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Original Article

Association of *Helicobacter pylori*-associated Duodenal Ulcer and Precancerous Findings with Toll-like Receptor-4 Asp299Gly and Toll-like Receptor-9 123T/C Polymorphism and Cag-A, Vac-A in Children

Çocuklarda Helicobacter pylori ile İlişkili Duodenal Ülser ve Prekanseröz Bulguların Toll-like Reseptör-4 Asp299Gly ve Toll-like Reseptör-9 123T/C Polimorfizmi ve Cag-A, Vac-A ile İlişkisi

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ABSTRACT

Objective: We aim to show whether endoscopic, histopathological and precancerous findings in childhood *Helicobacter pylori (H. pylori)* infection are associated with some changes in the host immune system and some virulence factors of the bacteria. For this purpose, we interpreted the changes in endoscopic and histopathological findings of *TLR-4* and *TLR-9* gene polymorphisms in the innate immune system of the host and cytotoxin associated gene A (Cag-A) and vacuolating cytotoxin A (Vac-A) positivity, the main virulence factors of the bacteria.

Method: Between April 2009 and October 2010, 100 *H. pylori*-positive and 100 *H. pylori*-negative cases were cross-sectionally selected by retrospectively reviewing the files of patients admitted to a tertiary hospital with dyspepsia. After obtaining informed consent, blood samples from these patients were analysed for *TLR-4 [Asp 299 Gly (rs4986790)]* and *TLR-9 [1237 TC (rs5743836)]* gene polymorphisms and for the presence of Cag-A and Vac-A in isolates obtained from pathological specimens.

Results: Poor socio-economic conditions were an important risk factor for *H. pylori.* The presence of Cag-A increased the likelihood of duodenal ulcer. There was no significant difference between *TLR-4 [Asp 299 Gly (rs4986790)]* gene polymorphism and endoscopic and histopathological findings. However, TLR-9 [-1237TC (rs5743836)] polymorphism increased precancerous intestinal metaplasia and atrophy.

Conclusion: The presence of Cag-A increases the risk of duodenal ulceration due to *H. pylori* infection. The TLR-9 [-1237TC (rs5743836)] polymorphism is associated with gastric atrophy and intestinal metaplasia in the pathogenesis of *H. pylori* infection. Studies in large groups of patients are needed.

Keywords: *H. pylori*, toll-like receptors, Vac-A, Cag-A

ÖZ

Amaç: Çocukluk çağı *Helicobacter pylori (H. pylori)* enfeksiyonunda endoskopik, histopatolojik ve prekanseröz bulguların konak immün sistemindeki bazı değişiklikler ve bakterinin bazı virülans faktörleri ile ilişkili olup olmadığını göstermeyi amaçladık. Bu amaçla, konağın doğuştan gelen bağışıklık sistemindeki *TLR-4* ve *TLR-9* gen polimorfizmleri ve bakterinin başlıca virülans faktörleri olan sitotoksin ilişkili gen A (Cag-A) ve vakuolleştirici sitotoksin (Vac-A) pozitifliğinin endoskopik ve histopatolojik bulgulardaki değişiklikleri yorumladık.

Yöntem: Nisan 2009 ve Ekim 2010 tarihleri arasında, dispepsi ile üçüncü basamak bir hastaneye başvuran hastaların dosyaları retrospektif olarak incelenerek 100 *H. pylori*-pozitif ve 100 *H. pylori*-negatif olgu kesitsel olarak seçilmiştir. Bilgilendirilmiş onam alındıktan sonra, bu hastalardan alınan kan örnekleri *TLR-4 [Asp 299 Gly (rs4986790)]* ve *TLR-9 [1237 TC (rs5743836)]* gen polimorfizmleri ve patolojik örneklerden elde edilen izolatlarda Cag-A ve Vac-A varlığı açısından analiz edilmiştir.

Bulgular: Kötü sosyo-ekonomik koşullar *H. pylori* için önemli bir risk faktörüydü. Cag-A varlığı duodenal ülser olasılığını artırmıştır. *TLR-4 [Asp 299 Gly (rs4986790)]* gen polimorfizmi ile endoskopik ve histopatolojik bulgular arasında anlamlı bir fark yoktu. Bununla birlikte, TLR-9 [1237TC (rs5743836)] polimorfizmi prekanseröz intestinal metaplazi ve atrofiyi artırmıştır.

Sonuç: Cag-A varlığı *H. pylori* enfeksiyonuna bağlı duodenal ülserasyon riskini artırmaktadır. TLR-9 [-1237TC (rs5743836)] polimorfizmi, *H. pylori* enfeksiyonunun patogenezinde gastrik atrofi ve intestinal metaplazi ile ilişkilidir. Bu konuda geniş hasta gruplarını içeren çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *H. pylori*, toll-like reseptörler, Vac-A, Cag-A

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INTRODUCTION

Helicobacter pylori (H. pylori), a gram-negative *microaerophilic* bacterium classified as a class I carcinogen by the World Health Organisation, infects 70-80% of the population in some developing countries and causes peptic ulcer, gastric cancer, or mucosa-associated lymphoid tissue lymphoma[1,2]. *H. pylori* causes severe mucosal inflammation and inhibits acid secretion of parietal cells, leading to gastric atrophy and hypochlorhydria[3]. This clinical outcome has been attributed to the interaction of several factors, including *H. pylori* virulence factors, genetic susceptibility of the host, local innate and adaptive immune responses, and environmental conditions[4]. It has been suggested that *cytotoxin associated gene A (Cag-A)* and *vacuolating cytotoxin A (Vac-A)* genes, which are among the *H. pylori* virulence factors, mainly play a role in epithelial cell damage and chronic inflammation, which may lead to an increase in the risk of gastric cancer[5,6]. Vac-A toxin iniitated the development of vacuoles in the cell membrane, and the presence of the *Cag-A* gene is associated with the development of ulcers, precancerous and cancerous lesions[7-9].

