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Decreased Activity and Genetic Polymorphisms of CYP2C19 in Behçet's Disease

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Abstract: Behçet's disease (BD) is a systemic autoimmune disorder. Cytochrome P450 enzymes (CYPs) are responsible for various drug metabolism reactions as well as those of endogenous substances which may be associated with autoimmune disease susceptibility. Recently, we reported that in patients with BD, CYP2C9 seems to be down-regulated due to inflammation. In the same Turkish patients with BD, we investigated whether also CYP2C19 activity is decreased. Lansoprazole (30 mg) was given as a probe drug to evaluate CYP2C19 activity in 59 patients with BD and 27 healthy control volunteers. An HPLC method was used to determine plasma lansoprazole and its metabolite, 5-hydroxy lansoprazole, concentrations. The genotyping for *CYP2C19 *2, *3* and **17* polymorphisms was made using PCR-RFLP. The median lansoprazole/5-hydroxy lansoprazole metabolic ratio (MR) in patients with BD was 2.6-fold higher as compared to the healthy control group (p = 0.001, 22.6 (1.3–26) and 8.8 (0.5–140) as median and range, respectively). The *CYP2C19*17*17* genotype frequency was found to be significantly less in the BD group as compared to the healthy controls (1.7% *versus* 14.8% in controls, p = 0.01). Additionally, colchicine treatment did not affect the CYP2C19 enzyme activity in six patients (p = 0.43). In conclusion, the patients with BD had lower CYP2C19 enzyme activity and lower frequency of the *CYP2C19*17* allele as compared to those of the healthy controls. Further studies are warranted on the mechanisms underlying this relation. This study should also be applied to other autoimmune diseases similarly characterized by local or systemic inflammation.

Autoimmune diseases (AD) are an important public health problem worldwide. AD are mainly characterized by inflammation, but many other genetic, immunological and environmental factors have roles in different degrees in their nature [1]. Scleroderma, rheumatoid arthritis, systemic lupus erythematosus, ancylosan spondilitis and Behçet's disease (BD) are main autoimmune diseases, which progress with systemic or local inflammation. BD is more common in Mediterranean and ancient Silk Road countries but also sporadically present in other countries as well [2]. The prevalence of the disease is around 80–420/100,000 in Mediterranean nations and about 13–20/100,000 in Far East countries [3,4]. BD is a multi-system disorder with its clinical features of skin, mucosal involvement, eye, joints, gastrointestinal and the central nervous system [5,6]. The aetiopathogenesis of the BD is yet unknown but the involvement of systemic inflammation, immunogenetics, immune regulation, vascular abnormalities or bacterial and viral infection may contribute to its development [7]. Colchicine, corticosteroids and cytotoxic agents are used for the treatment of BD.

Cytochrome P450 enzymes (CYPs) are the largest group of enzymes responsible for drug metabolism. One of these enzymes, CYP2C19, plays a role in the metabolism of many drugs like some of the proton pump inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors, benzodiazepines, barbiturates, S-mephenytoin, proguanil, nelfinavir and voriconazole [8–12]. Up to now, there are more than 40 alleles defined for *CYP2C19* (http://www.cypalleles.ki.se/cyp2c19.htm). Many of these polymorphisms cause a loss or decrease of the enzyme activity (**2, *3, *11, *13*, etc.). On the contrary, *CYP2C19*17* results in an increased metabolic capacity of the enzyme [9,13]. Validated probe drugs such as omeprazole, proguanil and S-mephenytoin have been used for the assessment of CYP2C19 activity [9,12,14–18]. 5-hydroxy lansoprazole formation from lansoprazole has recently been suggested for this purpose [19].

Few studies have addressed the interaction between these autoimmune diseases and the CYPs. A study by Aitken & Morgan [20] has shown a possible effect of the disease on the CYPs. An increased concentrations of circulating plasma pro-inflammatory cytokines (e.g. TNF-α and IL-6) have been linked to a significant decrease up to 50% in mRNA levels of CYP2C9 and CYP2C19 in human hepatocytes [20]. We have in a previous study in BD patients and healthy controls shown that the rate of metabolism of losartan by CYP2C9 in patients with BD presumably by down-regulation due to inflammation [21]. In that study, the same subjects also received lansoprazole to evaluate the activity of CYP2C19. In this study, we report the data of lansoprazole MR as the probe of CYP2C19 activity to investigate whether CYP2C19 is regulated similarly to CYP2C9 in BD.

