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Assessment of the role of EGF +61A/G and EGFR R497K polymorphism in patients with inflammatory bowel disease: A case-control study

İnflamatuar bağırsak hastalığında EGF +61A/G ve EGFR R497K polimorfizm rolünün değerlendirilmesi: Bir olgu-kontrol çalışması

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Abstract

Aim: Epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) play an important role in the regulation of cell growth, survival, migration, apoptosis, proliferation, and differentiation. We aimed to investigate the presence of EGF (+61A/G) and EGFR R497Kpolymorphisms in patients with inflammatory bowel disease (IBD) and their associations with clinical features of the patients.

Methods: This case-control study included 91 IBD patients (45 Crohn's disease (CD) patients and 46 ulcerative colitis (UC) patients) and 129 healthy controls (HC).EGF and EGFR were genotyped by polymerase chain reaction and restriction fragment length polymorphism techniques to elucidate their association with clinical outcomes. The disease activity for UC and CD were assessed by Truelove-Witts index (TW) and Crohn's disease activity index (CDAI), respectively. The Montreal classification was used for disease involvement and behavior.

Results: EGFR497 AA genotype was significantly decreased in patients with UC compared with CD and HC. In addition, the patients with UC who had EGF +61 A allele had increased risk of moderate and severe disease (p=0.28; OR= 3.13; 95% CI=0.34-28.73). The patients with CD who had the EGF61 AG genotype were found to increased risk for the presence of penetrating disease (p=0.14; $\chi 2$=5.59; OR=5.00; 95% CI=1.26-19.83). EGF +61 A genotype carriers also had higher CDAI scores (p=0.19; OR=4.00; 95% CI=0.44-36.14). In addition, A+ carriers were also found to have higher requirement for anti-TNF treatment (p=0.11; OR=5.0; 95% CI=0.56-44.4).

Conclusion: In this study, EGFR 497 AA genotype was found to decrease significantly in patients with UC compared to HC and CD patients. To enlighten the mechanism, further studies with larger sample groups are needed to clarify the role of the EGF (+61A/G) and EGFR R497K genes polymorphism, and development of the etiology and pathogenesis of IBD.

Keywords: EGF61, EGFR497, inflammatory bowel diseases, Crohn's disease, ulcerative colitis

Öz

Amaç: Epidermal büyüme faktörü (EGF) ve epidermal büyüme faktörü reseptörü (EGFR), hücre büyümesi, canlılığı, migrasyonu, apoptoz, proliferasyon ve farklılaşmasının düzenlenmesinde önemli bir rol oynamaktadır. İnflamatuar barsak hastalığı (İBH) olan hastalarda EGF (+61A/G) ve EGFR R497K polimorfizmlerinin varlığını ve hastalığın klinik özellikler ile ilişkisini araştırmayı amaçladık.

Yöntemler: Bu vaka kontrol çalışmasında 91 IBD hastası (45 Crohn hastalığı (CD) hastası ve 46 ülseratif kolit (UC) hastası) ve 129 sağlıklı kontrol (HC) vardı. EGF ve EGFR, polimeraz zincir reaksiyonu ve restriksiyon fragman uzunluğu polimorfizm teknikleri ile hastalık ve sağlıklı control grubu genotiplendirildi. Genotiplerinhastalık ve klinik özellikleri ile ilişkileri incelendi. UC ve CD için hastalık aktivitesi sırasıyla Truelove-Witts indeksi (TW) ve Crohn hastalığı aktivite indeksi (CDAI) ile değerlendirildi. Montreal sınıflandırması hastalık tutulumu ve davranışı için kullanılmıştır.

Bulgular: Ulseratif kolit hastalarında EGFR497 AA genotipi CD ve HC'ye göre anlamlı olarak azaldığı saptanmıştır. Ek olarak, EGF +61 A alleli olan UC'li hastalarda orta ve ciddi hastalık riski artmıştır (p = 0.28; OR = 3.13; % 95 CI = 0.34-28.73). EGF +61 AG genotipine sahip olan CD'li hastalarda penetran hastalık varlığı açısından artmış risk bulundu (p = 0.14; $\chi 2$ = 5.59; OR = 5.00; % 95 CI = 1.26-19.83). EGF +61 A alleli taşıyıcılarında daha yüksek CDAI skor riski saptandı (p = 0.19; OR = 4.00; % 95 CI = 0.44-36.14). Ek olarak, CD hastalarında EGF +61 A alleli taşıyıcılarının anti-TNF tedavi gereksinimi için artmış riske sahip olduğu bulunmuştur (p = 0.11; OR = 5.0; % 95 CI = 0.56-44.4).

