

CLINICAL STUDY

A pilot study of *Helicobacter pylori* genotypes and cytokine gene polymorphisms in reflux oesophagitis and peptic ulcer disease

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Abstract: *Helicobacter pylori* causes various diseases such as chronic gastritis, peptic ulcer and gastric cancer. While majority of the people infected with *H. pylori* is asymptomatic, 15–20 % of them develop such diseases. The main factors, which determine the development of *H. pylori* related diseases might be bacterial virulence, host genetic and environmental factors.

The aim of this study was to reveal the factors that play a role in the disease development in patients with reflux esophagitis and peptic ulcer, infected with *Helicobacter pylori*. Environmental factors such as medical agents, smoking and body mass index were evaluated. The factors specific to bacteria such as *vacA*, *CagA*, *babA* and *iceA* virulence genotypes and the host factors such as IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, interferon- γ , TNF- α , ve TGF- β_1 gene polymorphisms were compared between the two groups.

H. pylori infected twenty five patients with reflux esophagitis and peptic ulcer were enrolled in the study. There was no statistical difference between the two groups regarding environmental factors. IL-2 –330T + 166T ($p=0.037$) and IL10 –1082A; –819C ($p=0.049$) gene polymorphisms were significantly more common in the group of patients with peptic ulcer compared to the group with reflux esophagitis. In both groups of patients, either with reflux esophagitis or peptic ulcer, multiple *H. pylori* virulence genotypes (*cagA*, *vacA*, *babA*) (mean values 74 %, 78 %, 54 % respectively) were observed. In this study, we revealed that cytokine gene polymorphisms may play a role in the development peptic ulcer while *H. pylori* virulence genotypes seem to be crucial for the development of associated diseases (Tab. 4, Ref. 51). Text in PDF www.elis.sk.

Key words: *Helicobacter pylori* genotypes, single nucleotide polymorphisms, reflux oesophagitis, peptic ulcer.

Helicobacter pylori (*H. pylori*) infection is a gram-negative, microaerophilic bacteria which infects a majority of the world population (1). The most important diseases caused by *H. pylori* are duodenum and gastric ulcer, gastric adenocarcinoma and primary gastric lymphoma (2). In 1994, *H. pylori* was accepted as a 1st group carcinogen by the World Health Organization (3).

The reason why *Helicobacter pylori* infection leads to different clinical results is not exactly known, but primarily three mechanisms are blamed for pathogenesis. These are host components such as genetic predisposition to the diseases and cytokine gene polymorphism which are said to affect the clinical results (4), peripheral components such as smoking and food (5–7), and virulence genotypes of *Helicobacter pylori* strain (8). Evidences collected show that *H. pylori* infectious power has a high genetic

heterogeneity, which results in the emergence of clinical sequelae (9). It is thought that principally four of the bacterial virulence genotypes that have been identified are relevant with the clinical results. These are pore-forming toxin (*vacA*), cytokine-associated antigen (*cagA*), blood group antigen binding adhesin (*babA*) that allows *H. Pylori* to bind to Lewis b (a-1,3/4-difucosylated) blood group antigens in epithelial cells in the human stomach and *iceA* (induced by contact with epithelium). Virulence genotype called *IceA* is induced by the formation of a contact between *H. pylori* and human epithelial cells and was shown to be associated with peptic ulcer disease (10). Strains producing *VacA* genotype were shown to be associated with increased gastric damage and peptic ulcer (11). *CagA* positivity was identified in higher rates in patients with peptic ulcer disease and gastric adenocarcinoma (12–14).

The relation of *H. pylori* infection with gastroesophageal reflux disease (GERD) is a very disputed subject due to suggested conflicting epidemiological data (15–16). One of the suggested hypotheses proposes that the suppression of acid release by corpus gastritis which is caused by *H. pylori* infection and the neutralization of the gastric acid by the ammonia which is produced by the bacterium are protective against the development of gastroesophageal reflux. The studies, which support this view, reported a meaningful relationship between the absence of *H. pylori* infection and GERD symptoms (17–18). There are also publications stating that the development, relapse and symptoms of

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Funding sources: Karadeniz Technical University research fund and identification number is 2007.114.003.3.

the gastroesophageal reflux deteriorate with the eradication of *H. pylori* infection (19–20). In contrast with these studies, there are evidences that suggest no clinically significant effect on GERD symptoms with the eradication of *H. pylori* infection (21–24). It is stated that virulent *H. pylori* strains are not protective against GERD development (25–26). In recent prospective researches, it was shown that gastroesophageal reflux symptoms were ameliorated or pre-existing reflux esophagitis improved after *H. pylori* eradication in patients with duodenum ulcer (27–28). The geographical differences between distribution of virulent *H. pylori* strains as well as varying immune response to *H. pylori* have led to the occurrence of conflicting results in literature (29). This immune response, which develops subject to *Helicobacter pylori*, is determined by the virulence of the primary infectious strain, the genetic disposition of the host to the disease and peripheral factors. Certain cytokines such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumour necrosis factor (TNF) and interferon (IFN) which are released by the body during the immune response which develops in relation to *Helicobacter pylori* infection affect different mechanisms and lead to tissue destruction. As a result of the emerging inflammation, epithelial cell damage leads to various clinical pictures such as atrophy, dysplasia, ulceration and cancer (30).