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Table 1.
Primers and restriction enzymes used in the genotyping of *CYP2C19*2, *3* and **17* variants.

Polymorphism	Primer	Enzyme	PCR product (bp)	Cleavage products (bp)	References
*CYP2C19*2*	5′-CAGAGCTTGGCATATTGTATC-3′ 5′-GTAAACACACAACTAGTCAATG-3′	*Sma*I	321	109 + 212	Goldstein *et al.* [23]
*CYP2C19*3*	5′-AAATTGTTTCCAATCATTTAGCT-3′ 5′-ACTTCAGGGCTTGGTCAATA-3′	*Bam*HI	271	175 + 96	Goldstein *et al.* [23]
*CYP2C19*17 (-3402C > T)*	5′-AATAAAGATGACCTTGATCTGG-3′ 5′-GTCTCCTGAAGTGTCTGTAC-3′	*Mnl*I	504	280 + 224	Sim *et al.* [24]

Additionally, the association between the genetic polymorphisms of *CYP2C19* and BD has been reported.

Materials and Methods

The study was conducted at the Departments of Pharmacology and Rheumatology at Hacettepe University Hospital, Ankara, Turkey. Patients and controls were included after being informed about the study, and all subjects gave written informed consent. The study was approved by the local Ethics Committee at Hacettepe University (TBK11/14-15). The research was designed in accordance with the Declaration of Helsinki. All patients were diagnosed according to the International Study Group criteria for the diagnosis of BD [22]. The control group was selected among healthy persons without any known disease. Exclusion criteria in the controls were known hypersensitivity to lansoprazole, use of lansoprazole or any medication that may cause drug–drug interactions within 1 week prior to the study day, having any autoimmune disease and paediatric–geriatric age groups.

Recently, we reported the relation between CYP2C9 polymorphisms and BD [21]. In that study, the patients with BD and healthy controls received a 50-mg single oral dose of losartan before going to bed, and urine was collected for 8 hr after administration of the drug [21]. In the morning, the subjects brought the urine samples and additionally received 30 mg lansoprazole after an overnight fasting. Peripheral venous blood samples were taken 3 hr after lansoprazole to determine the *CYP2C19* genotype and to analyse plasma concentrations of lansoprazole and 5-hydroxy lansoprazole, in this study.

The blood samples were immediately centrifuged for 10 min. at 5000 × *g* for the analysis. The samples were stored at −20°C until analysis. Lansoprazole and the 5-hydroxy metabolite were kindly supplied by Takeda Pharmaceutical Company Limited (Osaka, Japan). Pantoprazole used as an internal standard was kindly provided by Astra Zeneca R&D (London, UK).

High-performance liquid chromatography analysis of lansoprazole and its 5-hydroxy metabolite in plasma. Lansoprazole and 5-hydroxy lansoprazole in plasma were analysed by high-performance liquid chromatography (HPLC) system as described by Gumus *et al.* [19]. Agilent Technologies 1,200 series (Waldbron, Germany) including a quaternary pump, diode array and multiple wavelength detector, vacuum degasser and autosampler were used for the analysis. Briefly, a 65/35 (v/v) mixture of phosphate buffer (NaH_2PO_4, 15 mM, pH = 2.8) and acetonitrile was used as mobile phase for the analysis of lansoprazole and its 5-hydroxy metabolite. Flow rate of mobile phase was calibrated to 1.2 ml/min. The column temperature was adjusted at 40°C and the auto-sampler temperature at 20°C. Ultraviolet detection at a wavelength of 290 nm was used.

