Microb Health Dis 2024; 6: e987

GENE EXPRESSION IN GASTRIC BIOPSIES FROM PATIENTS WITH *HELICOBACTER PYLORI* INFECTION

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Abstract – *Objective:* Heterogeneity among *Helicobacter pylori (H. pylori)* strains in the expression of CagA and outer membrane proteins (OMPs) is an important factor influencing clinical outcomes. We investigated the expression of *H. pylori cagA, omp6 (hopA), omp13 (oipA), omp18, and omp20 (alpA)* genes and their associations with clinicopathological findings.

Materials and Methods: Endoscopic biopsies from the gastric antrum and corpus of 120 patients were initially examined using a rapid urease test (RUT), histopathology, and culture. Biopsy specimens from 101 patients were analyzed for *H. pylori cagA, omp6, omp13, omp18, omp20*, 16S rRNA, and *ureA* gene expression by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from antral and corpus biopsies and cDNA was synthesized.

Results: Forty-eight patients were positive for *H. pylori* infection based on both RUT and histopathology or culture positivity. *H. pylori* was detected in 77 of 101 (76.2%) patients by RT-PCR. Of the 48 patients with *H. pylori* infection, 45 (93.7%) were RT-PCR positive. The gene expression frequencies of *cagA, omp6, omp13, omp18, and omp20* in the antrum and corpus of 45 patients were 30 (66.7%), 35 (77.8%), 37 (82.2%), 45 (100.0%), and 37 (82.2%), respectively. No significant association was found between gene expression frequency and endoscopic findings. However, the expression level of the *omp13* gene was significantly lower in patients with gastric ulcers than in patients with non-erosive gastritis. There was no association between gene expression frequency or level and histopathological parameters. The expression levels of the *omp6* and *omp13* genes were significantly higher in the corpus than in the antrum.

Conclusions: Neither *cagA* nor OMPs gene expression had a statistically significant impact on patient's clinical outcomes in our hospital. The *omp6* and *omp13* genes may play an important role in the pathogenesis of *H. pylori* infection.

Keywords: *Helicobacter pylori*, CagA, Outer membrane protein, Gene expression, RT-PCR.

INTRODUCTION

H. pylori infection is one of the most prevalent infectious diseases worldwide. *H. pylori* is closely associated with human diseases of the upper gastrointestinal tract, such as chronic

gastritis, peptic ulcer, and gastric adenocarcinoma. The role of bacterial virulence factors and pathophysiological mechanisms involved in the development of *H. pylori*-induced gastric cancer still remains to be elucidated. Host, environmental, and bacterial factors have been implicated in the pathogenesis of the disease[1-3]. Clinical isolates of *H. pylori* are highly polymorphic. Genetic diversity among *H. pylori* strains is known to be an important factor affecting the outcome of infection[3-5]. Cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) are the most important virulence factors of *H. pylori*. These proteins have been reported to be associated with an increased risk of developing peptic ulcer disease and gastric cancer[2,6,7]. It has been shown that some outer membrane proteins (OMPs) mediate the adherence of *H. pylori* to gastric epithelial cells[8-10]. Many studies[11-16] have focused on phase-variable genes, such as *oipA, hopZ, sabA/B,* and *babA/B*. Their significant role in the pathogenesis of *H. pylori* infection has been demonstrated. Little is known about *H. pylori omp6 (hopA), omp18,* and *omp20 (alpA)* genes and their association with gastroduodenal diseases[17-19]. The *omp13 (oipA, hopH)* and *omp18* genes have been linked to inflammation[9,20], while the *omp6* and *omp20* genes are supposed to be involved in host-bacterial interactions[19,21]. The *oipA* gene also plays a role in bacterial adhesion[2,8,15,22]. There are a few studies in Turkey, mainly focusing on genotyping of *H. pylori* virulence-associated genes[23,24]. The prevalence of *H. pylori* in Turkey has been reported to be as high as 86%[25].

We hypothesized that the differences in gene expression of *H. pylori cagA* and OMPs *(omp6, omp13, omp18, omp20)* may explain the different clinical outcomes of infection. In the present study, we used TaqMan Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) to determine *H. pylori* gene expression in gastric biopsy specimens. This study aimed to evaluate the association between bacterial gene expression and clinicopathological findings in patients with *H. pylori* infection. The reliability of the RT-PCR assay was evaluated.

MATERIALS AND METHODS

Patients and Biopsy Sampling

The study included 120 patients who were referred to the Endoscopy Unit at the Gastroenterology Polyclinic of Dokuz Eylül University Hospital due to upper gastrointestinal issues. An upper gastrointestinal system endoscopy was performed. Four sets of biopsy specimens were taken from the gastric antrum and corpus of each patient. The first set was used for rapid urease test (RUT); the second set was placed into 10% formalin and sent to the Pathology Laboratory for histopathological examination. The third set was immediately transported to the Microbiology Laboratory and processed for culture. Finally, the last set was snap-frozen in liquid nitrogen using a nitrogen storage tank (MVE Lab, Medford, NY, USA). The samples were thereafter stored at -80°C until total RNA extraction. To prevent nucleic acid contamination of fiber-optic endoscopes, the cleaning and disinfection procedures were followed after each use[26].

Patients were excluded if they had a history of gastric surgery and *H. pylori* infection, were receiving steroids or non-steroidal anti-inflammatory drugs, and had taken proton pump inhibitors and/or antibiotics within four weeks before endoscopy.