Sonuç: Bu çalışmada, UC'li hastalarda EGFR497 AA genotipinde HC ve CD'li hastalara kıyasla, anlamlı azalma saptandı. EGF +61A allele sahip hastalarda artmış aktivite riski saptanmıştır.IBD'nin etiyolojisi ve patogenezinde EGF (+ 61A / G) ve EGFR R497K gen polimorfizminin rolünü açıklığa kavuşturmak için daha geniş örnek gruplarıyla daha fazla çalışmaya ihtiyaç vardır.

Anahtar Kelimeler: EGF +61A/G, EGFR R497K, inflammatuar bağırsak hastalığı, Crohn's hastalığı, ülseratif kolit

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Introduction

The inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders of the alimentary tract with unknown etiology. Interactions between genetic and environmental factors and exaggerated immunologic response against several antigens have been accepted as included in the etiopathogenesis of IBD [1]. IBD mainly consists of two diseases according to clinical and histopathologic features: Crohn's disease (CD) and ulcerative colitis (UC).

The intestinal epithelial layer is a barrier that prevents the transport of several toxins, allergens, and microorganisms from the gut lumen into the circulation. Dysfunction of this barrier is associated with increased gut permeability, which is claimed as one of the factors in the etiopathogenesis of IBD. Several growth factors maintain gut mucosal integrity, including transforming growth factor β (TGF- β), insulin-like growth factor (IGF), and epidermal growth factor (EGF) [2].

Epidermal growth factor (EGF) is a mitogenic polypeptide that has 53 amino acids except for alanine, phenylalanine, and lysine [3]. EGF has been detected in a variety of body fluids and plays an important role in regulating cell growth, survival, migration, apoptosis, proliferation, and differentiation [4]. Another important function of EGF in the gastrointestinal tract (GI) is mucosal protection associated with intestinal maturation and maintenance of epithelial cell homeostasis in the small intestine [4]. Experimental studies have shown that EGF plays an important role in protecting the intestinal barrier function, and wound healing in necrotizing enterocolitis and ischemia-reperfusion injury models [5, 6]. It has been shown that EGF has anti-inflammatory effects in human fetal intestinal and colonic cells [7]. It has been determined that a single nucleotide polymorphism located in the 5' untranslated region at position 61 of the EGF gene affects expression levels of EGF [8, 9]. Shahbazi et al. [10] demonstrated that cells of individuals with the EGF 61 AA genotype produce less EGF compared with those with EGF 61 AG and GG genotypes. In addition, Wu G, et al. [11] noted an important role between EGF +61 GG genotype and the +61 G allele with the risk of colorectal cancer. Inflammation has been shown to induce genetic or epigenetic changes in cells, resulting in overexpression or persistent activation of endothelial growth factor receptors, thereby activating oncogenesis-related pathways [11].

EGF receptor (EGFR) is a 170-kDa transmembrane glycoprotein encoded by a gene located on chromosome 7p13-q22. EGFR serves as the common receptor for EGF and transforming growth factor-α (TGF-α) [5,12]. EGFR is a tyrosine kinase that manages cell survival, proliferation, barrier function, and ion transport of colon epithelial homeostasis [13]. The receptors could be found on a variety of cells such as fibroblasts, cornea, lens, glial cells, and epithelium of the small intestine [14, 15].

One of the major receptors of polymorphic EGFR has been identified; it plays its role as a single nucleotide change (G-A) belonging to an arginine to lysine substitution in codon 497, which is also called R497K, in the extracellular domain [16]. The EGFR R497K polymorphism has been shown to reduce EGFR activation and downregulate EGFR target genes. This has been demonstrated to be an important marker for the reduction of tumor recurrence in patients with colorectal carcinoma [17]. Animal models and cell culture studies revealed the anti-inflammatory role of EGF an EGFR in intestinal inflammation models [7, 18].

