

Bactericidal Permeability Increasing Protein Gene Polymorphism is Associated with Inflammatory Bowel Diseases in the Turkish Population

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ABSTRACT

Background/Aims: Inflammatory bowel disease, a chronic inflammatory disease with unknown etiology, affects the small and large bowel at different levels. It is increasingly considered that innate immune system may have a central position in the pathogenesis of the disease. As a part of the innate immune system, bactericidal permeability increasing protein has an important role in the recognition and neutralization of gram-negative bacteria. The aim of our study was to investigate the involvement of bactericidal permeability increasing protein gene polymorphism (bactericidal permeability increasing protein Lys216Glu) in inflammatory bowel disease in a large group of Turkish patients. **Patients and Methods:** The present study included 528 inflammatory bowel disease patients, 224 with Crohn's disease and 304 with ulcerative colitis, and 339 healthy controls. **Results:** Bactericidal permeability increasing protein Lys216Glu polymorphism was found to be associated with both Crohn's disease and ulcerative colitis ($P = 0.0001$). The frequency of the Glu/Glu genotype was significantly lower in patients using steroids and in those with steroid dependence ($P = 0.012$, OR, 0.80; 95% confidence interval [CI]: 0.68-0.94; $P = 0.0286$, OR, 0.75; 95% CI: 0.66-0.86, respectively). There was no other association between bactericidal permeability increasing protein gene polymorphism and phenotypes of inflammatory bowel disease. **Conclusions:** Bactericidal permeability increasing protein Lys216Glu polymorphism is associated with both Crohn's disease and ulcerative colitis. This is the first study reporting the association of bactericidal permeability increasing protein gene polymorphism with steroid use and dependence in Crohn's disease.

Key Words: Bactericidal permeability increasing protein, inflammatory bowel disease, single nucleotide polymorphism

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Inflammatory bowel disease (IBD) is a chronic inflammatory disease with unknown etiology, which affects the different layers of small and large intestine at different levels.^[1] It is known that a lot of environmental and genetic factors have a role in the development of the disease. Immunologic response is excessively increased against intestinal microorganisms in genetically susceptible persons. Healthy people have an

immune tolerance against commensal bacteria; however, there is a loss of immune tolerance in patients with IBD. It is not clear what mechanisms are responsible for this situation.^[2] Luminal antigens not contacting the immune system normally may trigger the immune response by crossing the epithelial barrier as a result of increased permeability of the intestinal epithelium.^[3,4] On the other hand, improper activation of pathogen recognition receptors that identify the pathogen microorganisms may be responsible for the excessive immune response.

As a part of the innate immune system, nucleotide-binding oligomerization domain-2 (NOD2) protein, whose function is to identify the bacterial motifs, was found to have three different mutations associated with susceptibility to Crohn's disease (CD).^[5-8] Thereafter, two different

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single nucleotide polymorphisms (SNP) associated with IBD were identified in toll-like receptor-4 (TLR4) recognizing lipopolysaccharide (LPS), an integral part of the gram-negative bacterial membrane.^[9] It is increasingly recognized that innate immune system may have a central position in the pathogenesis of the disease. Proteins that play a role in the recognition of pathogen microorganisms are believed to be associated with IBD pathogenesis.

Various antimicrobial peptides are secreted by the host against invasive pathogens. These peptides selectively identify and eradicate pathogen microorganisms before spreading all over the body. Bactericidal permeability increasing protein (BPI), one of the most important of these peptides, prevents inflammatory response by binding to LPS. Normally, it has an opposite effect to LPS binding protein (LBP) that induces acute inflammation. It is effective in the elimination of bacteria besides limiting inflammation at a focal area without progression to systemic inflammation.^[10-12]

Some authors have asserted that any impairment in the functions of BPI protein by virtue of BPI gene polymorphism may change the pathogen microorganism recognition ability of the innate immune system and eventually give rise to undesirable outcomes in IBD.^[13] The results of the studies so far, on the association between IBD and BPI polymorphism are not satisfactory, besides not compatible with each other. Akin *et al.*^[14] found that BPI gene polymorphism was significantly associated with UC and CD; however, their results conflicted with the current literature. It was thought that these conflicting results might be due to being a single-centre study and the small sample size of their study. The aim of our study was to investigate the involvement of protein (BPI) gene polymorphism (Lys216Glu) in a large group of Turkish IBD patients recruited from three geographically different regions, as a continuation of the previous study in Turkish population.

