The association between *cagL* and *cagA*, *vacAs/m, babA* genes in patients with gastric cancer, duodenal ulcer, and non-ulcer dyspepsia related to *Helicobacter pylori*

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Abstract

***Introduction* : As a component of the cag T4SS, the cagL gene is involved in the translocation of CagA into host cells and is essential for the formation of cag PAI-associated pili between H. pylori and gastric epithelial cells.**

***Aim* : We aimed to investigate the clinical association of the cagL gene with other virulence factors (VacA, CagA, EPIYA-C, and BabA protein) of H. pylori strains isolated from GC, duodenal ulcer (DU), and non-ulcer dyspepsia (NUD) cases.**

***Methods* : The patient group (PG), including 47 patients (22 GC and 25 DU) and a 25 control group (CG= NUD) were included. Amplification of the H. pylori cagL, cagA, vacA, and babA2 genes and typing of EPIYA motifs were performed by PCR methods.**

***Results* : Sixty-one (84.7%) H. pylori strains were detected with cagL (93.6% in SG, 68% in CG). We detected a significant difference between SG and CG for the presence of cagL (p=0.012) but no statistical comparison was done for (≥2) EPIYA-C repeats In the comparison of H. pylori strains with cagA/vacAs1m1 and cagA/vacAs1m2 and babA2 for the presence of cagL, we could not detect a significant difference (p=1).**

***Conclusion* : We detected a significant difference between groups for the presence of cagL genotype (p=0.012). The vacAs1m1 (OR: 2.829), genotypes increased the GC and DU risk by 2.8 times, while multiple (≥2) EPIYA-C repeats incresed the GC and DU risk by 3.524 times. Gender (to be female) (OR: 0.454) decreased the GC and DU risk by inversly decreased in the multivariate analysis.** (Acta gastroenterol. belg., **2020**, 83, **385-392**).

Key Words: *Helicobacter pylori*, cytotoxin-associated gene A (*cagA*), cytotoxin-associated gene L (*cagL*), vacuolating cytotoxin A (*vacA*).

Introduction

Helicobacter pylori (*H. pylori*) infects approximately half of the world's population and is usually acquired in childhood and persists for the duration of the lifetime. *H. pylori* has various virulence genes in order to survive in the acidic environment of the human stomach. However, these virulence genes not only allow bacteria to survive in this acidic environment but also make this bacterium one of the most well-adapted human pathogens that is capable of sustaining extremely efficient persistent infection (1). In particular, some genotypes of *H. pylori* have been associated with chronic gastritis, peptic ulcer, and gastric cancer (GC). *H. pylori* negatively impacts the balance between cell proliferation and apoptosis, which is important for the development of GC by its virulence factors. Cytotoxin-associated gene A (c*agA*), vacuolating cytotoxin A (*vacA*), and lipopolysaccharide (LPS) are the most important virulence factors of *H. pylori* (2). The Cag pathogenicity island (*cagPAI*) genes encode a type IV secretory system, forming a syringe structure that injects CagA protein into gastric epithelial cells (3). During infection, CagA is localized on the plasma membrane, where it is phosphorylated at specific Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs by host Src and Abl kinases. Four EPIYA motifs have been described as A, B, C, and D, and the biological activity of CagA depends on the number and types of the EPIYA motifs at the C-terminal region (4).

CagA as an oncoprotein may induce hypermethylation of tumor suppressor genes by injecting into host gastric epithelial cells and may trigger some signal transduction events – for example, proliferation and inflammation, induction of pro-inflammatory responses that lead to chronic inflammation of gastric mucosa, and induction of gastric carcinogenesis (5, 6). Translocation of CagA from *H. pylori* to the host cell cytosol is mediated by the Cag T4SS by a contact-dependent secretion system including a pilus and several ATPases that promote T4SS assembly, pilus formation, and/or CagA translocation (7). As a component of the cag T4SS, the *cagL* gene is involved in the translocation of CagA into host cells and is essential for the formation of e cag PAI-associated pili between *H. pylori* and gastric epithelial cells (8). CagL has also a role for the binding of the cag T4SS to the α5β1 integrin receptor on gastric epithelial cells (9). CagL is a pilus protein that interacts with host cellular a5b1 integrins through its arginine-glycine-aspartate (RGD)

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motif. By binding to integrin receptors, CagL may cause various cellular alterations, such as the stimulation of cell spreading, focal adhesion formation, and activation of focal adhesion kinase and the epidermal growth factor receptor (EGFR). These cellular changes are triggered by purified recombinant CagL alone, and this indicates the importance of CagL as a component of T4SS. Also, its activity varies according to the polymorphisms in the RGD motif (10).