In the present study, we aimed to investigate the presence of EGF +61A/G and EGFR 497 polymorphisms in patients with IBD and the association of these polymorphisms with the clinical features of the patients with CD and UC compared with healthy subjects.

Material and Methods

Subjects

In total, 220 Turkish subjects, 91 patients with IBD (45 CD and 46 UC) and 129 healthy control subjects were enrolled consecutively in the study. The characteristics of the patients with CD (Group 1) and UC (Group 2) and control group (Group 3) and demographic data are given in Table 1. This study was designed as a case-control study. The patient group was selected from patients diagnosed with UC and CD according to European Crohn's and Colitis Organization (ECCO-2010) criteria and followed up at the Gastroenterology Clinic of the Umraniye Education and Research Hospital (Istanbul-Turkey) between January 2012 and December 2013 [19]. The control group included age- and sex-matched volunteers from hospital staff and volunteers from among individuals who admitted to the Gastroenterology Clinic for dyspeptic complaints. Subjects in the control group were individuals not having an inflammatory disease or systemic disorder. After obtaining written informed consent from the participants and approval from Istanbul University's Ethics Committee, blood specimens were collected in tubes containing EDTA. DNA was ex¬tracted from peripheral blood lymphocytes using the salting-out procedure. Disease activity and severity were evaluated using the Truelove-Witts index (TW) in patients with UC and with the Crohn's Disease Activity Index (CDAI) in patients with CD [20]. Patients with CD were divided into three groups as mild (CDAI= 150-220), moderate (CDAI= 220- 450), and severe activity (CDAI> 450). The location and behavior of disease are classified according to the Montreal classification. [21]. The CD location is classified as L1 terminal ileum with or without cecum involvement (L1), colon (L2), ileocolon (L3). According to the CD behavior, three groups were separated as nonstricturing, nonpenetrating (B1), stricturing (B2) and penetrating (B3). Patients with UC were divided into three groups according to the extent of disease: distal (proctosigmoiditis) colitis was determined as inflammation limited to the rectum and sigmoid colon; left-sided colitis, determined as inflammation limited to distal of the splenic flexure; and extensive colitis, involvement exceeding the splenic flexure. Patients were also divided into two groups according to whether they had surgery or not. The patients were also divided into mesalazine, azathioprine and anti-tumor necrosis factor alpha drugs (infliximab and adalimumab) according to their treatment.

Polymorphism analysis and RFLP for EGFR R497K and EGF +61A/G

Genomic DNA was extracted from isolated lymphocytes using a standard nonorganic procedure. The

extracted DNA was used for characterization of the subsequent polymorphic genes. Polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP), was used for genotyping. Initially, PCR was performed to determine the polymorphic regions using suitable primers. PCR products of EGFR R497K and EGF +61A/G were further subjected to digestion using BstN1 and AluI restriction enzymes, respectively (Table 2). The PCR products were visualized using electrophoresis through a 3% agarose gel. The relative size of the PCR products was determined by comparison of the migration of a 50–1000 bp DNA molecular weight ladder. A permanent visual image was obtained using an ultraviolet (UV) illuminator. Two independent researchers read all genotypes. In the event of any conflicts, the genotypes were repeated.

Statistical analysis

SPSS 11.0 software was used for statistical analysis. The Chi-square test and Fisher's test were used to assess the differences of genotype and allele frequency between the two groups. Comparison of intergroup demographic data was determined using Student's t-test. ANOVA and t-test were used to compare averages of variables in more than two groups. The calculation of differences between sexes was made using the Chi-square test. For the assessment of correlation between the variables, Pearson's and Spearman's correlation analyses were conducted for parametric and nonparametric variables, respectively. Quantitative variables were expressed as mean ± SD (standard deviation) and median (Minimum / Maximum), and categorical variables were expressed as n (%). Variables were examined at 95% confidence interval. A p value of <0.05 was considered statistically significant.