The primary genetic factors that affect inflammatory response to *H. pylori* are cytokine gene polymorphisms. Polymorphism is a restricted change that may emerge in the DNA chain. Cytokine gene polymorphisms affect the protein structure of and release by the cytokines. Cytokines and cytokine receptor genes have high polymorphism capability. The studies conducted report that cytokine gene polymorphism impact the high mucosal cytokine release, the inflammatory response and bacterial colonization that develops in the stomach in case of *H. pylori* infection (31).

There is a limited number of studies on the effect of cytokine gene polymorphism on *H. pylori* related diseases. Cytokine gene polymorphism studies dealing with *Helicobacter pylori* infection frequently concentrate on peptic ulcer and gastric cancer, and increased hypochlorhydria and gastric cancer risk characterized by increase in proinflammatory cytokines caused by IL-1 α gene polymorphism was shown. Gene polymorphisms, which result in a reduced IL-10 expression, are associated with an increased peptic ulcer and gastric cancer risk. This risk is higher for much virulent *H. pylori* strains (31). In this study, we aimed to express the importance of the clinical results that we have obtained as a result of the analysis of *Helicobacter pylori* virulence genotypes (CagA, VacA, IceA, BabA) and cytokine gene polymorphisms which play an important role in the development of the disease in patients with reflux esophagitis and peptic ulcer.

Method

Subjects

50 patients who applied to Karadeniz Technical University (KTU) Faculty of Medicine, Gastroenterology Division out-patient clinic with gastrointestinal complaints and diagnosed with reflux esophagitis and peptic ulcer as a result of physical examination and

Tab. 1. Invitrogen cytokine gene polymorphism analysis table.

Plate1	Plate2	Cytokine Gene	Allelic characteristic	Base length	Internal Control
1	49	IL-1 α	T at pos-889	220	440
2	50	IL-1 α	C at pos-889	220	440
3	51	IL-1 β	C at pos-511	215	440
4	52	IL-1 β	T at pos-511	215	440
5	53	IL-1 β	T at pos+3952	336	440
6	54	IL-1 β	C at pos+3952	336	440
7	55	IL-1R	C at pos pst1 1970	288	440
8	56	IL-1R	T at pos pst1 1970	288	440
9	57	IL-1RA	T at pos mspa1 11100	297	440
10	58	IL-1RA	C at pos mspa1 11100	297	440
11	59	IL-4R α	G at pos +1902	143	440
12	60	IL-4R α	A at pos +1902	143	440
13	61	IL-12	C at pos-1188	802	440
14	62	IL-12	A at pos-1188	802	440
15	63	IFN- γ	A at pos-874	277	440
16	64	IFN- γ	T at pos-874	277	440
17	65	TGF- β	C at Codon 10;G at Codon25	80	440
18	66	TGF- β	C at Codon 10;C at Codon25	80	440
19	67	TGF- β	T at Codon 10;G at Codon25	80	440
20	68	TGF- β	T at Codon 10;C at Codon25	80	440
21	69	TGF- β	C at Codon 10	195	440
22	70	TGF- β	T at Codon 10	195	440
23	71	TNF- α	G at pos-308;G at pos-238	110	440
24	72	TNF- α	A at pos-308;G at pos-238	110	440
25	73	TNF- α	G at pos-308;A at pos-238	110	440
26	74	TNF- α	A at pos-308;A at pos-238	110	440
27	75	IL2	T at pos-330;G at pos+166	562	89
28	76	IL2	G at pos-330;G at pos+166	564	89
29	77	IL2	G at pos-330;T at pos+166	569	89
30	78	IL2	T at pos-330;T at pos+166	569	89
31	79	IL4	T at pos-1098;T at pos-590	557	89
32	80	IL4	T at pos-1098;C at pos-590	557	89
33	81	IL4	G at pos-1098;T at pos-590	557	89
34	82	IL4	G at pos-1098;C at pos-590	557	89
35	83	IL4	T at pos-590;T at pos-33	610	89
36	84	IL4	T at pos-590;C at pos-33	610	89
37	85	IL4	C at pos-590;T at pos-33	610	89
38	86	IL4	C at pos-590;C at pos-33	610	89
39	87	IL6	G at pos-174;G at pos nt565	427	89
40	88	IL6	C at pos-174;G at pos nt565	426	89
41	89	IL6	G at pos-174;A at pos nt565	428	89
42	90	IL6	C at pos-174;A at pos nt565	428	89
43	91	IL10	G at pos-1082;C at pos-819	305	89
44	92	IL10	G at pos-1082;C at pos-592	530	89
45	93	IL10	A at pos-1082;C at pos-819	305	89
46	94	IL10	A at pos-1082;T at pos-819	305	89
47	95	IL10	A at pos-1082;C at pos-592	530	89
48	96	IL10	A at pos-1082;A at pos-592	530	89