The vacuolating cytotoxin A (VacA) induces vacuole formation in eukaryotic cells and affects the release of organic anions and HCO3, promotion of immune tolerance and chronic infection, and modulation of autophagy (11, 12). There are variations in five *vacA* regions: s-region (s1 and s2), i-region (i1, i2, i3), m-region (m1 and m2), d-region (d1 and d2), and the recently identified c-region (c1 and c2) (12,13). The *vacAs2* variant is considered less pathogenic than *vacAs1*. *H. pylori* strains with *vacAs1/m1* genotype were commonly detected in patients with severe inflammation and gastric epithelial damage as well as peptic ulcer disease than in *vacAs2/m2*-positive patients. In Western countries and the Middle East, *vacA s1/m1* is associated with an increased risk of peptic ulcer disease, whereas in East Asia, most of the *H. pylori* strains have the *vacAs1/i1/m1* genotype (14).

The allelic types of the bab gene are *babA1*, *babA2*, and *babB*. The *babA2* gene encodes a blood group antigen binding adhesin (BabA), and it is a major adhesin on the outer bacterial membrane that enables binding of *H. pylori* to the mucosal Lewis b blood group antigens, thus facilitating colonization. The presence of the *babA2* gene increases peptic ulcer disease risk in Western populations (15,16). *H. pylori* strains carrying *cagA*, *vacAs1*, and *babA2* were associated with duodenal ulcer and adenocarcinoma, but *cagA*, *vacAs1/m1*, and *babA2* were found to work synergistically in causing intestinal metaplasia (17,18).

The aim of this study was to investigate the clinical association of the *cagL* gene with other virulence factors (VacA, CagA, EPIYA-C, and BabA protein) of *H. pylori* strains isolated from GC, duodenal ulcer (DU), and non-ulcer dyspepsia (NUD) cases in Turkish patients for the first time.

Material and methods

Study design and patients

This case-control study was conducted between 11 September 2015 and 6 July 2017. The patient group (PG), comprising a total of 47 patients (22 GC and 25 DU patients; 28 males, 19 females; mean age 56.45 years for GC and 43.31 years for DU patients; age range 19–79 years), and a control group (CG), comprising a total of 25 individuals [25 (NUD) patients; 7 male, 18 female; mean age 48.64 years; age range 22-68 years] were enrolled in this study. All PG and CG members had *H. pylori*. The CG was matched with the PG according to the age and gender distribution of the PG ($p > 0.05$). The antrum and corpus biopsy specimens of the PG and CG members by *H. pylori* were used in molecular studies. We excluded patients who were under 18 years old, had previous gastric surgery and *H. pylori* eradication treatment, or had a history of therapy with antibiotics, antisecretory drugs, bismuth salts, or sucralfate in the month prior to sampling. Two biopsies from the antrum and the corpus were collected and transferred immediately in Brucella broth to the laboratory. The study was approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (Ethical approval; Ethical approval No: A-15/2014), and recognized the standards of the Declaration of Helsinki. All patients gave informed consent to participate in the study.

Molecular methods

PCR

H. pylori DNA extractions were performed from the antrum and corpus biopsy specimens. Biopsies were homogenized using a Magna Lyser Homogenizer (Roche Diagnostic, Basel, Switzerland), and deoxyribonucleic acid (DNA) was extracted using the QIAamp DNA Mini Kit (QiagenGmbH, Hilden, Germany) according to the manufacturers' instructions.

ureC gene detection in *H. pylori*

The *H. pylori*-QLS 1.0 kit (Fluorion) was used to detect 156 bp of the ureC gene in *H. pylori* DNA extractions.

Amplification of the *H. pylori cagA, vacA*, and *babA2* genes

The *cagA*, *vacAs1/s2*, *vacAm1/m2*, and *babA2* genotypes determined using a molecular PCR technique by using specific primers. All primer sets used were selected from the published work and are shown in Table 1.