PATIENTS AND METHODS

Study population

The patients with IBD were recruited from three geographically different regions including Marmara University Faculty of Medicine Hospital, Balıkesir State Hospital and İzmir Katip Çelebi University Atatürk Training and Research Hospital. IBD patients who were admitted to the gastroenterology clinics of these centers between November 2009 and March 2010 were included in our study. CD and UC were defined using conventional clinical, radiological, endoscopic and histological criteria. The patients who had indeterminate colitis and those younger than 18 years were excluded from the study. Cases were phenotyped according to the Montreal classification systems that allow genotype-phenotype analysis. The study

population consisted of 528 IBD patients (304 UC, 224 CD) and 339 healthy controls. Clinical and demographic data of the study population were recorded. Informed consent was taken from all participants before participating.

DNA extraction

Venous blood samples taken from all patients and healthy controls via venipuncture were collected into sterile, ethylenediaminetetraacetic acid (K₃EDTA) containing tubes. Genomic DNA was isolated from collected blood samples by phenol-chloroform method.^[15] High-Pure polymerase chain reaction (PCR) Template Preparation Kit (Cat. No: 11 796 828 001, Roche, Mannheim, Germany) was used for DNA extraction.

Genotyping

The Lys216Glu (645 A/G) (rs4358188) SNP in BPI gene was genotyped with PCR and restriction fragment length polymorphism procedure (RFLP).^[16] PCR solution was prepared by using 100 ng genomic DNA, 1 × PCR buffer (Fermentas, Burlington, Ontario, Canada), 0.2 mM of each dNTP (Fermentas), 0.5 U *FastStart Taq* DNA polymerase (Fermentas), 5 pmol primer and 2.5 mM MgCl₂. The final volume of solution was increased to 20 µL with 12.5 µL distilled water. The solution prepared for PCR was heated for 15 minutes at 95°C, then after denaturation for 35 cycles at 94°C for 30seconds, and a gap of 30 seconds at 60°C, it was followed by 30 seconds at 72°C for annealing, and finally 10 minutes at 72°C for extension. After the PCR reaction was completed, amplification products were digested with *HindIII* restriction enzyme (Fermentas) for overnight at 37°C. The length of PCR products, primers and the heat of annealing are demonstrated in Table 1. For electrophoresis, 2.5% standard agarose gel was prepared with 100 cc 0.5X *Tris Borate* EDTA (TBE) and 20 µL (10 mg/mL) ethidium bromide. The resulting fragments were placed on agarose gel after staining with bromophenol blue, and were run on agarose gel at 80 volts for 20 minutes by electrophoresis. The resulting bands were read and compared with representative samples for BPI [Figure 1]. The length of restriction fragments and the restriction's heat are given in Table 1.

Statistical analysis

Chi-square test with Yates correction was used for the comparison of allele and genotype frequencies of disease and control groups. The Fisher's exact test was used at the appropriate conditions. Bonferroni correction was applied for multiple comparisons. Comparison of observed and predicted frequencies was analyzed by Hardy-Weinberg equilibrium. A *P* value < 0.05 was considered to be statistically significant. Statistical Package for the Social Sciences (SPSS) for Windows (version 16.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analysis in this study.

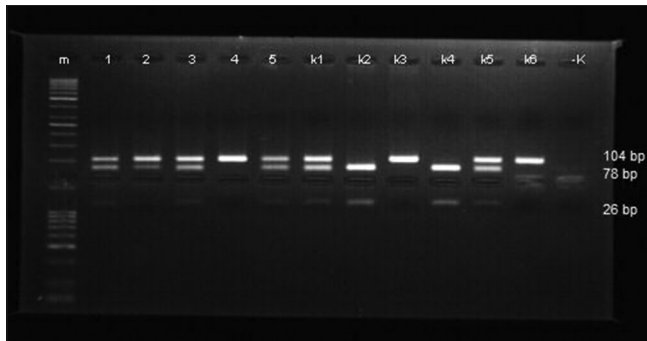


Figure 1: The resulting bands from polymerase chain reaction and restriction fragment length polymorphism procedure for bactericidal permeability increasing protein in study population. m: Marker, k: Control group, -k: Negative control

Ethics

The Ethics Committee of Marmara University Faculty of Medicine, Istanbul, Turkey approved this study (Istanbul 9. Clinical Research Ethics Committee; project no: 09.2009.0018; acceptance date: 07 Jan 2010).