assessment and upper gastrointestinal endoscopy were enrolled in this study. The blood, biopsy and faeces samples collected from these patients were analyzed in the microbiology laboratory of KTU, Faculty of Medicine. The age, gender, body mass index, drug use and smoking habit of each patient were determined. Patients with esophageal and gastric surgery history, esophageal and gastric cancer, who refused endoscopy, and who received *H. pylori* eradication treatment in the past were excluded. No patients had

Tab. 2. Primers used in detecting bacterial virulence factors.

Gene	Primer	Primer Sequence	PCR product (bp)
CagA	CagA1F(+)	GATAACAGGGCAAGCTTTTGAGG	349
	CagA2R(-)	CTGCAAAAGATTGATTGTTTGCAGAGA	
VacA s1/s2	VA1F(+)	ATGGAAATACAACAAACACAC	176/203
	VA1XR(-)	CCTGARACCGTTCCTACAGC	
VacA s1a	SS1F (+)	GTCAGCATCACACCGCAAC	190
	VA1R (-)	CTGCTTGAATGCGCCAAAC	
VacA s1b	SS3F (+)	AGCGCCATACCGCAAGAG	187
VacA s1c	S1C-F	CTYGTTTAGTRGGGYTA	214
BabA	LBF	AATCCAATTTAATCCAAA	190
	LBR	ATAGTTGTCTGAAAGATC	
IceA1	IceA1F	GTTGGGTAAGCGTTACAGAATTT	567
	IceA1R	CATTGTATATCCTATCATTAC	
IceA2	IceA2F	GTTGGGTATATCACAATTAT	334
	IceA2R	TTRCCCTATTTTCTAGTAGGT	

taken antibiotics or any kind of proton pump inhibitors within the eight weeks before admission. This study was performed according to the Declaration of Helsinki. The informed consent was obtained from all patients and research protocols were approved by the Ethics Committee of Karadeniz Technical University Hospital.

Two biopsy samples were collected from each corpus and antrum from the patients who were diagnosed with reflux esophagitis and peptic ulcer as a result of routine physical examination and assessment, and urease, culture and direct microscopy. The rating of reflux esophagitis was made using Los Angeles classification (32). For histopathological examination, samples were stained with hematoxylen eosine in the pathology department, and presence of helicobacter pylori was examined. While one of the samples was used to detect urease, other samples were cultivated in Brucella agar for culture. Two laminas were prepared for examination in direct microscopy. Also HpSA antigen was searched for in faeces samples of the same patients. Patients with positive results in any two of the urease, culture, direct microscopy or faeces tests were included in the assessment. Blood (6cc) was collected from these patients in EDTA tubes and mononuclear cells were separated. The cells so separated were stored in -20 °C until DNA isolation. DNA isolation was performed from mononuclear cells. For DNA isolation MagNA Pure LC (Roche) device and MagNA Pure Total Nucleic Acid Isolation Kit (Roche) were used and DNAs obtained. First, the presence of Beta actin gene was investigated in isolated DNAs using PCR, it was determined whether there was sufficient DNA in the sample, and cytokine gene polymorphism was examined in samples with sufficient DNA.

Genotyping of cytokine gene polymorphisms

For the genotyping of cytokine gene polymorphism, PCR-SSP (Single Strand Polymorphism) cytokine gene panel of Invitrogen was used (Tab. 1).

For PCR-SSP amplification of each patient a master mix with a total volume of 522.3 µl and a DNA mix (Buffer 140 µl, genomic DNA 50 µl, deionized water 329 µl, Taq Pol. (5 U/µl) 3.3 µl) were prepared. The master mixes prepared were slightly vortexed, and divided into 48 wells on plates in portions of 10 µl and then amplified. The PCR product that was obtained with a 2-minute

preliminary denaturation at 94 °C, followed by 10 cycles of denaturation for 15-seconds at 94 °C in the second round, 1 minute of binding at 65 °C, 20 cycles of denaturation for 15 seconds at 94 °C, 50 seconds of binding at 61 °C, and 30 seconds of extension at 72 °C was stored at 4 °C until use. 0.5X TBE-buffer containing 2 % agarose gel was prepared to electrophorese the amplified PCR products. Samples were loaded in 2 separate gels in 24 wells for each patient and were subject to electrophoresis. Results were evaluated in UV transilluminator. It was checked whether the positive controls in each well functioned or not, and wells showing double bands were accepted as positive and photographed.

