 7q22 that was associated with knee and/ or hand osteoarthritis at SNP rs3815148 [24]. Understanding the molecular genetic basis of a common disease casts light on the biological factors involved in disease initiation and progression, and may suggest new treatment strategies. Due to the limited research on *ACE* and MTHFR genes and osteoarthritis, present study provides an important contribution to the literature. Biological mechanism of these polymorphims; ACE is a metalloenzyme converts angiotensin I to a potent vasoconstrictor angiotensin II [26,27]. It also inactivates bradykinin which is a vasodilator of the kallikrein kinin system and has major implication in inflamatory process including OA [22]. Synovial fluid SF ACE activity is found higher in patients with OA than controls [28]. The inhibition of the enzyme MTHFR is responsible for the increas in homocystein level. The plasma homocystein levels is increas in arthritis [29].Genetic studies of patients with OA can help to understand the molecular mechanisms responsible for specific disease manifestations. Limitation of our study was possible interaction with other polymorphism may affect our results.

Our results suggest that, presence of D allele of the *ACE* gene and C allele of *MTHFR* gene may constitute a risk for developing osteoarthritis. Further work is required to confirm these findings in different study groups.

Conflicts of interest

None.

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