Rapid Urease Test

An in-house rapid urease test (RUT) was performed on gastric biopsy specimens. Biopsies were placed in 2 ml of a freshly prepared solution of 10% urea (Merck, Darmstadt, Germany) with 1% phenol red (Merck, Darmstadt, Germany) as a pH indicator. The presence of urease was detected by a color change from yellow to pink-red within minutes[27].

Histopathology

Histological sections from gastric biopsies were stained with hematoxylin and eosin (H&E), as well as Giemsa stains (Merck KGaA, Darmstadt, Germany). They were examined for the

presence of Helicobacter-like organisms (HLO). Histopathological parameters, including the density of lymphocytic infiltration and neutrophil activity, degree of intestinal metaplasia, and atrophy, were determined. All parameters were scored on a scale of 0 to 3 by a pathologist according to the updated Sydney system[28].

H. pylori Culture

Biopsy specimens were removed from the transport medium (Brain Heart Infusion broth) (Oxoid, Basingstoke, Hants, UK). They were immediately ground using a sterile glass tissue grinder and homogenized in 200 µl of phosphate-buffered saline (PBS, pH=7.2) (Biochrom AG, Berlin, Germany). The homogenates were inoculated onto Columbia Blood agar (Oxoid, Basingstoke, Hampshire, UK) containing 7% defibrinated horse blood (Oxoid, Basingstoke, Hampshire, UK) and *H. pylori*-selective supplement (Dent) (Basingstoke, Oxoid, UK). The plates were incubated at 37°C for 3-7 days under microaerophilic conditions using the GasPak Campy Container System (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Bacterial isolates with grey translucent colonies were identified as *Helicobacter pylori* by positive urease, oxidase, and catalase test results. *H. pylori* produced unique golden colonies on an indicator medium that contained 0.004% 2,3,5-triphenyl tetrazolium chloride (TTC) (PanReac AppliChem, Darmstadt, Germany) (Figure 1). Gram staining was performed on smears from colonies to examine Gram-negative spiral morphology[27,29,30].

Total RNA Extraction and cDNA Synthesis (Reverse Transcription)

Based on RUT, histopathology, and culture results, 101 out of 120 patients were included in the RT-PCR experiment. Total RNA was extracted from gastric biopsies using the TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Total RNAs were suspended in 50 µl of PCR grade water (Zymo Research, CA, USA) and the purity was checked (A260/A280 nm ratio) using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) was used to synthesize cDNA from total RNA according to the manufacturer's instructions. cDNAs were stored at -20°C until used.

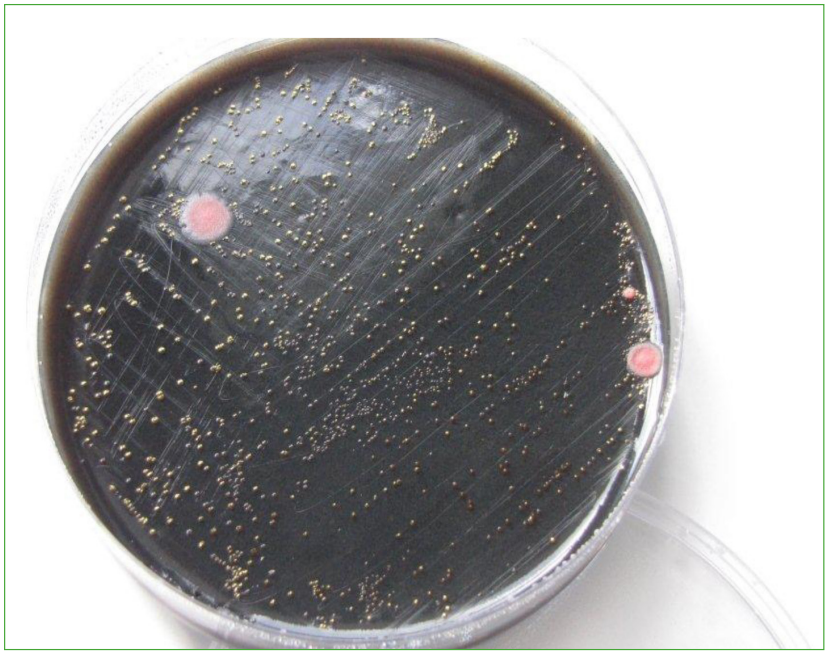

Figure 1. The colonies of *H. pylori* on indicator medium (Columbia Blood agar) supplemented with TTC.

Primer and Probe Design

The *cagA, omp6, omp13, omp18,* and *omp20* genes of *H. pylori* strain 26695, as well as the 16S rRNA and *ureA* housekeeping genes of *H. pylori* strains G27 and P12 were selected. The genome sequences of the strains were described in the GenBank database of the National Center for Biotechnology Information (NCBI). The Roche Universal Probe Library (UPL) online tool was used to find specific primers (TIB Molbiol, Berlin, Germany), and TaqMan probes (Roche Applied Science, Penzberg, Germany) (Table 1). The specificity of primers was further tested using NCBI Primer-BLAST tool.

TaqMan Real-Time RT-PCR Experiment

Since all the primers had almost the same melting temperature, a 96-well plate format was used. Therefore, multiple gene expression assays were run simultaneously. Primer and probe concentration and the amount of probe master (hot start Taq DNA polymerase) were adjusted. Optimization procedures were applied following the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The master mix was prepared by adding 10 µl of Taq DNA polymerase, dNTPack, 2 µl of each primer, 2 µl of TaqMan probe, and 2 µl of PCR grade water; then, it was dispensed into each well in the rows of a plate. Finally, 2 µl of cDNA was added to each well in the columns to achieve a total reaction volume of 20 µl. PCR-grade water was also included. The plate was sealed, centrifuged at 4,500 rpm (Eppendorf, Enfield, CT, USA), and loaded into the LightCycler 480 II/96 (Roche Diagnostics Ltd., Rotkreuz, Switzerland). It was programmed with the following cycling conditions: initial denaturation at 95°C (10 min) to activate hot start Taq DNA polymerase; followed by 50 cycles of denaturation at 95°C (10 s); annealing at 55°C (50 s); extension at 72°C (1 s); finally cooling at 40°C (30 s)[31].