Polymerase Chain Reaction for cag A, vacA, ice A and babA genes

Samples that multiply in culture were taken to determine bacterial virulence factors and kept in 10 % glycerol-containing brucella liquid medium at -20 °C until use. The biopsies of the samples that did not show any multiplication were kept in 0.9 % NaCl at -20 °C. To obtain *H. pylori* DNA from these samples, the boiling method was used, and PCR was produced from the DNA obtained using the PCR materials from Promega (Tab. 2).

For the detection of VacA and CagA, 10 mmol/L Tris-HCl (Ph8), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, deoxynucleotides with a concentration of 200 µmol/L, 25 pmol forward and reverse primers, and 1.5 U Tag DNA polymerase were used for each PCR reaction. Master mixes prepared in specified quantities were slightly vortexed, and divided into small eppendorfs in portions of 40 µl. 10 µl DNA sample was added and amplified.

The PCR program consisted of 3 min of pre-incubation at 95 °C, followed by 38 cycles of 1 min at 95 °C, 1 min at 55 °C for cagA detection or at 50 °C for vacAs and m genotyping, and 1 min at 72 °C. Final extension was performed for 5 min at 72 °C. The PCR products were inspected by electrophoresis on 2 % agarose gels. Water for cell culture grade (Sigma Chemical, UK) was used as negative controls.

For the detection of babA, polymerase chain reactions (PCRs) were performed in a volume of 50 L containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 M of each dNTP, 2 L of genomic DNA released by GR, 2.5 U of Taq DNA polymerase,

Tab. 3. Comparison of cytokine gene polymorphisms results of patients with reflux esophagitis and peptic ulcer.

	Cytokine gene	Allelic characteristic	bp	Reflux esophagitis		Peptic ulcer		P value
				n	%	n	%	
1	IL-1 α	T at pos-889	220	21	84	21	84	1
2	IL-1 α	C at pos-889	220	24	96	24	96	1
3	IL-1 β	C at pos-511	215	23	92	22	88	0.637
4	IL-1 β	T at pos-511	215	20	80	16	64	0.208
5	IL-1 β	T at pos+3952	336	20	80	19	76	0.733
6	IL-1 β	C at pos +3952	336	20	80	24	96	0.189
7	IL-1R	C at pos pst1 1970	288	23	92	25	100	0.49
8	IL-1R	T at pos pst1 1970	288	16	64	16	64	1
9	IL-1RA	T at pos mspa1 11100	297	24	96	24	96	1000
10	IL-1RA	C at pos mspa1 11100	297	17	64	21	84	0.185
11	IL-4Ra	G at pos+1902	143	6	24	9	36	0.355
12	IL-4Ra	A at pos +1902	143	25	100	25	100	1
13	IL-12	C at pos-1188	802	4	16	5	20	1
14	IL-12	A at pos-1188	802	10	40	11	44	0.774
15	IFN- γ	A at pos-874	277	20	80	18	72	0.508
16	IFN- γ	T at pos-874	277	16	64	18	72	0.544
17	TGF- β	C at Codon 10;G at Codon25	80	18	72	17	68	0.758
18	TGF- β	C at Codon 10;C at Codon25	80	3	12	3	12	1
19	TGF- β	T at Codon 10;G at Codon25	80	16	64	20	80	0.345
20	TGF- β	T at Codon 10;C at Codon25	80	0	0	0	0	0
21	TGF- β	C at Codon 10	195	18	72	14	56	0.239
22	TGF- β	T at Codon 10	195	15	60	18	72	0.37
23	TNF- α	G at pos-308;G at pos-238	110	24	96	24	96	1
24	TNF- α	A at pos-308;G at pos-238	110	16	64	20	80	0.345
25	TNF- α	G at pos-308;A at pos-238	110	11	44	15	60	0.258
26	TNF- α	A at pos-308;A at pos-238	110	6	24	4	16	0.725
27	IL2	T at pos-330;G at pos+166	562	14	56	19	76	0.136
28	IL2	G at pos-330;G at pos+166	564	17	68	13	52	0.248
29	IL2	G at pos-330;T at pos+166	569	0	0	1	4	1
30	IL2	T at pos-330;T at pos+166	569	2	8	9	36	0.037
31	IL4	T at pos-1098;T at pos-590	557	9	36	11	44	0.564
32	IL4	T at pos-1098;C at pos-590	557	13	52	15	60	0.776
33	IL4	G at pos-1098;T at pos-590	557	2	8	1	4	1
34	IL4	G at pos-1098;C at pos-590	557	6	24	4	16	0.725
35	IL4	T at pos-590;T at pos-33	610	10	40	8	32	0.769
36	IL4	T at pos-590;C at pos-33	610	8	32	6	24	0.754
37	IL4	C at pos-590;T at pos-33	610	8	32	4	16	0.321
38	IL4	C at pos-590;C at pos-33	610	11	44	10	40	1
39	IL6	G at pos-174;G at pos nt565	427	16	64	18	72	0.762
40	IL6	C at pos-174;G at pos nt565	426	6	24	7	28	1
41	IL6	G at pos-174;A at pos nt565	428	1	4	1	4	1
42	IL6	C at pos-174;A at pos nt565	428	7	28	10	40	0.551
43	IL10	G at pos-1082;C at pos-819	305	20	80	21	84	1
44	IL10	G at pos-1082;C at pos-592	530	16	64	18	72	0.762
45	IL10	A at pos-1082;C at pos-819	305	18	72	24	96	0.049
46	IL10	A at pos-1082;T at pos-819	305	20	80	16	64	0.345
47	IL10	A at pos-1082;C at pos-592	530	14	56	21	84	0.062
48	IL10	A at pos-1082A at pos-592	530	14	64	16	64	0.773