Results

The demographic and laboratory data of patients with CD, UC, and the control group are presented in Table 1. There were no significant differences between the three groups in terms of age and sex. Patients with UC and CD did not differ in terms of the disease duration. Table 3 summarizes the distributions of genotypes and alleles of EGF +61A/G and EGFR R497K genes in patients with IBD including CD and UC and healthy controls.

The EGFR497 AA genotype was significantly decreased in patients with UC compared with HC (p=0.002 and CD (p=0.027). Nevertheless, there was no statistically significant difference in EGF +61 genotype frequencies between the three groups (all p>0.05). EGF +61A/G and EGFR497 polymorphisms were compared in terms of disease localization, severity, anti-TNF drug use, and operative status in both disease groups (Table 4, 5). The patients with CD who had EGF +61 AG genotype were found to have a 5-fold increased risk for the presence of penetrating disease (p=0.14; $\chi 2$=5.59; OR=5.00; 95% CI=1.26-19.83). EGF +61 A allele carriers also had higher CD activity index (CDAI >220) scores (p=0.19; OR=4.00; 95% CI=0.44-36.14). In addition, A+ carriers were also found to have five times higher requirement for anti-TNF treatment (p=0.11; OR=5.0; 95% CI=0.56-44.4). Based on the extension of CD, EGF +61 AG genotype carriers had a 2.5-fold higher risk of ileocolonic involvement (p=0.14; OR=2.56; 95% CI=0.66-9.96). The patients with UC who had the EGF +61 A allele had increased risk of moderate and severe disease (p=0.28; OR= 3.13; 95% CI=0.34-28.73).

Table 1: Characteristics of patients with CD and UC and control.

	Group1	Group 2	Group 3	P1	P2	P3
Number (n)	45	46	129			
Age (year) [β]	39.4±11.6	42.0±11.8	42.8±14.8	0.182	0.085	0.560
Sex (Female/Male)	23/22	26/20	79/50	0.540	0.060	0.190
Disease duration (month) [β]	38.2±47.0	56.1±50.0		0.085		
BMI (kg/m^2) [β]	24.4±6.4	25.3±5.6	26.3±3.8	0.264	0.084	0.498
CRP (mg/dl) [β]	1.57±3.56	0.74±1.0	0.47±0.3	0.084	0.046	0.140
CD Behavior			NA			
Nonstr-nonpenet.	22	NA				
Stricturing	11	NA				
Penetrating	12	NA				
CD Location			NA			
Ileal	20	NA				
Ileocolon	18	NA				
Colon	7	NA				
Disease Activity Mild/Moderate /Severe	23/14/8	22/21/3	NA			
UC Location			NA			
Proctitis	NA	14				
Left-sided	NA	11				
Extensive	NA	21				
Treatment (n (%))			NA			
Mesalazine [α]	38 (77)	56 (100)				
Azathioprine [α]	39 (80)	18 (32)				
Anti-TNF [α]	13 (20)	0				

[β]:Mean±standard deviation, [α]: n(%)

Group1: Crohn's disease group, Group 2: ulcerative colitis group, Group 3: healthy control, CD: Crohn's disease, CRP: C-reactive protein, BMI: body mass index, p1: p value between group 1 and group 2, p2: p value between group 1 and group 3, p3: p value between group 2 and group 3.

Table 2: Polymerase chain reaction and restriction fragment length polymorphism methods

Gene Variants	Primers	Enzymes
EGFR R497K	5'-TGCTGTGACCCACTCTGTCT-3'5'CCAGAAGGTTGCACTTGTCC-3'	BstN1
EGF +61	5′-TGTCACTAAAGGAAAGGAGGT-3′, 5′-TTCACAGAGTTTAACAGCCC-3′	AluI