H. pylori NCTC 11637 (*cagA-, vacA-, oipA-,* and *babA*-positive strain) was used as a positive control. Due to the use of TaqMan hydrolysis probes, the melting curve analysis was not feasible to perform. To confirm the specificity of the RT-PCR reaction, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and a clinical isolate of *Campylobacter jejuni* were used. The colonies from bacterial subcultures were harvested and suspended in PBS

TABLE 1. PRIMERS AND PROBES USED FOR REAL-TIME RT-PCR.

Gene	Primer	Sequences	Tm	Probe
cagA (cag26) [HP0547]	F (21 nt) R (25 nt)	5´ACCACCGACATACAAGGCTTA 3´ 5´ATTACCCCTTTCATCAAGTAAATCC 3´	59°C 59°C	TaqMan #124
omp6 (hopA) [HP0229]	F (18 nt) R (22 nt)	5´TTAATGCAAGCGGGAACG 3´ 5´TCATGCTAGTGATGGCGTTAAA 3´	60°C 60°C	TaqMan #145
omp13 (oipA) [HP0638]	F (23 nt) R (21 nt)	5´GCCTTAAATGCAGTGAAAGATTC 3´ 5´CCCAAAGCTGAGCTCATTAAA 3´	59°C 59°C	TaqMan #138
omp18 [HP1125]	F (22 nt) R (20 nt)	5´TGCAAAAAGCTAAAGAAAACCA 3´ 5´CGCTAGAGCCAAATTCATCG 3´	59°C 60°C	TaqMan #97
omp20 (alpA) [HP0912]	F (20 nt) R (22 nt)	5´TGTGAAATTGCATGGGAAAA 3´ 5´TCATCCTCATACCGAAGTCAAA 3´	59°C 59°C	TaqMan #154
16S rRNA [HPG27_1093]	F (25 nt) R (24 nt)	5´TTTATTGCGATTTAGTAGGGCTTAG 3´ 5´CCTTTGGATTTCTATGACTTTTCC 3´	59°C 59°C	TaqMan #31
ureA [HPP12_0077]	F (21 nt) R (22 nt)	5´GCTTCAATACCCACTTCATGG 3´ 5´GCACTCTTTTAAAACCGGATGA 3´	59°C 60°C	TaqMan #158

F: Forward; R: Reverse; Tm: Melting temperature; nt: nucleotide.

(pH=7.2). RNA was extracted from bacterial suspensions that contained ~10^8 CFU/ml of *H. pylori* positive control and ~10^7 CFU/ml of other bacteria. RNA extraction and cDNA synthesis were performed according to the protocols mentioned above.

Detection of *H. pylori* Gene Expression Frequency and Level

The cycle threshold (Ct) value for each gene was recorded. The Ct value was defined as the cycle number at which the fluorescent signal crossed the fluorescence threshold. The expression of the target genes *cagA, omp6, omp13, omp18, omp20*, and housekeeping genes (16S rRNA, *ureA*) was determined based on the Ct values obtained in the RT-PCR experiment. Housekeeping genes were used to normalize gene expression data. The relative gene expression for each target gene was calculated as follows:

ΔCt = Ct (target gene) **–** Ct (housekeeping gene)
Gene expression level (normalized **ΔCt**) = $2^{-\Delta Ct}$.
It was calculated using Roche relative expression software[31,32].

H. pylori Infection Status

Patients were considered to have *H. pylori* infection if both rapid urease test (RUT) and histopathology or culture were positive. Gene expression was evaluated in patients infected with *H. pylori*.

Statistical Analysis

SPSS version 20.0 software was used for statistical analysis. Frequency data were analyzed using Pearson's chi-square test, Fisher's exact test, and McNemar's chi-square test; continuous data were compared using the Mann-Whitney U test, Wilcoxon signed-rank test, and *t*-test. Spearman's correlation test was also used. Sensitivity and specificity ratios were calculated from the contingency table. The *p*-value for statistical significance was defined as $p<0.05$.

RESULTS

Endoscopic Findings

Of the 120 patients (71 females and 49 males; mean age 50.58 ± 14.34; age range 20-83) who underwent upper endoscopy, 11 (9.2%) were diagnosed with gastric ulcer/erosion (GU/E), 10 (8.3%) with duodenal ulcer/erosion (DU/E), 28 (23.3%) with erosive gastritis (EG), 66 (55.0%) with non-erosive gastritis (NEG), 3 (2.5%) with normal mucosa, and 2 (2.7%) with gastric cancer (GC). RUT was positive in 79/120 (65.8%) patients. Two patients with gastric cancer were examined using microbiological, histopathological, and molecular methods. However, these data were not included in the statistical analysis.

Histopathologic Findings

H. pylori infection was significantly associated with infiltration of polymorphonuclear and mononuclear leukocytes (PNL and MNL) in the gastric mucosa (antrum: $p=0.006$ and $p=0.001$; corpus: $p=0.001$ and $p=0.010$, respectively). There was no association between *H. pylori* infection and intestinal metaplasia ($p>0.05$). There was a moderate positive correlation between *H. pylori* density (HLO) and PNL levels (antrum: r=0.575, $p=0.001$). No correlation was found between HLO and MNL levels ($p>0.05$). The MNL levels were significantly higher than the PNL levels (antrum: $p=0.013$; corpus: $p=0.036$).