and 25 pmol of specific forward and reverse primers. Master mixes prepared in specified quantities were slightly vortexed, and divided into small eppendorfs in portions of 40 μ l. 10 μ l DNA sample was added and amplified.

The PCR program for babA comprised 30 cycles of 1 min at 94 $^{\circ}$ C, 1 min at 38 $^{\circ}$ C, and 1 min at 72 $^{\circ}$ C. After amplification 10 mmol/L of PCR product was electrophoresed on 1.7 % agarose gel and examined under UV illumination.

For Ice A1, IceA2, 10 mmol/L Tris-HCl (Ph8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, deoxynucleotides with a concentration of 200 μ mol/L, 0.5 μ M primers, and 2.5 U Tag DNA polymerase were used. The amplification cycles (iceA) consisted of an initial denaturation of target DNA at 94 $^{\circ}$ C for 9 min and then denaturation at 94 $^{\circ}$ C for 1 min, primer annealing at 60 $^{\circ}$ C for 1 min and extension at 72 $^{\circ}$ C for 1 min (35 cycles). The final cycle included an extension step for 5 min (6, 9, 14 PCR) products were

Tab. 4. Comparison of the bacterial virulence factors in reflux esophagitis and peptic ulcer patients.

	Reflux esophagitis		Peptic ulcer		P value
	n	%	n	%	
cagA positive	19	76	18	72	0.747
vacA s1 positive	19	76	20	72	0.733
vacA s2 positive	6	24	5	20	0.733
Ice A1 positive	10	40	8	32	0.556
Ice A2 positive	7	28	6	24	0.747
Bab A positive	13	52	12	48	0.777

visualised by agarose 2 % gel electrophoresis. 0.5X TBE-buffer containing 2 % agarose gel was prepared and used to electrophorese the amplified PCR products. Results were evaluated in UV transilluminator. Wells that showed bands were accepted as positive and photographed.

Data analysis

The statistical analysis of cytokine gene polymorphism was performed using the SPSS Windows 9.0 program. Statistical significance between the groups were calculated with chi-square test. The mean values were compared using the Student's t-test. Age, gender and accompanying diseases were evaluated with univariate analysis. $p < 0.05$ value was considered as statistically significant.

Results

This study was carried out between March 2007 and November 2007 at Karadeniz Technical University, Faculty of Medicine, Gastroenterology Department, Pathology Department, Microbiology and Clinical Microbiology Department. A total of 50 patients comprised of 25 patients with esophagitis and 25 patients with peptic ulcer having positive results from at least any two of *H. pylori* diagnosis tests were enrolled in the study.

The ages of the patients varied between 19–70. The mean age of patients with reflux esophagitis was 41.80 ± 12.98 . The mean age of patients with peptic ulcer was 41.56 ± 13.65 . When the mean age was examined in both patient groups, no statistical difference was identified ($p = 0.949$). The number of female subjects in reflux esophagitis group was 13 (52 %) while the number male subjects was 12 (48 %). Number of female subjects in reflux esophagitis group was 14 (56 %) while the number male subjects was 11 (48 %). No statistical difference was identified between the two groups in terms of distribution of genders ($p = 0.777$).