Described primers in other studies were used for the detection of *H. pylori* virulence factors (Table 1). The study protocol was as follows: initial denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 30 s, 45 s at 53 °C, and 45 s at 72 °C. The final longation was performed for 5 min at 72 °C.

Amplification and typing of EPIYA motifs in the cagA 3'variable region

Primers [forward (cagA28F) and reverse (cagAP1C, cagA-P2CG, cagA-P2TA, and cagA-P3E)] were used to amplify DNA for EPIYA-A, -B, -C, and –D, respectively (Table 1).[21] The PCR protocol was as follows: initial denaturation at 95 °C for 2 min, followed by 50 cycles of 30 s at 95 °C, 45 s at 57 °C, and 35 s at 72 °C. The final elongation step was performed for 5 min at 72 °C. After the PCR amplification, PCR products were sequenced bidirectionally using a Sequence Reagent Mix

Table 1. — **PCR primers for amplification of *cagA, vacA, babA2,* EPIYA repeats and *cagL* gene sequences**

Gene	Primer	Primer sequence (5¢-3¢)	Refrences
cagA[a]	Forward Reverse	GAT AAC AGG CAA GCT TTT GAG G CTG CAA AAG ATT GTT TGG CAG A	19
vacAs1/s2[b]	Forward Reverse	ATG GAA ATA CAA CAA ACA CAC CTG CTT GAA TGC GCC AAA C	19 19
vacAm1/m2	Forward Reverse	CAA TCT GTC CAA TCA AGC GAG GCG TCT AAA TAA TTC CAA GG	19 19
babA2[c]	Forward Reverse	AAT CCA AAA AGG AGA AAA AGT ATG AAA TGT TAG TGA TTT CGG TGT AGG ACA	19
cagL	Forward Reverse	AGC CAA TTT TGA AGC GAA TG CAA GCG TCT GTG GAA GCA GTG	20
cagA28F	Forward	TCTCAAAGGAGCAATTGGC	21
cagA28F	Reverse	GTCCTGCTTTCTTTTTATTAACTTKAGC	21
cagA-P1C	Reverse	TTTAGCAACTTGAGCGTAAATGGG	21
cagA-P2TA	Reverse	TTTAGCAACTTGAGTATAAATGGG	21
cagA-P3E	Reverse	ATCAATTGTAGCGTAAATGGG	21

[a]cag : cytotoxin-associated gene. [b]vac : vacuolating cytotoxin gene. [c]bab : blood group antigen-binding adhesin

Table 2. — **Baseline characteristics of patient and control group**

	Endoscopic Findings		
	GC	**DU**	**NUD**
Number of patients	22	25	25
Mean age±SD(min-max)	56.95±12.92 (31-79)		48.64±14.38 (22-68)
Male/Female	15/7	13/12	7/18
***H. pylori* (+) GC**	100%	100%	100%
Cardia cancer (n,%)	3(13.6%)	-	-
İntestinal-type (n,%)	17(77.5%)	-	-
Diffuse-type (n,%)	2(9.1%)	-	-
Duodenal Ulcer	-	25	-
Non-athrophic Gastritis	-	-	25
***H. pylori* virulence factors**			
CagA	22(100%)	25(100%)	19(76%)
vacAs1	18(81.8%)	21(84%)	15(60%)
vacAs2	4(18.2%)	4(16%)	10840%)
vacAm2	8(36.4%)	12(48%)	9(36%)
vacAs1m1	14(63.6%)	13(52.7%)	16(64%)
vacAs1m2	7(31.8)	11(44%)	3(12%)
babA2	11(50%)	10(40%)	12(48%)
multiple (≥2) EPIYA-C repeats	16(72.7%)	10(40%)	9(36%)
cagL	13(59.1%)	7(28%)	1(4%)
total *cagL*	21(95.4%)	23(92%) 61/72 = 61(84.72%)	17(68%)

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia.

kit (DYEnamic ET Terminator Cycle Sequencing kit; GE Healthcare) with an ABI 310 (Applied Biosystems) automatic sequencing machine.