Plasma (500 µl) was extracted after adding pantoprazole (50 µg/ml) as an internal standard. Diethyl ether–dichloromethane mixture (70:30, v/v) was used as an extraction solvent. After shaking (15 min.) and centrifuging (1700 × *g* for 5 min. at 4°C), the organic phase was evaporated in vacuo at 45°C at 20 min. to dryness (Vacuubrand MZ 2C + AK + EK, Wertheim, Germany). The residue was dissolved in 200 µl of the mobile phase of which 40 µl was injected to Agilent Eclipse XDB-C18 (5 µm, 4.6 × 150 mm) column. The inter- and intraday coefficients of variation (CV) for lansoprazole and 5-hydroxy lansoprazole were <4.5% and <4%, respectively. The ratio of lansoprazole and 5-hydroxy lansoprazole was calculated (metabolic ratio, MR) and used as a marker for the activity of the CYP2C19. ChemStation for LC-3D systems (Rev.B.03.01.317), Agilent Technologies 2007 software (Agilent Technologies) were used for data evaluation.

Genetic analysis of CYP2C19 **2, *3 and *17.* Genomic DNA was isolated from the peripheral venous blood cells using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). The *CYP2C19*2, *3* and **17* allelic variants were analysed by polymerase chain reaction (PCR) and endonuclease digestion methods (table 1) as described previously with minor modifications [19,23–25].

Polymerase chain reaction amplification was performed in 25 µl of reaction mixture that included 200 ng DNA, 0.25 mM of each deoxynucleotide, 2 mM magnesium, 0.5 U of Taq polymerase (New England Biolabs GmbH, Frankfurt, Germany) and 1.25 µM of each primers for the *CYP2C19*2, *3* and **17* (table 1). Amplification was performed using a P × 2 Thermal Cycler (Thermo Electron Co., Waltham, MA, USA). Primers and restriction enzymes used to determine polymorphisms and fragments generated by each PCR-RFLP and PCR conditions are summarized in tables 1 and 2. The resulting DNA fragments were separated by gel electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining.

Effects of colchicine on CYP2C19 activity. To evaluate the effect of colchicine, CYP2C19 activity was assessed in six patients with rheumatologic diseases before starting the colchicine treatment and after using the colchicine for 2 weeks. These patients were diagnosed as BD (n = 2), arthritis (n = 2), familial Mediterranean fever (n = 1) and aphthous stomatitis (n = 1). MRs analysed at two different occasions were statistically compared in the same subjects.

Table 2.
PCR conditions for the analysis of *CYP2C19 *2, *3* and **17* variants.

	*CYP2C19 *2* and **3*		*CYP2C19*17 (-3402C>T)*	
PCR conditions	Temperature	Duration	Temperature	Duration
Initial denaturation	94°C	5 min.	94°C	1 min.
Denaturation	94°C	20 sec.	94°C	30 sec.
Annealing temperature	55°C	30 sec.	52°C	30 sec.
Extension temperature	72°C	20 sec.	72°C	30 sec.
Final extension temperature	72°C	5 min.	72°C	7 min.
Number of cycles	37		35	

Statistical analysis. Differences in allele frequencies were determined using Fisher's exact chi-square test. Differences in plasma drug and metabolite concentrations of the drug and its metabolite in the patients with BD and healthy volunteers were compared with the Kruskal–Wallis test. Two groups were compared with the Mann–Whitney *U*-test. The MRs of the six subjects before and after the colchicine treatment were compared with Wilcoxon's test. The $p < 0.05$ was assumed as statistically significant. GraphPad Prism version 6.01 for Windows (San Diego, CA, USA) was used for the statistical comparisons. The power of the study was calculated as described by Dupont WD and Plummer WD [26].

Results

Fifty-nine Behçet patients (31 men, 28 women) and 27 healthy volunteers (24 men, three women) were recruited in this study. The mean age of the patients was 41.5 (range between 20 and 73) years, and the mean time after the onset of the disease was 10.9 (range between 1 and 43) years. The mean age of the control group was 32.7 (range between 26 and 47) years.