When body mass indexes of patients with reflux esophagitis and peptic ulcer were evaluated, BMI was found to be 23.18 ± 3.16 in the patient group with reflux esophagitis and 23.20 ± 3.54 in the patient group with peptic ulcer. No statistically significant difference was identified between the two groups in terms of body mass index ($p = 0.832$).

When smoking rates were evaluated in patients with reflux esophagitis and peptic ulcer, number of smokers was found to be 7 in the reflux esophagitis group and 8 in the peptic ulcer group. No statistically significant difference was identified between the two groups in terms of smoking rate ($p = 0.758$).

None of the patients with reflux esophagitis and peptic ulcer used corticosteroids, nonsteroidal anti-inflammatory and anticoagulant agents as a drug.

Findings related to cytokine gene polymorphism in reflux esophagitis and peptic ulcer patients

When cytokine gene polymorphisms of patients with reflux esophagitis and peptic ulcer are compared, statistically significant differences were detected with IL-2 -330T +166T ($p = 0.037$) and IL10 -1082A; 819C ($p = 0.049$). IL-2 -330T +166T polymorphism was determined as positive in 2 reflux esophagitis patients and in 9 peptic ulcer patients. IL10 -1082A; -819C was detected as positive in 18 patients with reflux esophagitis and in 24 patients with peptic ulcer. When other polymorphisms are viewed, IL-1 α -889T, IL-1 α -889C, IL-1 β -511C, IL-1 β -511T, IL-1 β +3962T, IL-1 β +3962C, IL-1R pst1 1970C, IL-1RA mspa1 11100T, IL-4Ra +1902A, IL-4Ra +1902G, TNF- α -308G; -238G and IL10 -1082G; -819C were detected in both patient groups concurrently with a positivity rate as high as above 80 %. In contrast IL-12 -1188C, TGF- β 10 codonC; 25 codonC, TGF- β 10 codonT; 25 codonC, TNF- α -308A; -238A, IL-2 -330G; +166T, IL-4 -1098G; -590T, IL-4 -1098G; -590C and IL-6 -174G; nt565A were detected in both groups concurrently with a positivity rate below 20 %. In terms of TGF- β 10 codonC; 25 codonC no positivity was detected in any of the two groups (Tab. 3).

Findings related to bacterial virulence factors in reflux esophagitis and peptic ulcer patients

The findings were obtained by boiling the DNA of the bacteria obtained using the culture method or direct biopsy in patients with reflux esophagitis and peptic ulcer who proved to be positive in any two of *Helicobacter pylori* diagnosis tests, and bacterial virulence factors were examined using PCR method (Tab. 4).

CagA, a bacterial virulence factor, was detected as positive in 19 of the patients with reflux esophagitis (76 %) and in 18 of the patients with peptic ulcer (72 %). No statistical difference was identified between the two patient groups in terms of CagA as a virulence factor ($p = 1.000$).

VacA, a *H. pylori* virulence factor, is divided into two as s1ve s2 as a signal sequence. S1 is separated into three as s1a, s1b and s1c. s1 and s2 were detected as positive in respectively 19 (76 %) and 6 (24 %) of the patients with reflux esophagitis, whereas s1 and s2 were detected as positive in respectively 20 (80 %) and 5 (20 %) of the patients with peptic ulcer. 19 of the patients with reflux esophagitis (76 %) and 20 of the patients with peptic ulcer (80 %) in who S1 was detected were found to have s1a. 6 of the patients with reflux esophagitis (24 %) and 5 of the patients with peptic ulcer (20 %) in who S1 was detected were found to have sb1. S1c was not detected as positive in any of the patients. No statistical difference was detected between the two groups in terms of s1, s1a, s1b, and s2 ($p = 0.733$).

When we examined IceA1 and IceA2 virulence factors, IceA1 was detected as positive in 10 of the patients with reflux esophagitis (40 %) and in 8 of the patients with peptic ulcer (32 %). In the case of IceA2 positivity was detected in 7 of the patients with

reflux esophagitis (28 %) and in 6 of the patients with peptic ulcer (24 %). From the results obtained, no statistically significant difference was determined between the two patient groups (IceA1 $p=0,556$, IceA2 $p=0,747$).

BabA positivity rate was detected in 13 of the patients with reflux esophagitis (52 %) and in 14 of the patients with peptic ulcer (56 %), however this result is not statistically significant ($p=1.000$).

Discussion

Helicobacter pylori is a gram-negative microorganism that may lead to various diseases such as chronic gastritis, peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer. A majority of the people infected with *Helicobacter pylori* is asymptomatic, but the above mentioned diseases can be seen in up to 15–20 % of this population. Basic factors that play a role in the development of diseases associated with *Helicobacter pylori* are specific virulence factors, genetic factors of the host and peripheral factors (33).