RESULTS

The present study included 528 patients with IBD and 339 healthy controls. The demographic and clinical characteristics of the study groups are given in Table 2. UC, CD and control groups were equally distributed in terms of age and gender. Statistical analysis revealed that BPI Lys216Glu (645 A > G) SNP was found to be associated with both CD and UC ($P = 0.0001$) as compared with healthy controls [Table 3]. It was demonstrated that both *Glu/Glu* genotype and *Glu* allele frequencies were significantly increased in the UC group ($P = 0.0006$, OR = 1.862 95% CI = 1.302-2.662; $P = 0.0003$, OR = 1.504 95% CI = 1.207-1.875, respectively), however only *Glu* allele frequencies were shown to be increased in the CD group as compared with healthy controls ($P = 0.0013$, OR = 1.491 95% CI = 1.172-1.895). On the other hand, *Lys/Lys* genotype and *Lys* allele frequencies were significantly decreased both in the UC ($P = 0.0113$, OR = 0.686 95% CI = 0.422-0.897; $P = 0.0003$, OR = 0.664 95% CI = 0.533-0.828, respectively), and CD groups ($P = 0.0001$, OR = 0.405 95% CI = 0.256-0.640; $P = 0.0013$, OR = 0.670 95% CI = 0.527-0.852, respectively). Genotype and allele frequencies of patient and control groups are shown in Table 3. When the association of corticosteroid use and corticosteroid dependence in IBD with BPI gene polymorphism were investigated separately, the frequency of *Glu/Glu* genotype was found to be significantly lower in CD patients using corticosteroids and in those with corticosteroid dependence ($P = 0.0127$, OR = 0.80 95% CI = 0.68-0.94; $P = 0.0286$, OR = 0.75 95% CI = 0.66-0.86, respectively) [Table 4]. No association was found for other drug usage, disease phenotype and localization, duration of

Table 1: Polymerase chain reaction and restriction fragment length polymorphism procedure conditions, primers and resulting products

Primer (BPI; Lys216Glu)	F: 5'-CACTATGGGAAGACCTTACTGATTAC-3' R: 5'-CAGAGTCTGGAAATAAGGTTGAAGC-3'
PCR product (bp)	104
Heat of annealing (°C)	60
Restriction fragments (bp)	G allele: 104, A allele: 78+26
Heat of restriction (°C)	37

BPI: Bactericidal permeability increasing protein, PCR: Polymerase chain reaction

Table 2: Demographic and clinical characteristics of the patients

	UC (n=304)	CD (n=224)
Age, years	45.7±14.9 (18-81)	39.9±13.4 (18-86)
Female	44.8	45.1
Localization UC		
Extensive colitis	99 (32.6)	-
Left colitis	160 (52.6)	-
Proctitis	45 (14.8)	-
Phenotype CD		
Inflammatory	-	136 (60.7)
Fibrostenotic	-	31 (13.8)
Fistulizing	-	57 (25.5)
Localization CD		
Ileal	-	67 (29.9)
Ileocolonic	-	101 (45.1)
Colonic	-	56 (25.0)
Corticosteroid use	77 (26.3)	81 (40.9)
Corticosteroid dependence	11 (3.8)	20 (10.1)
Azathioprine	44 (15.1)	99 (52.1)
AntiTNF-α	3 (1.0)	32 (16.2)
Resection surgery	17 (5.8)	22 (11.2)
Family history	33 (11.1)	19 (10.0)
Smoking	104 (35.7)	93 (48.9)

Data are presented as mean±standard deviation (range) or (%) or n (%), where appropriate. UC: Ulcerative colitis, CD: Crohn's disease, AntiTNF-α: Anti-tumor necrosis factor-α

inflammation, family history, extraintestinal manifestations, bowel resection and smoking.