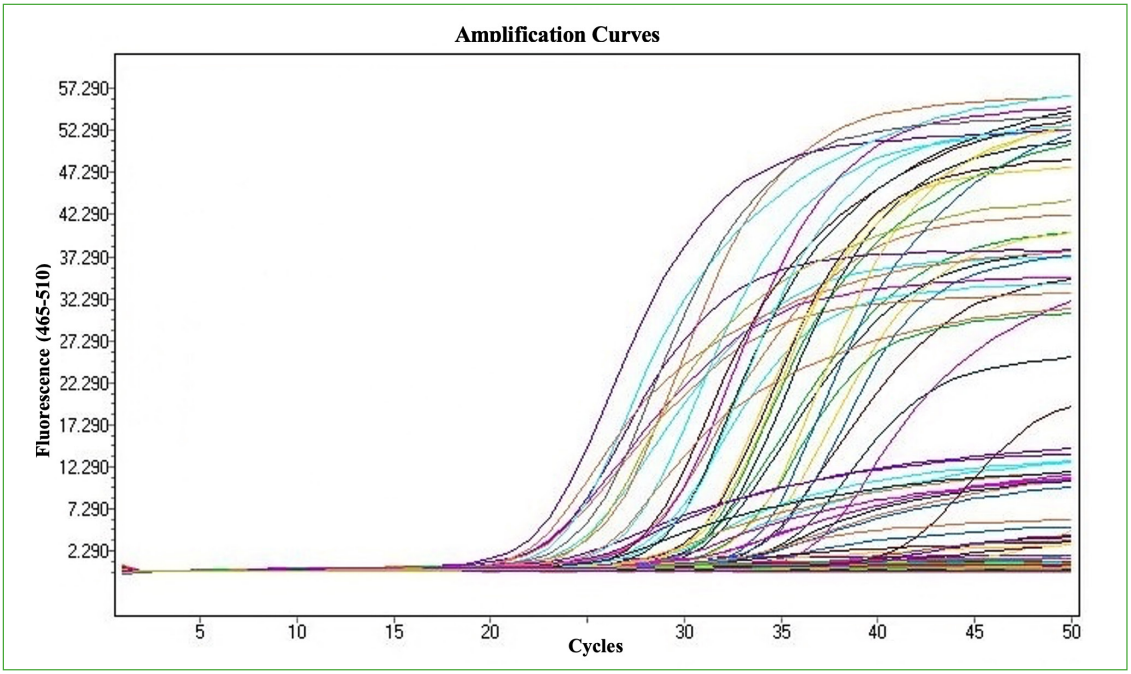

Figure 2. Real-Time RT-PCR amplification curves of *H. pylori cagA*, *omp6*, *omp13*, *omp18*, *omp20*, 16S rRNA, and *ureA* gene expression in gastric biopsy specimens of patients.

Microbiologic Findings

H. pylori was found positive by culture in 50/120 (41.6%) patients. Culture had a sensitivity of 82.0% and a specificity of 90.0% compared with both RUT and histopathology results.

Real-Time RT-PCR and Gene Expression Findings

Of the 101 patients, 77 (76.2%) were positive for *H. pylori* by RT-PCR based on Ct values obtained for both 16S rRNA and *ureA* housekeeping genes. RT-PCR showed a sensitivity of 94.0% and a specificity of 60.0% compared with both RUT and histopathology or culture results. No cross-reactivity was observed with *E. coli*, *S. aureus*, and *C. jejuni*, whereas a strong signal was detected with *H. pylori* NCTC 11637 positive control. In order to validate the precision of the RT-PCR assay, the experiment was repeated with randomly selected total RNAs. The coefficient of variation (CV%) was calculated, and the mean value of 1.70% was obtained. The amplification curves are shown in Figure 2.

The gene expression frequencies of *cagA, omp6, omp13, omp18,* and *omp20* in the antrum and corpus of 45/48 (93.7%) patients were 30 (66.7%), 35 (77.8%), 37 (82.2%), 45 (100.0%), and 37 (82.2%), respectively. The expression rate of *cagA* gene (66.7%) was lower than that of the *omp6, omp13, omp18,* and *omp20* genes. The *omp18* gene was expressed in all gastric biopsy specimens from 45 patients (Table 2).

TABLE 2. EXPRESSION OF *cagA* AND OMP GENES IN GASTRIC BIOPSIES FROM 45 PATIENTS INFECTED WITH *H. PYLORI*.

cagA		***omp6***		***omp13***		***omp18***		***omp20***	
(+)	**(–)**	**(+)**	**(–)**	**(+)**	**(–)**	**(+)**	**(–)**	**(+)**	**(–)**
30 (66.7%)	15 (33.3%)	35 (77.8%)	10 (22.2%)	37 (82.2%)	8 (17.8%)	45 (100.0%)	0 (0.0%)	37 (82.2%)	8 (17.8%)

TABLE 3. EXPRESSION OF *H. PYLORI cagA* AND OMP GENES IN GASTRIC BIOPSIES FROM PATIENTS WITH DIFFERENT CLINICAL OUTCOMES.