Empty-site PCR

All strains negative for EPIYA PCR were confirmed as being true CagA negative by performing an empty-site-positive PCR assay. To confirm *cagPAI* in all of the strains, amplification was performed with two primers (forward 468 HP519 and reverse 496 HP549) located in the two genes flanking the *cagPAI* in the reference strains (HP519 and HP549) (Table 1, *cag* empty PCR). The PCR protocol was as follows: initial denaturation at 95 °C for 2 min followed by 40 cycles of 30 s at 95 °C, 30 s at 57 °C, and 20 s at 72 °C. The final elongation step was performed for 5 min at 72 °C.

CagL amplification by PCR

CagL DNAs of biopsy samples were amplified by PCR using primers cagL sense (F-5'-AGC CAA TTT TGA AGC GAA TG-3') and *cagL* antisense (R-5'CAA GCG TCT GTG GAA GCA GTG-3') (Table 1). Extracted DNA from the biopsy specimens were mixed as follows: the reaction mixture had a final volume of 25 µL and contained 2 µl $MgCl_2$, 2 µl dNTP's, 2.5 µl 10x PCR Gold Buffer, 1 µl primer-F, 1 µl primer-R, 0.3 µl of Taq recombinant DNA polymerase (Invitrogen, Massachusetts, USA), 11.2 µl sH2O, and 5 µl of DNA. The conditions used were: 1 cycle at 95 °C for 10 min; 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and 1 cycle at 72 °C for 15 min in a thermal cycler (Biorad T100). Each reaction included a positive (DNA from strain 26695) and a negative (DNA was substituted with water) control. All reactions were performed in a Mastercycler Ep gradient thermocycler (Eppendorf, Hamburg, Germany). PCR products were analyzed by agarose gel electrophoresis at 2% and stained with ethidium bromide. Positive samples were visualized under ultraviolet light.

Statistical analyses

The Pearson x^2 test (Table 4, 5a, 5b, and 6) and Fisher's exact test (Table 3) were used to compare the PG and CG for the presence of *cagL*. The risk factor determination of the PG (GC and DU cases) was performed according to the multivariate analysis. All analyses were performed using the SPSS 21.0 (SPSS) package program. The gender, age, *cagL*, *vacAs1m1*, *vacAs1m2*, multiple (≥2) EPIYA-C repeats, and *babA* were included as independent variables in the multivariate analysis using the logistic regression test (Table 7). The odds ratio (OR) was calculated to evaluate the increase in risk of DU or GC. Significance values were defined as P<0.05.

Results

The mean±SD (min-max) age of GC, DU, and NUD cases were 56.95±12.92 (31-79), 43.31±10.95 (19-67), and 48.64±14.38 (22-68), respectively. Sixty-one (84.7%) *H. pylori* strains were detected with *cagL* (93.6% in SG, 68% in CG) out of 72 *H. pylori* strains isolated from the GC, DU, and NUD patients. All *H. pylori* strains isolated from patients were GC *cagL*-positive except one strain, and two and eight strains isolated from DU and NUD patients, respectively, were *cagL*-negative among NUD patients. The baseline characteristics and the frequency of *H. pylori* virulence factors are shown in Table 2.

By comparing the study group (GC+DU) with the control group (NUD), we detected a significant difference between groups for the presence of *cagL* (p=0.012) (Table 3).

When we compared study and control groups with multiple (≥2) EPIYA-C repeats together with *cagA* positivity for the presence of *cagL* positivity, 13 *H. pylori* strains and 7 *H. pylori* strains were positively detected in the GC and DU groups, respectively. Meanwhile, only one strain has this property in the NUD group, and all of

Table 3. — **The comparison of the study(GC+DU) and control (NUD) groups for the presence of cagL**

***cagL* presence**	**Study Group (n:47)**	**Control group (n:25)**	**p value**
cagL +	44(93.6%)	17(68%)	0.012*
cagL -	3(6.4%)	8(32%)	

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia. *Fisher's Exact test.

Table 4. — **The comparison of the study (GC+DU) and control (NUD) groups with *cagA/*(≥2) EPIYA-C repeats for the presence of *cagL***

***cagL* presence**	**Patient group+ (≥2) EPIYA-C repeats (n:20)**	**Control group+ (≥2) EPIYA-C repeats (n:1)**
cagL +	20(100%)	1(100%)
cagL -	0(0%)	0(0%)

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia. No statistics are computed because *cagL* is constant.