Lansoprazole MR in patients with Behçet and controls.
The median lansoprazole/5-hydroxy lansoprazole metabolic ratio (MR) in the patients with BD was found to be 2.58-fold higher compared to the healthy control subjects as shown in fig. 1. Lansoprazole MR (as median and range) was 22.62 (1.27–260) and 8.75 (0.52–140.0) in the patients with BD and controls, respectively ($p = 0.001$). There was about a 6.4-fold increase in the lansoprazole MR of the BD patients with *CYP2C19*1*2* genotype as compared to that of the controls

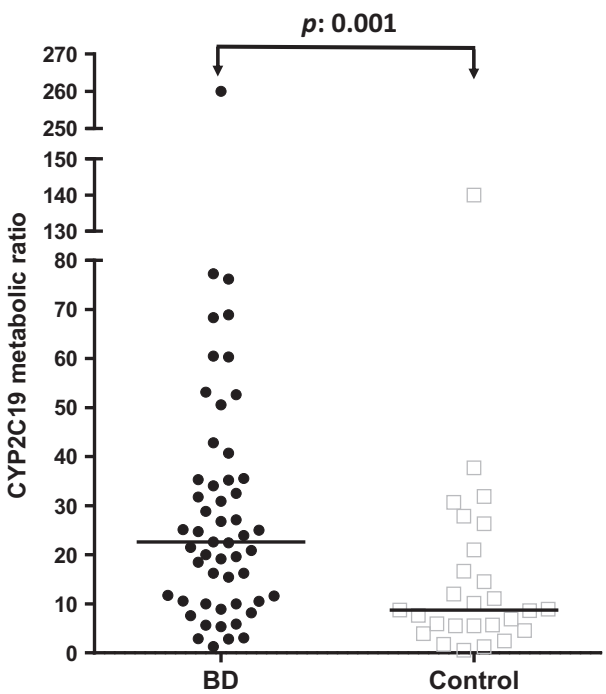

Fig. 1. Comparison of the lansoprazole/5-hydroxy lansoprazole metabolic ratios in patients with BD and in the healthy control subjects. n = 53 in BD and n = 27 in the control groups. Median values are indicated.

with the same genotype (table 3, $p = 0.002$). Lansoprazole and 5-hydroxy metabolite were detected in all of the healthy controls. However, in six patients with BD, neither the main drug nor its metabolite was detected in blood. A most probable explanation for the absence of detection could be that these six patients did not swallow the lansoprazole capsule.

The MR was higher overall in the whole patient group but was not significantly different between subgroups of the *CYP2C19*1*1, *1*17* and the **2*17* genotyped subjects (table 3). Large variations in the subgroups were thought to be the reason for this incompatibility.

The median MRs were similar among the male and female Behçet patients (20.3 *versus* 26.8, respectively, $p = 0.76$). The MRs of the male and female subjects when compared between BD and control groups were not significantly different either ($p = 0.98$ and $p = 0.68$, for male and female individuals, respectively). The age did not have a significant effect on MR in the groups. The MRs of the patients between the over 40 and <40 years age groups were similar (23.8 *versus* 22.4).

Effects of colchicine on CYP2C19 activity.
In six patients who were prescribed colchicine, lansoprazole/5-hydroxy lansoprazole MR was calculated before starting the treatment and 2 weeks after the treatment. The median (range) metabolic ratios were 20.0 (0.8–36.1) and 17.2 (1.5–37.6) before and after the colchicine treatment ($p = 0.43$).

CYP2C19 *genetic polymorphism analysis.*
Chi-square test on the observed and expected values supports the hypothesis that the population is in Hardy–Weinberg equilibrium for the *CYP2C19* gene. The frequencies of the *CYP2C19 *1*, **2* and **17* alleles in the patients with BD were 73.7%, 11.9% and 14.4%, respectively, while **3* allele was not found among the patients with BD. In the controls, the frequencies of the **1*, **2* and **17* alleles were 57.4%, 11.1% and 29.6%, respectively, while the **3* allele was only present in only one subject. In the patients with BD, **1* allele frequency was about 73.7% higher than that of the controls ($p = 0.03$) while the **17* allele frequency was about twofold lower ($p = 0.01$) as summarized in table 4. Also the *CYP2C19 *17*17* genotype frequency in the patients with BD (1.7%) was significantly lower as compared to that of the controls (14.8%, $p = 0.01$). Considering the *CYP2C19* genetic analysis, six different genotypes were determined in the patients and controls. Allele and genotype frequencies evaluated by the chi-square test were in compliance with the Hardy–Weinberg equation. The allele (**2* and **3*) and genotype (**1*1*, **1*2, *1*3, *1*17, *2*2* and **2*17*) frequencies were similar in the patients with BD and the controls (table 4).