EGF: epidermal growth factor, EGFR: epidermal growth factor receptor

Table 3: Distributions of genotypes and alleles of EGF61 and EGFR497

Polymorphism	Group 1 n=45		Group 2 n=46		Group 3 n=129		p_1	p_2	p_3
	n	%	n	%	n	%			
EGF(+61A/G)									
GG	11	24.4	13	28.3	28	21.7	0.704	0.367	0.680
AA	17	37.8	14	30.4	34	26.4	0.147	0.595	0.460
GA	17	37.8	19	41.3	67	51.9	0.102	0.215	0.731
GG+AG vs AA	28	62.2	32	69.6	95	73.6	0.147	0.595	0.460
AA+AG vs GG	34	75.6	33	71.7	101	78.3	0.704	0.367	0.680
EGFR R497K									
AA	7	15.6	1	2.2	25	19.4	0.569	0.002	0.027
GG	20	44.4	20	43.5	57	44.2	0.976	0.934	0.926
AG	18	40.0	25	54.3	47	36.4	0.670	0.034	0.170
AA+AG vs GG	25	55.6	26	56.5	72	55.8	0.976	0.934	0.926
GG+AG vs AA	38	84.4	45	97.8	104	80.6	0.569	0.002	0.027

Group1: Crohn's disease group, Group 2: ulcerative colitis group, Group 3: healthy control group, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, p1: p-value between healthy control group and CD group, p2: p-value between healthy control group and UC group, p3: p-value between CD group and UC group

Discussion

EGF exerts effects on cell proliferation and differentiation by binding to a tyrosine kinase receptor EGFR. It is well known that EGF and its receptor have roles on the immune system, cell proliferation, and apoptosis. The interaction of EGF and its receptor activates intracellular signaling pathways and has a mitogenic effect. The disruption of this regulation causes various cancers including colon cancer.

Table 4: Evaluation of EGF +61A/G and EGFR 497 polymorphisms in patients with CD in relation to disease type, localization, activity, treatment and operative status.

	EGF61		GG N%		GA+AA N%		p
CD	Localization	Ileal	7	35.0	13	65.0	0.172
		Ileocolonic	4	22.2	14	77.8	
		Colonic	0	0.0	7	100.0	
CD	Disease	Non-stricturing, non-penetrating	5	22.7	17	77.3	0.529
	behaviour	Stricturing	4	36.4	7	63.6	
		Peetrating	2	16.7	10	83.3	
CD	Disease	Mild	7	30.4	16	69.6	0.178
	Activity	Moderate	1	7.1	13	92.9	
	(CDAI)	Severe	3	37.5	5	62.5	
CD	Surgery	No	7	23.3	23	76.7	0.709
		Yes	4	28.6	10	71.4	
CD	Anti-TNF	No	8	25.0	24	75.0	0.607
	treatment	Yes	3	23.1	10	76.9	
CD	Azathioprine	No	6	37.5	10	62.5	0.130
		Yes	5	17.2	24	82.8	
	EGFR497		AA N	AA %	GG+AG N	GG+AG %	p
CD	Localization	Ileal	3	15.0	17	85.0	0.555
		Ileocolon	2	11.1	16	88.9	
		Colon	2	28.6	5	71.4	
CD	Disease	Non-stricturing, non-penetrating	4	18.2	18	81.8	0.723
	Behavior	Stricturing	2	18.2	9	81.8	
		Penetrating	1	8.3	11	91.7	
CD	Disease	Mild	3	13.0	20	87.0	0.715
	Activity	Moderate	2	14.3	12	85.7	
		Severe	2	25.0	6	75.0	
CD	Surgery	No	4	13.3	26	86.7	0.392
		Yes	3	21.4	11	78.6	
CD	Anti- TNF	No	5	15.6	27	84.4	0.680
	treatment	Yes	2	15.4	11	84.6	
CD	Azathioprine	No	3	18.8	13	81.2	0.484
		Yes	4	13.8	25	86.2	

CD, Crohn's disease; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor, TNF, tumor necrosis factor; CDAI, Crohn's disease activity index

Table 5: Evaluation of EGF +61A/G and EGFR 497 polymorphisms in patients with UC in relation to disease type, localization, activity, treatment and operative status.