Gene exp	GU/E n= 6	NEG n = 6	DU/E n = 9	EG n = 24	OR (95% CI)	*p*-value
cagA +	2 (33%)	6 (100%)	5 (55%)	17 (71%)	[a]OR = 0.20 (0.03-1.4)	$p = 0.105$
–	4 (67%)	0 (0.0%)	4 (45%)	7` (29%)	[b]OR = 5.57 (0.27-112)	$p = 0.261$
					[c]OR = 0.51(0.10-2.50)	$p = 0.410$
omp6 +	3 (50%)	6 (100%)	5 (55%)	21 (87%)	[b]OR = 2.12 (0.09-46.5)	$p = 0.634$
–	3 (50%)	0 (0.0%)	4 (45%)	3 (13%)	[c]OR = 0.18 (0.03-1.07)	$p = 0.058$
omp13 +	5 (83%)	4 (67%)	6 (67%)	22 (92%)	[a]OR = 0.45 (0.03-6.05)	$p = 0.550$
–	1 (17%)	2 (33%)	3 (33%)	2 (8%)	[b]OR = 0.18 (0.02-1.70)	$p = 0.134$
					[c]OR = 0.18 (0.02-1.35)	$p = 0.095$
omp18 +	6 (100%)	6 (100%)	9 (100%)	24 (100%)	*OR = Not calculated	–
–	0 (0.0)	0 (0.0%)	0 (0.0%)	0 (0.0%)	*OR = Not calculated	–
					*OR = Not calculated	–
omp20 +	5 (83%)	5 (83%)	6 (67%)	21 (87%)	[a]OR = 0.71 (0.06-8.4)	$p = 0.789$
–	1 (17%)	1 (17%)	3 (33%)	3 (13%)	[b]OR = 0.71 (0.06-8.4)	$p = 0.789$
					[c]OR = 0.28 (0.05-1.8)	$p = 0.182$

GU/E: Gastric ulcer/Erosion; DU/E: Duodenal ulcer/Erosion; EG: Erosive gastritis; NEG: Non-erosive gastritis. [a]OR= Comparison between patients with GU/E and NEG; [b]OR = Comparison between patients with DU/E and NEG; [c]OR= Comparison between patients with EG and NEG. *Due to the expression of *omp18* in all patients, the expression status could not be statistically analyzed.

TABLE 4. *H. PYLORI cagA* AND OMP GENE EXPRESSION LEVELS IN GASTRIC BIOPSIES FROM PATIENTS WITH DIFFERENT CLINICAL OUTCOMES.

	cagA		***omp6***		***omp13***		***omp18***		***omp20***	
Endoscopic finding	**Med**	**Min-Max**	**Med**	**Min-Max**	**Med**	**Min-Max**	**Med**	**Min-Max**	**Med**	**Min-Max**
GU/E	2.85	0.04-5.66	0.06	0.00-0.54	0.03	0.01-0.46	1.96	0.07-6.23	34.54	9.12-61.89
NEG	3.65	0.01-77.49	0.37	0.00-1.53	0.51	0.06-1.55	1.17	0.02-8.08	21.78	0.46-99.13
p-value	n=19 *p*=0.399		n=24 *p*=0.714		n=27 ***p*=0.005**		n=30 *p*=0.795		n=26 *p*=0.345	
DU/E	4.83	0.64-72.68	0.36	0.06-1.04	0.43	0.06-1.47	0.92	0.34-1.99	9.26	0.00-19.84
NEG	3.65	0.01-77.49	0.37	0.00-1.53	0.51	0.06-1.55	1.17	0.02-8.08	21.78	0.46-99.13
p-value	n= 23 *p*=0.507		n=27 *p*=0.496		n=26 *p*=0.816		n=30 *p*=0.254		n=26 *p*=0.085	
EG	1.81	0.01-14.58	0.20	0.00-0.49	0.12	0.00-0.98	1.73	0.08-4.82	15.51	0.20-51.44
NEG	3.65	0.01-77.49	0.37	0.00-1.53	0.51	0.06-1.55	1.17	0.02-8.08	21.78	0.46-99.13
p-value	n=22 *p*=0.345		n=26 *p*=0.585		n=28 *p*=0.051		n=33 *p*=0.936		n=27 *p*=0.414	

GU/E: Gastric ulcer/Erosion; DU/E: Duodenal ulcer/Erosion; EG: Erosive gastritis; NEG: Non-erosive gastritis.

H. pylori Gene Expression and Endoscopic/Histopathologic Findings

Gene expression frequencies showed no significant differences between patients with non-erosive gastritis (NEG) and those with gastric ulcer/erosion (GU/E) or duodenal ulcer/erosion (DU/E) (p>0.05). There was no association between *H. pylori cagA* or OMPs gene expression and clinical outcome (Table 3). The expression level of the *omp13* gene was significantly lower in patients with GU/E compared to those with NEG (p=0.005) (Table 4).

The gene expression frequency and level of *cagA, omp6, omp13,* and *omp20* were higher among patients with pangastritis than among patients with antral-predominant gastritis. However, there was no statistically significant difference (p>0.05) (Tables 5 and 6). There was no relationship between the expression frequency or level of the examined genes and histopathological parameters (*H. pylori* density, PNL, MNL) (p>0.05).

TABLE 5. EXPRESSION OF *H. PYLORI cagA* AND OMP GENES IN GASTRIC BIOPSIES FROM PATIENTS WITH DIFFERENT PATTERNS OF GASTRITIS.

Pattern of gastritis	*cagA* n (%)		*omp6* n (%)		*omp13* n (%)		**omp18* n (%)		*omp20* n (%)	
	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)
Pan-gastritis	16 (59.3)	7 (50.0)	18 (56.3)	5 (55.6)	20 (60.6)	3 (37.5)	23 (56.1)	0 (0.0)	19 (54.3)	4 (66.7)
Antral gastritis	11 (40.7)	7 (50.0)	14 (43.7)	4 (44.4)	13 (39.4)	5 (62.5)	18 (43.9)	0 (0.0)	16 (45.7)	2 (33.3)
n = 41 *p*-value	p = 0.571		p = 0.970		p = 0.237		––		p = 0.572	

*Due to the expression of *omp18* in all patients, the expression status could not be statistically analyzed.