In this study, which we conducted on patients with peptic ulcer and gastroesophageal reflux, infected with *Helicobacter pylori*, we aimed to assess the basic factors that were thought to play a role in the development of the disease. For this reason, use of drugs, smoking and body mass index were evaluated as peripheral factors. VacA, CagA, BabA and Ice A virulence genotypes, which are the specific virulence factors of the bacterium were studied with cytokine gene polymorphisms of IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, interferon- γ , TNF- α , and TGF- β 1 that are known to affect the immune response in the patients.

CagA positivity was detected as 76 % in the reflux esophagitis group, 72 % in the peptic ulcer group, and 74 % as average when all patients are taken together. In studies conducted in our country, cagA positivity is reported to be in the range of 59 % and 78 %. Various studies conducted show that cagA positivity is associated with peptic ulcer formation. In a study conducted by Aydin et al. in our country, it was reported that cagA positivity was associated with an increased duodenal ulcer and gastric cancer risk (34). In another study performed by Leodolter et al, cagA positivity was detected to be higher in patients with duodenal ulcer and GERD than in patients with functional dyspepsia (respectively, 40 %, 83 %, 80 %), and this result was similar to our data (26).

VacA, a *H. pylori* virulence factor, is divided into two subtypes as s1ve s2 as a signal sequence. In our study, no statistically significant was detected in terms of vacA s1, vacA s1a, vacA s1b and vacA s1c and vacA s2 between the two groups with reflux esophagitis and peptic ulcer ($p=0.733$). S1c was not detected as positive in any of the patients.

When all patients are evaluated together, iceA1 was detected in the rate of 36 %, iceA2 was detected in the rate of 26 %. Erzin et al on patients with dyspepsia, iceA1 was determined as 74.7 and IceA2 as 25.3 %, iceA1 rates in our region were found to be lower than in Istanbul. However, iceA1 is much dominant compared to iceA2 in our country, and is consistent with Holland (35), Japan (36) and Hong Kong (37) findings. Studies conducted in USA (38), Columbia (39) and Brazil showed that iceA2 was dominant

(40). Peek et al (41) and van Doorn et al (35) reported meaningful association between iceA1 genotype and peptic ulcer, and no statistically significant difference was found in our study between the two groups with reflux esophagitis and peptic ulcer in terms of iceA1 and iceA2 (IceA1 $p=0,556$, IceA2 $p=0,747$).

BabA was detected as positive in 13 of the patients with reflux esophagitis (52 %) and in 14 of the patients with peptic ulcer (56 %). The mean babA positivity was reported as 52.5 %, similar to babA positivity in our study which was detected as 54 % in a study in Turkey (42). In our study no statistically significant difference was found between the two groups with reflux esophagitis and peptic ulcer in terms of babA, and a babA positivity of average 54 % was detected ($p=1.000$).

In this study, where we assessed reflux esophagitis and peptic ulcer patients in terms of bacterial virulence genotypes, it was found that the subjects expressed multi *Helicobacter pylori* virulence genotypes (cagA, vacA, babA). Similar to our findings, in the study performed by Leodolter et al, cagA, vacA, and babA positivity was shown to be higher in patients with duodenal ulcer and gastroesophageal reflux disease than in patients with functional dyspepsia. The literature stresses the importance of multi virulence genotype expression, which increases inflammation and plays role in the development of diseases associated with *Helicobacter pylori* (43).

In this study, we have performed in the East Black Sea Region, the results we have achieved in terms of expression rates of virulence genotypes show similarity to studies conducted in other regions of our country.

Cytokines are regulators of host immune response in situations such as infection, inflammation and trauma. In such situations, some cytokines increase inflammation and are called proinflammatory cytokines. Primary proinflammatory cytokines are IL-1, IL-2, TNF α , IL-6, IL-8, IL-11, IL-12 and chemokines. Some cytokines are responsible for suppressing proinflammatory cytokine activity. These are called anti-inflammatory cytokines. Primary anti-inflammatory cytokines are IL-4, IL-10, IL-13, IL-16, IFN- α .

Studies on cytokine polymorphisms associated with *Helicobacter pylori* frequently focus on gastric cancer, and increased risk of hypochlorhydria and gastric cancer characterized with increase in proinflammatory cytokines as a result of IL-1 β gene polymorphism was shown (44). Predisposition to increased gastric atrophia and gastric cancer associated with IL-2 gene polymorphism has been reported in the literature. Won HH et al reported that interleukin 10 polymorphisms differentially influence the risk of gastric cancer in East Asians and Caucasians. There is a limited number of studies relating to gastroesophageal reflux disease and cytokine gene polymorphism. Muramatsu et al reported increased IL-1 β -511C/T polymorphism in patients with reflux esophagitis (40).