EGF61		GG		GA+AA		p
		N	%	N	%	
Localization	Proctitis	4	28.6	10	71.4	0.224
	Left-sided	1	9.1	10	90.9	
	Extensive	8	38.1	13	61.9	
Disease Activity (TW)	Mild	6	27.3	16	72.7	0.975
	Moderate	6	28.6	15	71.4	
	Severe	1	33.3	2	66.7	
Azathioprine treatment	No	8	28.6	20	71.4	0.953
	Yes	5	27.8	13	72.2	
EGFR 497		AA	AA	GG+ AG	GG+ AG	p
		N	%	N	%	
Localization	Proctitis	0	0.0	14	100.0	0.554
	Left-sided	0	0.0	11	100.0	
	Extensive	1	4.8	20	95.2	
Disease Activity (TW)	Mild	0	0.0	22	100.0	0.544
	Moderate	1	4.8	20	95.2	
	Severe	0	0.0	3	100.0	
Azathioprine treatment	No	1	3.6	27	96.4	0.609
	Yes	0	0.0	18	100.0	

UC: ulcerative colitis, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, TW: Truelove-Witts activity index

In this study, we investigated the presence of EGF +61A/G and EGFR R497 polymorphisms in patients with IBD and the association of these polymorphisms with the clinical features of patients with CD and UC. In our study, the EGFR 497 AA genotype and A allele were significantly decreased in patients with UC compared with controls and patients with CD. However, there was no statistically significant difference between the three groups in EGF +61 genotype frequencies. Geng et al. [22] suggested as a result of their animal studies that EGF helped to recover damage resulting from intestinal ischemia and the reperfusion process. Even though IBD has unknown and unclear etiology to understand the disease, it has multifactorial mechanism including genetic, environmental and immunological mechanisms [23, 24]. In addition, Menard et al. [25] have worked with human fetal intestine culture and they have found the EGF regulates the genes which are related with inflammation process. Bedford et al. [26] have pointed out EGF therapy has the ability to increase the expression of interleukin 13 as an anti-inflammatory cytokine. Therapeutic effects in experimental colitis models and the positive effects of necrotizing enterocolitis treatment have led to the use of EGF in the treatment of IBD.[27] EGF enema treatment was also found effective on the left colon and distal type UC[11]. The data coming from studies on the EGF +61A/G polymorphism in CRC showed that the G + allele and G/G genotype were related with the presence of CRC and more advanced disease [11]. In addition, Shahbazi et al. [10] demonstrated that cells of individuals with the EGF +61 AA genotype produced less EGF compared with individuals who had EGF +61 AG and GG genotypes. Shahbazi et al. [10] also found that position on EGF +61, G allele carriers express significantly more than A allele carriers.

In our study, the results indicate that the EGF +61 A allele is related with particularly active CD. In patients with CD with EGF +61 A alleles, there is a greater risk of increased disease activity index. The risk of using anti-TNF agents was also found to be increased. It was also found that patients with EGF +61AG polymorphism increased the risk of penetrating disease. In patients with UC, there was an increase in the risk of moderate and severe disease. This may be due to the low expression of EGF in patients with alleles of EGF +61 A and consequent deterioration of the mucosal barrier and healing process. In this regard, there is a need for further studies.

EGFR plays an important role in the homeostasis of the colon epithelium, cell proliferation, barrier functions, and ion transport. In a recent study, it was found that microbial products such as lipopolysaccharide caused EGFR activation in macrophages, resulting in decreased anti-inflammatory cytokines such as interleukin (IL)-10 [28]. It has been found that colitis is exacerbated and healing is impaired. In addition to the present study, selective EGFR-depleted macrophages have been shown to increase IL-10 release resulting in the recovery of intestinal inflammation due to proinflammatory cytokine depletion [28].

EGFR R497K polymorphism leads to decreased intracellular signaling pathways by changing some processes such as cell growth factor and ligand binding, and decreased tyrosine kinase activation [17, 22, 28]. In our study, the EGFR497 AA genotype was significantly decreased in patients with UC compared with controls and patients with CD. The EGFR497 AA genotype has more attenuated functions than the GG polymorphism in terms of ligand binding, growth stimulation, and tyrosine kinase activation [28, 29].

In conclusion, this study was a preliminary study that EGF +61 and EGFR497 gene variants in patients with UC and CD. The EGFR 497 AA genotype was significantly decreased in patients with UC compared with controls and those with CD. Further studies with larger sample groups are needed to clarify the role of the EGF +61 and EGFR 497 polymorphisms, and the development of the etiology and pathogenesis of IBD.

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