Table 5a. — **The comparison of the study(GC+DU) and control (NUD) groups with *cagA/vacAs1m1* for the presence of *cagL***

***cagL* presence**	**Patient group+ *cagA/vacAs1m1* (n:18)**	**Control group+ *cagA/vacAs1m1* (n:2)**	**p value**
cagL +	16(88.9%)	2(100%)	
			1*
cagL -	2(11,1%)	0(0%)	

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia. *Fisher's Exact test

Table 5b. — **The comparison of the study(GC+DU) and control (NUD) groups with *cagA/vacAs1m2* for the presence of *cagL***

***cagL* presence**	**Patient group+ *cagA/vacAs1m2* (n:21)**	**Control group+ *cagA/vacAs1m2* (n:9)**	**p value**
cagL +	20(95.2%)	6(166.7%)	
			0.069*
cagL -	1(4.81%)	3(33.3%)	

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia. *Fisher's Exact test

Table 6. — **The comparison of the study (GC+DU) and control (NUD) groups with multiple *babA2* for the presence of *cagL***

***cagL* presence**	**Patient group+ *cagA/vacAs1m2* (n:26)**	**Control group+ *cagA/vacAs1m2* (n:9)**	**p value**
cagL +	25(96.2%)	8(88.9%)	
			1*
cagL -	1(3.8%)	1(11.1%)	

Abbreviations : GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia. *Fisher's Exact test

Table 7. — **Logistic regressions of variables in patient (GC+DU) group**

Variables in the Equation							
	B	S.E.	p value	Wald	df	Exp (B)	95%C.I
vacA/s1m1	1.040	0.436	0.017	5.701	1	2.829	1.205-6.642
Gender	-0.789	0.332	0.018	5.636	1	0.454	0.237-0.872
(≥2) EPIYA-C repeats	1.260	0.562	0.025	5.021	1	3.524	1.171-10.606
cagL	0.743	0.419	0.076	3.150	1	2.103	0.925-4.779
Constant	1.655	0.702	0.018	5.551	1	5.232	-

Abbreviations: GC, gastric cancer, DU, duodenal ulcer, NUD, non-ulcer dyspepsia.B, beta regression coefficient; Wald, test statistics used for the determination of the meaning of variables; d.f., degrees of freedom; exp(B), exponential.C.I, confidence interval.

these *H. pylori* strains also had *cagL*. Therefore, it was not possible to make a statistical comparison between the study and control groups, because *cagL* is a constant in each strain (Table 4).

The most frequently encountered *vacA* genotype in the GCs is *vacAs1m1*, but *vacAs1m2* was the highest detected *vacA* genotype in this study. Therefore, we used both *vacAs1m1* and *vacAs1m2* genotypes to compare the groups for the presence of *cagL*. In the comparison of *H. pylori* strains with *cagA/vacAs1m1* for the presence of *cagL* between the study and control groups, we could not detect a significant difference (p=1) (Table 5a). On the other hand, when we compared *H. pylori* strains with *cagA/vacAs1m2* for the presence of *cagL* between the study and control groups, we also did not detect a significant difference (p=0.069) (Table 5b).

Infection with *H. pylori* strains “triple-positive” for *cagA*, *vacAs1*, and *babA2* genes significantly correlates to the development of peptic ulcer. In order to evaluate the effect of *babA2* with *cagA* and *vacA*, we created various combinations with *cagA/vacA* plus *babA2*, but the number of cases were decreased, and no statistical measurements were possible. Therefore, we decided to use *babA2* alon for the comparisons of groups for the presence of *cagL*. When we compared *H. pylori* strains with *babA2* for the presence of *cagL* between the study and control groups, we did not detect a significant difference (p=1) between groups (Table 6).