Discussion

In this study, we found that CYP2C19 activity of the patients with BD was significantly lower than of healthy subjects. Additionally, distribution of the *CYP2C19* alleles and genotypes was significantly different in the two groups. Patients

Table 3.
Lansoprazole/5-hydroxy lansoprazole metabolic ratio in BD and healthy controls with respect to *CYP2C19* genotypes. Metabolic ratios are presented for 53 of 59 patients with BD, because six of them did not take lansoprazole for phenotyping purposes.

CYP2C19 Gene	BD n (%)	Metabolic Ratio Median (Range)	Control n (%)	Metabolic Ratio Median (Range)	$p^{\#}$
**1*1*	29 (54.7)	19.6 (1.3–260.0)	10 (37)	9.5 (1.7–31.9)	0.08
**1*2*	10 (18.9)	51.6 (25.1–68.8)	4 (14.8)	8.1 (5.5–16.7)	0.002
**1*3*	0		1 (3.7)	30.7	
**1*17*	11 (20.8)	20.9 (5.4–53.1)	6 (22.2)	9.0 (0.5–140)	0.56
**2*2*	1 (2.4)	77.3	0		
**2*17*	2 (4.8)	5.6 (3.1–8.2)	2 (7.4)	0.2 (0.1–0.4)	0.33
**17*17*	0		4 (14.8)	5.6 (1.3–26.3)	
$p^{\#\#}$		0.001		0.2	

[#]Two-group comparisons with Mann–Whitney *U*-test.
[##]Multi-group comparisons with Kruskal–Wallis test.

Table 4.
Comparison of the *CYP2C19* allele and genotype frequencies between the patients with BD and the controls.

CYP2C19 Alleles	Behçet patients N (%)	95% CI	Controls N (%)	95% CI	*p* Value
**1*	87 (73.7)	64.5–83	31 (57.4)	40–74.8	0.03
**2*	14 (11.9)	0–28.8	6 (11.1)	0–36.3	0.88
**3*	0		1 (1.9)	0–28.3	0.13
**17*	17 (14.4)	0–31.1	16 (29.6)	7.3–52	0.01
Total	118 (100)		54 (100)		
Genotypes	N (%)	95% CI	N (%)	95% CI	
**1*1*	32 (54.2)	37–71.5	10 (37.1)	7–67	0.13
**1*2*	10 (17)	0–40.2	4 (14.8)	0–49.6	0.80
**1*3*	0		1 (3.7)	0–40.7	0.13
**1*17*	13 (22)	0–44.6	6 (22.2)	0–55.5	0.98
**2*2*	1 (1.7)	0–27	0		0.49
**2*17*	2 (3.4)	0–28.5	2 (7.4)	0–4.7	0.41
**17*17*	1 (1.7)	0–27	4 (14.8)	0–49.6	0.01
Total	59 (100)		27 (100)		

with BD had lower presence of the **17* allele than that of the healthy controls. As we know, this is the first study that evaluated CYP2C19 phenotypes and genotypes in the patients with BD.

The relationship between different autoimmune diseases and polymorphisms of the genes encoding drug-metabolizing enzymes has been inconclusively reported in different studies [27–29]. However, the relationship between the patients with BD and polymorphisms of the genes encoding drug-metabolizing enzymes has not been investigated to the full. In this study, patients with BD had about 2.5-fold increased lansoprazole metabolic ratio compared to the controls suggesting that CYP2C19 activity is markedly reduced in these patients. Consistent with this finding, in our previous study [21], we reported a significantly decreased CYP2C9 activity in the same patients with BD. Immune cytokine reactions in BD might have caused the down-regulation of CYP2C9 as well as of the CYP2C19 activity.