TABLE 6. *H. PYLORI cagA* AND OMP GENE EXPRESSION LEVELS IN GASTRIC BIOPSIES FROM PATIENTS WITH DIFFERENT PATTERNS OF GASTRITIS.

Pattern of gastritis	*cagA* Med (min-max)	*omp6* Med (min-max)	*omp13* Med (min-max)	*omp18* Med (min-max)	*omp20* Med (min-max)
Pan-gastritis	5.13 (0.01-72.68)	0.37 (0.00-1.40)	0.51 (0.00-1.50)	1.53 (0.02-4.82)	26.23 (0.46-99.13)
Antral gastritis	3.55 (0.01-77.49)	0.14 (0.00-1.53)	0.25 (0.03-1.55)	1.05 (0.07-8.08)	14.30 (0.00-64.40)
p-value	p = 0.824 n = 25	p = 0.327 n = 30	p = 0.280 n = 31	p = 0.401 n = 41	p = 0.312 n = 35

H. pylori Gene Expression (Antrum Versus Corpus)

Biopsy specimens from the antrum and corpus were examined for differences in gene expression. The mean Ct values of *cagA, omp6, omp13, omp18*, *omp20*, 16S rRNA, and *ureA* genes were lower in the antrum than in the corpus. Only the difference in mean Ct values for the 16S rRNA gene was statistically significant (p=0.020). A low Ct value indicates high gene expression. The gene expression frequencies of *cagA, omp6, omp13, omp18*, and *omp20* did not show significant and large differences between the antrum and corpus. These rates were 58.3%, 70.8%, 83.3%, 100.0%, 75.0% in the antrum and 50.0%, 62.5%, 83.3%, 100.0%, 83.3% in the corpus, respectively. When gene transcripts were normalized to the housekeeping gene *ureA*, we found that the expression levels of the *omp6* and *omp13* genes were significantly higher in the corpus than in the antrum (p=0.014 and p=0.026, respectively) (Table 7).

TABLE 7. COMPARISON OF EXPRESSION LEVELS OF *H. PYLORI cagA* AND OMP GENES IN BIOPSIES FROM THE GASTRIC ANTRUM AND CORPUS.

cagA		***omp6***		***omp13***		***omp18***		***omp20***	
Antrum	**Corpus**	**Antrum**	**Corpus**	**Antrum**	**Corpus**	**Antrum**	**Corpus**	**Antrum**	**Corpus**
Med (min-max) 0.32 (0.06-5.24)	Med (min-max) 0.24 (0.03-28.25)	Med (min-max) 0.01 (0.00-0.15)	Med (min-max) 0.01 (0.00-0.04)	Med (min-max) 0.01 (0.00-9.51)	Med (min-max) 0.00 (0.00-0.04)	Med (min-max) 0.05 (0.00-0.54)	Med (min-max) 0.3 (00.0-21)	Med (min-max) 0.63 (0.17-3.39)	Med (min-max) 0.50 (0.50-2.22
$p = 0.097$ n = 23		**$p = 0.014$** n = 27		**$p = 0.026$** n = 32		$p = 0.083$ n = 39		$p = 0.140$ n = 31	

DISCUSSION

Genetic diversity among *H. pylori* isolates, geographic variation, and bacterial strain distribution may explain differences in the prevalence and incidence of *H. pylori*-associated gastroduodenal diseases. Analysis of virulence genes is important to determine predictive markers to identify patients at high risk of developing more severe disease. It may also provide information to better understand the pathogenesis of *H. pylori* infection[2,5,33].

In the present study, we found that *cagA* gene expression was not associated with clinicopathological parameters. The *cagA* gene is located in the *cag* pathogenicity island (*cag* PAI) of *H. pylori* and encodes the CagA protein. Tyrosine phosphorylation of CagA EPIYA motifs (Glu-Pro-Ile-Tyr-Ala) can trigger multiple signaling proteins that cause morphological changes, inflammation, and epithelial cell damage. In Western countries, *cagA* has been associated with peptic ulcer and/or increased risk of gastric cancer. In East Asia the presence or absence of *cagA* has not been defined as a marker of disease due to the presence of the gene in almost all *H. pylori* strains. It has been shown that the differences in the pathogenicity of CagA can be explained by four different types of EPIYA segments, named EPIYA-A, B, C, and D. *H. pylori* strains with multiple EPIYA-C segments (ABCCC versus ABC) are associated with increased risk of gastric cancer[2,34].

In our study, the expression rate of the *cagA* gene was 66.7%. The *cagA* was frequently expressed in patients with both severe (duodenal ulcer) and mild (non-erosive gastritis) clinical outcomes. The expression of the *cagA* gene was not associated with gastric ulcer or duodenal ulcer. The association between *cagA* and peptic ulcer disease, as well as increased risk of gastric cancer has been well documented worldwide[2,6,7,10,22,25]. Some studies[25,35] conducted in Turkey have shown that *cagA*-positive strains are associated with peptic ulcer disease. In contrast, other studies have failed to find an association between the *cagA* gene or CagA seropositivity and clinical outcome[36-38]. These contradictory results may be explained by differences in virulence between *cagA*-positive *H. pylori* strains and geographical distribution of the genetically distinct groups[39]. Further studies are needed to characterize the CagA EPIYA variants among *cagA*-positive isolates.