The gender, age, *cagL*, *vacAs1m1*, *vacAs1m2*, multiple (≥2) EPIYA-C repeats, and BabA were included as independent variables in the multivariate analysis using the logistic regression test. The result of *cagL* was not significant, but the p value is very near to the 0.005 cut-off value (0.076). *vacAs1m1* [p= 0.017, OR: 2.829, 95 % CI (1.205-6.642)], gender [p= 0.018, OR: 0.454, 95 % CI (0.237-0.872], and multiple (≥2) EPIYA-C repeats [p =0.025, OR: 3.524, CI (1.171-10.606]. The *vacAs1m1* genotypes increased the GC and DU risk by 2.8 times, while multiple (≥2) EPIYA-C repeats incresed the GC and DU risk by 3.524 times. Gender (to be female) decreased the GC and DU risk by inversly decreased (Table 7).

Discussion

Most *H. pylori*-infected individuals are asymptomatic, but clinical outcomes of *H. pylori* infections may vary from mild gastritis to peptic ulcers as well as gastric malignancy due to the virulence characteristics of infected *H. pylori* strains (22). In this study, we aimed to investigate the clinic relevance of *cagL* positivity with the other virulence factors of *H. Pylori*. We detected 61 (84.7%) *H. pylori* strains with the *cagL* genotype out of a total of 72 *H. pylori* strains isolated from the symptomatic GC, DU, and NUD patients. All of the *H. pylori* strains isolated from patients with GC were of the *cagL* genotype except one strain, and two and eight strains isolated from DU and NUD patients, respectively, were cagL-genonegative among the NUD patients. Meanwhile, in the other studies, a high prevalence (96.7 %) of cagL genotypes were found from 61 *H. pylori* isolates as reported by Yadegar et al., (23) and Yeh et al., (20) reported 98.6 %. In another study, 86.6 % (91/105) of the *H. pylori* strains from GC, peptic ulcer disease (PUD), and NUD patients were reported positive for the *cagL* genotype by Shukla et al., (24) The *cagL* genopositivity was detected as 71.6% in GC and PUD patients infected with *H. pylori* in another study in Iran (25). Moreover, Raei et al., (26) reported 98.1% and 82.9% cagL genepositivity in PUD and GC patients, respectively. As seen in the aforementioned studies, the frequency of cagL genopositivity is very high in virulent *H. pylori* strains, which is in accordance with the present study (88.8%).

When we compared our study group (GC+DU) with the control group (NUD), we detected a significant difference between the groups for the presence of *cagL* genotype (p=0.012). However, Yadegar et al., (23) did not detect any significant difference between their study groups for *cagL* presence, and also, no significant correlation was found between the various genotypes and clinical outcomes in their study. They only found a significant correlation between the presence of *cagL* gene and cagA positivity (p = 0.02), which was obvious as was also in this study. In the present study, all *H. pylori* strains isolated from GC and DU patients were both *cagA* and *cagL* positive except for one strain in the DU group.

Meanwhile, 14 of the 17 of the 19 *cagA*-positive strains of the NUD group were *cagL* positive. This means that there is a very high correlation between *cagA* and *cagL*.

The association between multiple EPIYA-C phosphorylation sites and GC was associated with the number of EPIYA-C sites, and there are numerous reports that have investigated this association in the literature. As already reported, *cagA* with multiple EPIYA-C repeats bound SHP-2 more robustly than *cagA* with a single EPIYA-C (27). Additionally, the ability of *cagA* is enhanced with multiple EPIYA-C phosphorylation sites to induce cellular phenotypic changes. Hence, strains with multiple EPIYA-C sites are associated with GC risk (28). The number of EPIYA-C with multiple repeats was significantly higher for the GC and DU cases than for the CG, and in GC patients, the number of EPIYA-C with multiple repeats was significantly higher than one repeat in a study from Turkey (21). When we compared our study and the CG's *H. pylori* strains with multiple (≥2) EPIYA-C repeats together with CagA positivity for the presence of *cagL* positivity, 13 *H. pylori* strains and 7 *H. pylori* strains were positively detected in the GC and DU groups, respectively. Meanwhile, only one strain had this property in the NUD group, and all of these *H. pylori* strains were also *cagL* genopositive. Therefore, it was not possible to make a statistical comparison between the study and control groups, because *cagL* is a constant in each of the *H. pylori* strains. There was no similiar study in the literature with which to compare our results. On the other hand, it is obvious that multiple (≥2) EPIYA-C repeats together with *cagA* positivity is dominant in GC and DU cases. Therefore, this close association of multiple (≥2) EPIYA-C repeats and GC suggests that *cagL* contributes to this association.