In this study, that we have conducted on patients with reflux esophagitis and peptic ulcer, IL-2 -330T +166T polymorphism was determined as positive in 9 peptic ulcer patients and only in 2 reflux esophagitis patients. IL-2 T/T polymorphism was much frequently detected in patients with peptic ulcer than in patients with reflux esophagitis, and this difference was found to be statistically significant ($p=0.037$).

T cell response, which develops in subject to *Helicobacter pylori*, is dominantly seen as T helper 1 (Th1) response in humans (46). Th1 cells produce IL-2 and such responses are associated with the expression of proinflammatory cytokines such as IFN- γ and TNF- α . IL-2 is a strong proinflammatory cytokine. Macrophages secrete proinflammatory factors when induced by Th1 cells. IL-2 induces antibody production with the proliferation of active T and B cells. They induce IL-3, IL-4, IL-5, IL-6, GM-CSF, INF- γ , TNF- β and TGF- β syntheses. Cytotoxic T lymphocytes ensure the activation of NK cells and neutrophils. Increased IL-2 inhibits gastric acid secretion with Th1 response, and results in precancerous gastric atrophia, metaplasia and dysplasia. In their study, Togawa et al determined thrice increase in gastric atrophia prevalence in 2T/T genotype compared to IL 2G/G genotype⁴⁷. Increased IL-2 T/T cytokine gene polymorphism we have determined in patients with peptic ulcer in this study is supported by immunologic evidences, which play a role in peptic ulcer development.

In our study, IL10 -1082A; -819C polymorphism was detected as positive in 18 patients with reflux esophagitis and in 24 patients with peptic ulcer. IL-10 -1082A; -819C polymorphism was slightly much more detected in patients with peptic ulcer than in patients with reflux esophagitis, and this difference was found to be statistically significant ($p=0.049$).

IL-10 is a multi-functional anti-inflammatory cytokine. IL-10 is secreted from Th 2 cells, thymocytes, monocytes, macrophages, B cells and Langerhans cells. Th 1 helps to suppress cell response and inhibit IL-2 and IFN synthesis. IL-10 induces B cell proliferation and differentiation. It inhibits IL-1, IL-6, IL-8, GM-CSF, G-CSF synthesis, suppresses inflammation and has a suppressive effect on type Th1 immune response. Low IL-10 production in patients infected with *Helicobacter pylori* results in increased gastric inflammation intensity, hypochlorhydria and increased risk of gastric atrophia and gastric cancer. Lu et al (48) reported increased risk of gastric cancer and peptic ulcer associated with IL-10 gene polymorphism. In another study conducted by El-Omar, it is stated that anti-inflammatory cytokines such as IL-10 have two-fold more effect in the formation of extracardiac gastric cancer (44).

In conclusion, this preliminary study establishes the importance of multi bacterial virulence factors and the cytokine gene polymorphisms that shape the immune response in the host on the development of peptic ulcer in patients with reflux esophagitis and peptic ulcer, infected with *Helicobacter pylori*. It was found that the subjects with reflux esophagitis and peptic ulcer expressed multi *Helicobacter pylori* virulence genotypes (cagA, vacA, iceA). In Turkey, *H. pylori* infection caused by these multi genotypes are much prevalent compared to western countries (49–50). This result may be related to a wide prevalence of *H. pylori* and childhood infections in Turkey. *H. pylori* may undergo genetic changes during the long-term colonization starting from the childhood (51). It was concluded that prevalence of multi-strain colonization should be taken into account when planning treatment and also researches relating to the pathogenesis of *H. pylori* infection.

IL-2 T/T polymorphism and IL-10 -1082A; -819C polymorphism, which was detected in patients with peptic ulcer, are considered to have an effect on peptic ulcer formation.

As a result, consistent with literature, multi *Helicobacter pylori* virulence genotype expression was found to be important in the development of diseases associated with *Helicobacter pylori*. However, it was concluded that cytokine gene polymorphisms may also play a role in peptic ulcer development. It is considered that a detection of multi virulence genotype expression and cytokine gene polymorphism may provide guidance in detecting prognosis in patients infected with *Helicobacter pylori* in studies to be conducted in the future years.

Which clinical pictures of *Helicobacter pylori* will result is determined primarily by the bacterial virulence factors and the immune system of the host, and it has been concluded that broad-scale meta-analyses will be required where HLA tissue groups, inflammation mediators, parietal cell antibodies, acid secretion and infection age, which were shown to influence this result have to be evaluated together.

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