Our results showed that there was a significant inverse correlation between the expression level of the *omp13 (oipA)* gene and gastric ulcer/erosion, but a positive correlation with non-erosive gastritis. The expression of the *oipA* gene is regulated by the slipped-strand mispairing (SSM) mechanism. This is based on the number of CT dinucleotide repeats in the 5´end of the gene (switch “on” = functional *oipA* and switch “off “ = non-functional *oipA*)[2,13]. Yamaoka et al[9,40] found a significant association between OipA-positive status and duodenal ulcer, gastric cancer, high *H. pylori* density, and inflammatory cells. Outer inflammatory protein A (OipA) is a low molecular weight protein encoded by the *oipA* gene and correlates with *cagA*-positive status. It has been hypothesized that there is a synergistic interaction between CagA, OipA, and VacA. In addition to its role in inflammation, there is evidence that OipA also functions as an adhesion factor[2,13,15].

Phase-variable genes that encode OMP adhesins play an important role in the pathogenesis of *H. pylori* infection. Modulation of *H. pylori* adherence by host-bacterial interactions facilitates *H. pylori* persistence and evasion of host immune responses[4]. Horridge et al[41] demonstrated that the functional status of *oipA* was significantly associated with increased bacterial adherence to human gastric cancer cells (AGS). They reported a significant increase in *oipA* transcription in adherent *H. pylori* cells compared to non-adherent bacteria. This increase also occured in *H. pylori* population with non-functional *oipA* status. They pointed out that the expression of the *oipA* gene is regulated by host cell contact. A recent study[42] employing gastric cell lines revealed that *H. pylori* strains with *oipA* “off “-status may trigger apoptosis more rapidly than those with *oipA* “on”-status.

In our study, sequencing was not performed due to the small and very low amount of amplicons generated during the RT-PCR experiment. Therefore, we could not determine the functional status of the *omp13* gene. Increased expression levels of the *omp13* gene in patients with non-erosive gastritis may be associated with the early stages of *H. pylori* infection. Considering the chronic nature of the infection, the *omp13* gene may play a critical role in adhesion and persistent colonization of *H. pylori*. Further studies should be conducted to determine the functional status of the gene. This allows the assessment of gene expression status in patients with different clinical outcomes.

We found higher expression levels of the *omp6* and *omp13* genes in the corpus compared to the antrum. Omp6 (HopA) is a low molecular weight protein that belongs to the Hop family of *H. pylori* OMPs and functions as a porin. The absence of Omp6 within bacterial outer membrane vesicles (OMVs) appears to be associated with strains that cause duodenal ulcers[18,43]. Kim et al[21] demonstrated the up-regulation of *omp6* in *H. pylori* upon bacterial adherence to AGS cells. It has been shown that the expression of 7% of *H. pylori* genome is affected by changes in pH. The expression of the *cagA, vacA, omp6*, and *omp13* genes is suppressed, while the expression of the *ureA* and *ureB* genes increases upon exposure to acid stress[44]. Some *in vitro* studies have demonstrated the effect of high-salt concentration on the expression of *H. pylori cagA, hopA (omp6),* and other adhesion genes[45,46]. Bugaytsova et al[47] reported that the binding affinity of *H. pylori* adhesin BabA to gastric mucosa was affected by pH. The acid sensitivity of BabA varies depending on bacterial strain and geographic region. A recent study conducted by Jeske et al[48] showed a high seroprevalence of HopA (Omp6) and CagA in patients with non-cardia gastric cancer (NCGC) compared to controls. They identified HopA as a novel seromarker for NCGC. The up-regulation of gene and antibody production in response to changes in gastric pH may reflect the adaptation of *H. pylori* to pathological alterations.

We concluded that the high expression levels of *omp6* and *omp13* genes in the corpus of patients may be related to changes in intragastric pH or physiological conditions. The gastric antrum is a preferred niche for *H. pylori* because it is less acidic than the gastric corpus[47]. It is unclear whether gene expression was affected by low or high pH, as there was no information about the gastric pH of the patients. Our results are consistent with those of Merrell et al[44] who showed that *H. pylori* gene expression is regulated by pH. The increased expression levels of adhesion genes in the corpus may be clinically important. Prolonged colonization with *H. pylori* can lead to the development of serious diseases such as peptic ulcer and gastric cancer.

In this study, gene transcripts for all genes examined were higher (lower Ct values) in the antrum than in the corpus. This may be due, in part, to differences in bacterial load and pH between the antrum and corpus. The 16S rRNA and the *ureA* genes were used to normalize the target gene expression data. The mean Ct value of the 16S rRNA gene was significantly higher than that of the *ureA* gene in the antrum (16S rRNA: 30.14 ± 2.41; *ureA*: 25.80 ± 3.34, $p<0.001$) and corpus (16S rRNA: 31.78 ± 3.16; *ureA*: 26.75 ± 3.88, $p<0.001$). The *ureA* gene had significantly higher transcript levels than 16S rRNA in both the antrum and corpus. This is in agreement with the findings of Boonjakuakul et al[49], who demonstrated the highest expression levels of *cag1, cag25,* and genes encoding urease and catalase in human gastric mucosa. It is well known that urease and catalase are important for bacterial homeostasis[49].

Histopathological examination of patients revealed two main topographic patterns of gastritis: antral-predominant gastritis and pangastritis. We were interested to know whether these patients had differences in gene expression. The gene expression frequency and level of *cagA* and OMPs were higher in patients with pangastritis than in patients with antral-predominant gastritis, but it was not statistically significant.