According to epidemiologic studies, GC risk is highest when an individual is infected by *H. pylori* strain producing multiple host-interactive components (type *s1-i1-m1 vacA*, *cagA*, the *cagT4SS*, and certain strain-specific OMPs). *H. pylori* strains that do not produce these components are associated with a lower level of GC risk (12, 29). It was reported that *H. pylori* strains with the *vacAs1* allele were more virulent strains than having the *vacAs2* allele, and *vacAs1* is mostly reported to be related with peptic ulceration, gastric atrophy, and gastric adenocarcinoma (30). Also, it was reported that vacuolating activity is very high in *s1/m1* genotypes, intermediate in *s1/m2* genotypes, and absent in *s2/m2* genotypes (31). In a meta-analysis, it was reported that patients infected with *H. pylori* having *vacAs1* or *m1* has an increased risk of GC in Western populations (OR = 2.50–5.32) (32). Besides that, in a long-term follow-up cohort, *cagA*-positivity together with *vacAs1/m1* was associated 4.8-fold risk of progression of gastric precancerous lesions compared to those infected with *cagA*-negative/*vacAs2/m2* strains (33). In the light of aforementioned studies, we compared *H. pylori* strains with *CagA/vacAs1m1* for the presence of *cagL* genotype between study and control groups, but we could not detect a significant difference (p =1) between the study and control groups. However, the number of *cagL*-genopositive *H. pylori* strains with *cagA/vacA s1m1* in the control group (NUD) was too small to be conclusive. On the other hand, when we compared *H. pylori* strains with *cagA/vacAs1m2* for the presence of *cagL* genotype between the study and control groups, we also did not detect a significant difference (p=0.069). There is also no similiar study with which to compare the groups in this context, and we suggest that large serial studies may exert a significant difference for *cagL* genopositivity. The ratio of cagL genopositivity in the *cagA/vacAs1m1* group is very high in our study group (88.9%); hence, the same ratio for *cagA/vacAs1m2* is also very high (95.2%). Unfortunately, the small positive strains with *vacA* positivity in our control group makes it impossible to draw conclusions. In this study, the frequency of *H. pylori* strains with *vacAs1m1* (n:18) and *vacA s1m2* (n:21) are similiar in our study (GC and DU, respectively) group, but *vacAs1m2* (n.9) were higher than *vacAs1m1* (n:2) in the control group. Yadegar et al., (23) detected a predominance of *s1* and *m2* allelic types in their study groups as similar to Asian populations.

Infection by *babA*-expressing *H. pylori* strains was reported with higher *H. pylori* density and more severe injury in the gastric mucosa of *H. pylori*-infected patients. As is already known, BabA protein encoded by the babA2 gene is a major adhesin on the outer membrane of *H. pylori*, which recognizes Lewis b blood group antigens on the gastric mucosal cells (14). In a case-control study, infection with *H. pylori* strains "triple-positive" for *cagA, vacAs1*, and *babA2* genes was found to be significantly correlated to the development of peptic ulcer ($p < 0.0001$) and adenocarcinoma ($p = 0.014$) (17). In light of the aforementioned studies, we compared our study's *H. pylori* strains with *babA2* for the presence of *cagL* between the study and control groups, and we did not detect a significant difference ($p = 1$) between these groups. The reason behind this may be the small positive number of *H. pylori* strains with *babA2* in the NUD group, but there were no other similiar studies in the literature with which to compare our results. Triple positive *cagA/vacA/babA2* genopositive *H. pylori* strains are associated with PU and GCs in Western populations than Eastern poulations. The reason behind this contraversy may be explained since the incidence of *cagA*- or *babA2*-genopositive *H. pylori* strains is nearly 100% in the Eastern population, and it is not possible to assess the clinical outcomes of *H. pylori* strains with these genotypes in the Eastern population (20). Turkish studies including our recent studies showed that the genotypic characteristics of Turkish *H. pylori* strains have Western-type characteristics, and therefore, we suggest that to evaluate Western-type characteristics in *H. pylori* strains may be helpful to screen virulent *H. pylori* strains related to gastric malignicies in Turkish populations in contrast to Eastern populations (19, 21). We also created a subgroup with *cagA/vacA s1m1/babA2* and *cagA/vacA s1m2/babA2*, but the number of *H. pylori*

strains with the sum of these virulence factors were too small, and it was not possible to make a comparison between groups.