DISCUSSION

To the best of our knowledge, four studies were performed on the association between IBD and BPI gene polymorphism [Table 5]. The first study was performed in a German population in 2004 by Török *et al.*^[16] This study found no significant association between IBD and BPI gene polymorphism. However, it was determined that concurrent presence of TLR4, which is another pathogen recognition molecule, and BPI (*Lys/Glu* genotype) gene

polymorphisms significantly increased the risk of CD. Another study in German population was conducted by Klein *et al.*^[13] in 2005. In variance with the results of Török *et al.*,^[16] he found that *Glu/Glu* genotype was protective against CD. The discrepancy between the results of the two studies performed in the same population can be attributed to the number of patients participated in the studies. In another study, performed by Petermann *et al.*^[17] in a New Zealand population, a significant association was shown only between *Lys* allele and ileocolonic CD. On the other hand, it was found that the combination of CD14 and BPI gene polymorphisms was higher in UC. Lastly, Akin *et al.*^[14] investigated BPI gene polymorphism in Turkish IBD patients in 2008 and found BPI gene polymorphism to be significantly associated not only with CD but also with UC, as compared with the other studies performed in different populations. Contrary to the study by Klein *et al.*,^[13] Akin *et al.*^[14] found that both *Glu* allele and *Glu/Glu* genotype were risk factors for both UC and CD. Moreover, the association between BPI polymorphism and UC was reported for the first time in the Turkish population.^[14] The number of patients included in our study was much greater than that in the study by Akin *et al.*^[14] and we showed a stronger association in the same population. Contrary to the results of the study by Akin *et al.*,^[14] we found that both *Lys* allele and *Lys/Lys* genotype were protective against UC and CD, but weak association between *Glu/Glu* genotype and CD in Akin's study lost its significance. It can be related to the number of patients involved in the studies. While *Glu/Glu* genotype was protective against CD with regard to the study by Klein *et al.*,^[13] it was found to be a risk factor for both UC and CD in the study of Akin *et al.*^[14] As for our study, it is only a risk factor for UC. On the other hand, Petermann *et al.*^[17] studied the polymorphism in patients of European origin. The results of two studies performed in the German population were neither similar to each other nor to those of Petermann *et al.*^[17] The results of the Turkish studies partially resemble each other, but they are quite different from the results of the other studies. Differences in the results of these studies may be explained by ethnic differences rather than methodological reasons, as all the studies used the same methodological procedure. As a result, it brings to mind that different mechanisms may play a role in the etiology of IBD in different populations. Because we showed the association between BPI and IBD in our population, we can say that BPI seems to have a role in the etiology of UC in Turkish population.

In addition, our study is the only report, which determined an association between corticosteroid use and dependence in CD, and BPI gene polymorphism. The frequency of *Glu/Glu* genotype was significantly lower both in CD patients who were using steroids and in those with steroid dependence [Table 4]. We infer that *Glu/Glu* genotype is

Table 3: Genotype and Allele frequencies of Lys216Glu (645 A>G) polymorphism in bactericidal permeability increasing protein gene

BPI Lys216Glu	Genotype frequencies n (%) [*]			Allele frequencies (%) [*]	
	Lys/Lys	Lys/Glu	Glu/Glu	Lys (allele)	Glu (allele)
UC (n=304)	56 (18.4) [‡]	150 (49.3)	98 (32.2) [§]	43 ^{**}	57 ^{††}
CD (n=224)	29 (12.9) [‡]	136 (60.7)	59 (26.3)	43 ^{‡‡}	57 ^{§§}
Control (n=339)	91 (26.8)	179 (52.8)	69 (20.4)	53	47

^{*}Chi-square test for multiple groups; $P<0.0001$, SD: 4, [†]Chi-square test for multiple groups; $P<0.0001$, SD: 2. After Chi-square test for multiple groups, two-groups Chi-square test was performed; [‡]($P=0.0113$, OR=0.686; 95% CI=0.422-0.897) [§]($P=0.0006$, OR=1.862; 95% CI=1.302-2.662). ^{‡‡}($P=0.0001$, OR=0.405; 95% CI=0.256-0.640). ^{**}($P=0.0003$, OR=0.664; 95% CI=0.533-0.828). ^{††}($P=0.0003$, OR=1.504; 95% CI=1.207-1.875). ^{‡‡}($P=0.0013$, OR=0.670; 95% CI=0.527-0.852). ^{§§}($P=0.0013$, OR=0.491; 95% CI=1.172-1.895). BPI: Bactericidal permeability increasing protein, UC: Ulcerative colitis, CD: Crohn's disease