The pattern of gastritis has been shown to be important in determining clinical outcomes. *H. pylori*-induced antral-predominant gastritis is associated with duodenal ulcers due to hypergastrinemia and high acid production. Pangastritis or corpus-predominant gastritis with decreased gastric acid levels is linked to gastric ulcer, corpus atrophy, and increased risk of gastric cancer[50,51]. Sepulveda et al[52] showed that young patients with a family history of gastric cancer develop pangastritis. Pangastritis was associated with high *H. pylori* density and dense lymphatic aggregates in the corpus of young patients[52]. *H. pylori* colonization density has been found to be low in patients with chronic hypochlorhydria[53].

We found small differences in *H. pylori* density, PNL, and MNL levels between patients with pangastritis and patients with antral gastritis. In patients with pangastritis, HLO density was slightly lower in the antrum and higher in the corpus compared to patients with antral gastritis. This may be related to the diffuse and localized nature of these patterns. Our results suggest that factors besides bacterial load may also influence *H. pylori* gene expression. Further studies are needed to confirm these preliminary results and identify factors affecting gene expression.

In our study, no association was found between *omp20 (alpA)* gene expression and clinicopathological findings. *H. pylori alpA* and *alpB* have been found to play a role in host cell adhesion and bacterial colonization. An association between genes and inflammatory responses has also been reported. Most of these results have been obtained through *in vitro* and *in vivo* animal studies[8,10,54,55].

We were unable to evaluate the expression status of the *omp18* gene in our patients because it was expressed in all gastric biopsies. This suggests that the *omp18* gene can be used as an internal control (IC) in RT-PCR experiments. Gene expression levels did not correlate with inflammatory cells or disease severity. Omp18 is a low molecular weight OMP encoded by the *omp18* gene. It belongs to the family of peptidoglycan-associated lipoproteins (PALs). All *H. pylori* strains express this protein[18,20]. Omp18 has been shown to induce dendritic cell maturation and inflammatory responses in mice[20,22]. Shan et al[56] demonstrated that Omp18 is a strong antigen and stimulates the production of interferon-gamma (IFN-γ) in mice. Omp18 may be involved in the persistent colonization of *H. pylori* by modulating IFN-γ signaling.

Mononuclear leukocytes were predominant in our patients, indicating the chronic nature of *H. pylori* infection. Schneider et al[57] showed that high levels of mononuclear cell infiltration are associated with DNA hypermethylation and increased risk of gastric cancer. Patients infected with *cagA+/vacA* s1m1 *H. pylori* strains are at high risk for the disease. In fact, chronic inflammation can promote abnormal DNA methylation.

CONCLUSIONS

Gene expression analysis of *H. pylori cagA, omp6, omp13, omp18,* and *omp20* by Real-Time RT-PCR revealed that neither *cagA* nor OMPs gene expression had a statistically significant impact on clinical outcome of the patients in our hospital. The genetic diversity and geographic variation of *H. pylori* strains may explain differences in virulence and infection outcomes between bacterial strains. Increased expression levels of the *omp13* gene in patients with non-erosive gastritis suggest that this adhesion-related gene may play an important role in the pathogenesis of *H. pylori* infection. Gastric pH or physiological conditions can influence *H. pylori* gene expression. High expression levels of the *omp6* and *omp13* genes in the corpus of patients may have clinical significance. Modulation of *H. pylori* adherence through host-bacterial interactions may allow *H. pylori* to adapt efficiently to changes in the gastric mucosal environment. This can lead to persistent infection and the development of more severe disease. It appears that bacterial virulence genes, in conjunction with host and environmental factors, contribute to the risk of developing gastroduodenal disease. Therefore, microarray analysis of both *H. pylori* and host genetic factors may reveal the mechanisms and roles of these factors in the development of *H. pylori*-related diseases. Real-Time RT-PCR was found to be a sensitive and reliable method that can be used in pathogenesis-related gene expression studies. However, studies with larger sample sizes and more advanced molecular methods should be considered.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors would like to give special thanks to Professor David Y. Graham at Baylor College of Medicine, Houston, and Professor Francis Mégraud at the University of Bordeaux for their critical reading of the manuscript and for providing constructive comments. We thank Genmar Laboratories, Izmir, Turkey, for their kind collaboration and the facilities provided.

Informed Consent

All patients gave written informed consent for participation in this study.

Ethics Statement

This study was approved by the Ethics Committee of the Faculty of Medicine of Dokuz Eylül University (Reference: 148/04.25.2008). In carrying out this study, all regulations regarding ethical principles and standards were observed in accordance with the standards of the relevant ethics committee based on the Declaration of Helsinki.

Funding

This research project was carried out as part of a doctoral thesis and was funded by the Scientific Research Projects Coordination Unit of Dokuz Eylül University with grant number 2008.KB.SAG.037.

Authors' Contribution

The design and construction of the study was carried out by Özlem Yılmaz. Endoscopic diagnosis was made by Hale Akpınar, İlkay Şimşek, and Müjde Soytürk. Histopathological diagnosis was made by Sülen Sarıoğlu. Microbiological and molecular studies were carried out by Daryoush Davoudi Oskouei under the supervision of Özlem Yılmaz. Statistical data analysis was performed by Pembe Keskinoğlu and Hülya Ellidokuz. All authors discussed the results and contributed to the preparation of the final manuscript.

Data Availability

Data are available on request from the Institute of Health Sciences of Dokuz Eylül University of Izmir/Turkey through the link Graduate School of Health Sciences (https://saglikbil.deu.edu.tr/en/).

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