In the present study, *CYP2C19*1* allele frequency in the BD group was higher, while the **17* allele frequency was lower, than those of the control group. This is the first study investigating the *CYP2C19*17* polymorphism in BD. A previous study by Tursen *et al.* [30] reported the genetic polymorphisms of *CYP2C19*2, *3* in BD. In their report, *CYP2C19*2* frequency was higher in the patients with BD when compared to the controls [30]. The low frequency of the *CYP2C19*17* polymorphism in the BD patients suggests that the frequency of this polymorphism in the other autoimmune diseases should also be investigated, because these diseases have similar aetiological factors.

The *CYP2C19*1*2* carriers in the BD group had about a 6.4-fold higher MR as compared to the healthy subjects with the same genotype. About a twofold higher MR was observed for *CYP2C19*1*1* carriers. These findings suggest a deceased CYP2C19 activity independent of genotype.

Previous studies found that pathogenesis of autoimmune diseases might be related to increased reactive oxygen species (ROS). A study by May *et al.* [29] reported that decreased cytochrome P-450 activity caused decline in ROS metabolism and this alteration might have a key role in scleroderma pathogenesis. Some evidence was found in the literature that autoimmune diseases such as rheumatoid arthritis may be associated with reactive metabolite substances resulting from exogenous and endogenous compounds metabolism by CYP1A2 [31]. A study by Tursen *et al.* found that the *CYP2C19*2* allele may be associated with BD pathophysiology [30]. Furthermore, we suggest that *CYP2C19*17* might be a preventive factor for BD as well as for other autoimmune diseases as they are of similar nature.

The elevated pro-inflammatory cytokines that have appeared in the course of the disease may be the cause of decreased enzyme activity in BD. In the literature, some studies reported that CYP activity was down-regulated when several pro-inflammatory cytokines (e.g. TNF-α and interleukin-6) increased and these findings support our hypothesis [32,33]. In an *in vivo* human study by Frye *et al.* [34], there was a reverse association between plasma TNF-α, interleukin-6 levels and CYP2C19 activity. In the same study, however, no association was found between these cytokines and CYP1A2, 2D6 and 2E1 activities. Another study reported that patients with *CYP2C19*2* polymorphism have higher IL-6 and CRP

levels [35]. After *in vivo* administration of IL-10, the activity of CYP3A was decreased but not that of the CYP1A2, 2C9 and 2D6 [36]. We did not analyse the levels of pro-inflammatory cytokines, and therefore, it is difficult to correlate the decreased enzyme activity with these cytokines.

In the present study, about 95% of the patients were using colchicine during the trial. Significantly decreased CYP2C19 activity in the patients with BD might have been due to the inhibitory effect of colchicine on enzyme activity. In the literature, no study demonstrated a possible interaction between colchicine and substrates metabolized by CYP2C19. In an *in vitro* study in human hepatocytes, Dvorak *et al.* [37] showed that colchicine did not have significant effect on CYP2C19 expression at up to 10-μM concentrations. In another *in vivo* study, colchicine did not significantly change CYP2C19 activity in rat [38]. We also recently showed that colchicine does not have any significant effect on losartan metabolism, which is a probe reaction for phenotyping of the CYP2C9 *in vivo* in human beings [21]. In this study, we demonstrated that colchicine did not exert any change in the CYP2C19 activity of six patients who used the drug for 2 weeks.

The main purpose of this study was to compare the CYP2C19 enzyme activity between the patients with BD and healthy controls, and the statistical power of this comparison was sufficient for a proper analysis. On the other hand, we could not reach the desired number of subjects for the genotype comparisons in the groups. The estimated sample sizes with a power of 0.80 (α:0.05) were 132 patients for the comparison of the CYP2C19**1*1* genotype and 68 patients for the comparison of the **17*17*. This is a drawback in our study but, even with these small number of genotype groups, we could demonstrate a statistically significant differences for *CYP2C19*17* allele.

In conclusion, we showed that CYP2C19 activity of the patients with BD was significantly lower as compared to healthy subjects. Frequency of the *CYP2C19*17* allele was significantly lower in the patients with BD. Further studies are needed on the mechanisms underlying this relation. This study should also be applied to other autoimmune diseases similarly characterized by local or systemic inflammation.

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