The *vacAs1m1* [p= 0.017, OR: 2.829, 95 % CI (1.205-6.642)] genotype increased the GC and DU risk by 2.8 times, while multiple (≥2) EPIYA-C repeats [p =0.025, OR: 3.524, CI (1.171-10.606] incresed the GC and DU risk by 3.524 times. Gender (to be female) [p= 0.018, OR: 0.454, 95 % CI (0.237-0.872] decreased the GC and DU risk due to the multivariate logistic regression analyses.

The *H. pylori cagT4SS* proteins CagL, CagA, CagY, and CagI interact with integrin α5β1 receptor to inject the CagA into gastric epithelial cells (34). The CagL RGD motif is known to be involved for binding to integrin α5β1 receptor (35). The RGD motif which is located in the fibronectin flexible loop (36). Widemann et al., (37) reported that the RGD motif of CagL is involved in translocatation of CagA and upregulatation of gastrin expression on gastric epithelial cells, resulting in hypergastrinanemia and GC. Consequent host signaling pathways activation by *cagA* is known to stimulate the transcription factor NF-kB and upregulate host cells' proinflammatory responses (25). CagL has an elongated 4-helix bundle that includes α1, α2, α5, and α6 with 2 short perpendicular helices that include α3 and α4 (38). The RGD motif at residues 76 to 78 of CagL is located in the center of the α2 helix. The flexibility of the α2 helix is important for binding to integrin and a flexible hinge region exists between helices α1 and α2 that contains a hypervariable amino acid sequence at residues 58 to 62 (39). Polymorphisms at these amino acid sequences may affect the CagL-binding affinity to the α5β1 integrin and sequence variations of *cagL* correlate with gastroduodenal disorders in different geographic regions (38, 40, 41). Yeh and et al., (20) reported that the *cagL* amino acid polymorphism Y58E59 was present in Taiwanese GC patients but D58K59 and N58 *cagL* polymorphisms were significantly linked with GC in Indian and Mexican patients, respectively (24, 42). As a limitation of the study, we did not evaluate the polymorphisms of the *cagL* gene; therefore, we will investigate polymorphisms of the *cagL* gene in future studies. CagL protein is responsible for *H. pylori*-induced IL-8 expression, which is related to gastric inflammation (43). CagL is also thought as a good vaccine target for being surface-expressed and well conserved among the *H. pylori* strains with T4SS (44, 45). In a CagL-immunized mice, the reduction in gastritis, follicles, and epithelial erosion was obvious, and it was believed that immunity for CagL may be effective in the treatment of *H. pylori*-induced gastric inflammation (46).

In conclusion, we detected a significant difference between groups for the presence of *cagL* genotype (p=0.012), but we could not detect a significant difference when we used the *H. pylori* strains with multiple (≥2) EPIYA-C repeats, *cagA/vacA s1m1*, *cagA/vacA s1m2*, and *babA2* for the presence of cagL genopositivity. The reason for this may be the small number of positive *H. pylori* strains in the control group (NUD). On the other hand, multiple (≥2) EPIYA-C repeats (OR: 3.524) increased the GC and DU risk by 3.524 times. The *vacAs1m1*

(OR: 2.829) genotype increased the GC and DU risk by 2.8 times, while gender (to be female) decreased (OR: 0.454) the GC and DU risk in the multivariate logistic regression analyses.

The number of *H. pylori* isolates included in this study is relatively small. Therefore, future studies with larger sample sizes and *H. pylori* genotypes from different regions of the world will be helpful for further evaluating the relationships between certain genotypes or genotype combinations with disease outcomes.

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Conflict of interests statement

We, all authors declare that to disclose any potential conflict of interest including any financial activities, additional affiliations, personal or other relationships with other people or organizations that could influence, or be perceived to influence, their work, such as employment, consultancies, stock ownership, honoraria, patent applications/registrations, grants or other funding.

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