Table 4: The association between corticosteroid use and dependence in inflammatory bowel disease and bactericidal permeability increasing protein gene polymorphism

	Genotype frequency n (%)			P value [*]
	Lys/Lys	Lys/Glu	Glu/Glu	
Cs use				
CD				
available (n=81)	13 (16.0)	55 (67.9)	13 (16.0) [†]	0.033
absent (n=117)	14 (12.0)	65 (55.6)	38 (32.5)	
UC				
available (n=77)	11 (14.3)	43 (55.8)	23 (29.9)	0.447
absent (n=216)	43 (19.9)	105 (48.6)	68 (31.5)	
Cs dependence				
CD				
available (n=20)	5 (25.0)	14 (70.0)	1 (5.0) [‡]	0.046
absent (n=178)	22 (12.4)	106 (59.6)	50 (28.1)	
UC				
available (n=11)	3 (27.3)	3 (27.3)	5 (45.5)	0.291
absent (n=282)	51 (18.1)	145 (57.4)	86 (30.5)	

^{*}After Chi-square test for multiple groups, two-groups Chi-square test was performed. [†]($P=0.0127$, OR=0.80; 95% CI=0.68-0.94). [‡]($P=0.0286$, OR=0.75; 95% CI=0.66-0.86). Cs: Corticosteroid, UC: Ulcerative colitis, CD: Crohn's disease

associated with mild CD. This finding may help us plan the treatment strategy in patients carrying this polymorphism.

In a recent study, the concentration of BPI protein was demonstrated to be increased in the intestinal tissue specimens of patients with ulcerative colitis (UC).^[18-20] In addition, it was shown that the level of autoantibodies for BPI protein was increased in direct proportion to increase of the severity of IBD.^[21] Although it was shown that BPI was associated with IBD, we do not know what are the modifications, what effect BPI gene polymorphism has on BPI molecule, and which mechanisms are involved in this process. Further research is required to explain this obscurity.

Table 5: Genotype and allele frequencies of BPI (Lys216Glu) polymorphism studies in IBD patients from different populations

BPI studies (Nations)	Török ^[16] (German)	Klein ^[13] (German)	Petermann ^[17] (New Zealand)	Akin ^[14] (Turkish)	Can (Turkish)
Patients, n					
Crohn's disease	102	265	382	116	224
Ulcerative colitis	98	207	400	122	304
Control	145	608	282	197	339
Genotype frequencies, n (%)					
Crohn's disease					
Lys/Lys	18 (18)	66 (25)	88 (23)	9 (7.8)	29 (12.9)
Lys/Glu	58 (57)	143 (54)	198 (51.8)	77 (66.4)	136 (60.7)
Glu/Glu	26 (25)	56 (21)	96 (25.2)	30 (25.9)	59 (26.3)
Ulcerative colitis					
Lys/Lys	17 (17)	45 (22)	88 (22)	20 (16.4)	56 (18.4)
Lys/Glu	50 (51)	106 (51)	200 (50)	61 (50)	150 (49.3)
Glu/Glu	31 (32)	56 (27)	112 (28)	41 (33.6)	98 (32.2)
Control					
Lys/Lys	32 (22)	140 (23)	55 (19.5)	66 (33.5)	91 (26.8)
Lys/Glu	72 (50)	298 (49)	150 (53.2)	100 (50.8)	179 (52.8)
Glu/Glu	41 (28)	170 (28)	77 (27.3)	31 (15.7)	69 (20.4)
Allele frequencies, (%)					
Crohn's disease					
Lys	46	52	49	40.1	43
Glu	54	48	51	59.1	57
Ulcerative colitis					
Lys	43	47	47	41.4	43
Glu	57	53	53	58.6	57
Control					
Lys	47	47	46	58.9	53
Glu	53	53	54	41.1	47

BPI: Bactericidal permeability increasing protein, IBD: Inflammatory bowel disease

In conclusion, our study provided a larger data to evaluate the association between BPI and IBD in the Turkish population, and it demonstrated that there was a strong association between BPI and UC, while the previously shown association between BPI and CD was weakened. BPI may be more important in the etiology of UC in the Turkish population than the other populations. It was shown for the first time that BPI is associated with drug usage and with mild CD. Ethnic differences may play a major role in the conflicting results obtained in different studies. Further large-scale studies in different populations are necessary to explore this association.

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