Toll-like receptors (TLRs), which constitute an important part of the innate immune system of the host, recognize structures that are foreign to the body (such as TLR5 flagella, TLR4 lipopolysaccharide, TLR9 unmethylated CpG oligonucleotides) and activate NF-κB and mitogen-activated protein kinases that trigger a common signaling pathway, enabling the immune system to resist the microorganism by increasing the secretion of various cytotoxins such as interleukin (IL)-1, 6, and 8[10-13]. It has been reported that some mutations in TLR4 and TLR9, which are receptors that play an important role in recognizing microorganisms and activating the immune system, are associated with inadequate host response to *H. pylori* infection[14,15]. Two single-nucleotide polymorphisms (SNPs) in the *TLR4* gene, i.e., D299G and T399I, have been linked to hyporesponsiveness and reduced cytokine production in response to endotoxin challenge. The TLR9-1237 TC (rs5743836) variant allele, which has a cytosine to tyrosine substitution in the proximal promoter region, has been associated with the development of *H. pylori*-induced gastric premalignancy[16,17].

To examine the relationship between *H. pylori* infection and the frequency of precancerous lesions in childhood and whether these lesions are caused by the weapons of the microorganism such as Vac-A, Cag-A or by immunity such as TLR receptor [TLR4 Asp299Gly (rs4986790) and TLR9 1237 TC (rs5743836)]. We tried to investigate whether development of these lesions is related to defects in important components of the system.

MATERIALS and METHODS

Between April 2008 and October 2010, using simple random sampling method 200 children were selected for this cross-sectional retrospective study from patients who applied to Celal Bayar University Medical Faculty Pediatric Gastroenterology Clinic and Underwent Gastric Endoscopies. Patient records were examined retrospectively and according to histopathological examination results, 100 *H. pylori*-positive children were classified as Group 1; hundred *H. pylori*-negative children were included in Group 2 (gastritis group). When the patients came for their routine polyclinic check-ups after their treatment was achieved based on their established diagnoses, written information, and consent forms were given to their parents to be read, and signed by them voluntarily if they approved the conduction of the study. Inclusion criteria in our study were as follows: presence of gastrointestinal (GI) symptoms (nausea, heartburn, abdominal pain, vomiting, etc.) consistent with acute gastritis, age between 5 and 18 years, absence of symptoms consistent with infectious disease, and chronic diseases. Exclusion criteria were as follows: age under 5 years, use of antibiotics, proton pump inhibitors or H_2-receptor blockers within the previous 4 weeks, clinical signs and symptoms consistent with infectious disease (fever, diarrhoea), incomplete clinical or laboratory data, parental/guardian refusal to undergo upper GI endoscopy and/or to sign the informed consent form for the participation of their children in the study.

DNA Isolation

DNA was isolated from 2 cc ethylenediaminetetraacetic acid treated blood samples collected from patients and controls. Vitek DNA Identification Kit (Germany) was used for isolation of DNA.

TLR4Asp299Gly (rs4986790) Polymorphism Genotyping

The desired region in the obtained DNAs was amplified by polymerase chain reaction (PCR). Allele-specific PCR method was used for genotyping. This method uses a common reverse primer to distinguish between mutant and normal sequences and allele-specific oligonucleotides designed to amplify normal and mutant sequences. PCR was performed using forward-

C+reverse and forward-G+reverse primer pairs for each sample. PCR products were evaluated by loading them onto a 2% agarose gel. Primer sequences obtained from Thermo Electron Corporation (Rosemount, Minnesota, USA) were used.

TLR9-1237 TC (rs5743836) Polymorphism Genotyping

The desired region was amplified by PCR in the DNA samples. The following primers used in the PCR products were evaluated by loading on a 2% agarose gel. Then, restriction fragment length polymorphism process was applied to PCR products.

Isolation of *H. pylori* from Histopathology Preparations

DNA isolation was performed in 94 of the gastric biopsy samples obtained using High Pure PCR Template Preparation Kit (Roche Diagnostics Co., Germany). The presence of *H. pylori* and the bacterial virulence markers Vac-A and Cag-A in the extracts were assessed by real-time PCR (Roche Light Cycler® 480 II, Germany). The 16S rRNA of *H. pylori* was isolated. The specific regions of the *Vac-A* and *Cag-A* genes were examined.

All endoscopies of the upper digestive tract were performed by the same trained and experienced gastroenterologist. For the histopathological examination, the Giemsa stain was used to detect *H. pylori*, and all of these examinations were also carried out by the same histopathologist.

Ethical Considerations

All the parents/caregivers signed the informed consent for the participation of their children in the study, which was performed in compliance with the World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. The study was approved by the Ethics Committee of the Manisa Celal Bayar University (approval number: 27, date: 17.03.2012).

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) v.26.0. Categorical data of pediatric patients were given as numbers and percentages. Data related to the age variable were given as mean, standard deviation, median, minimum and maximum. The suitability of the age variable of the patients to the normal distribution was decided by assessing the skewness and kurtosis values, and it was seen that the data fit to the normal distribution. The reference value taken in normal distribution varied between ±1.5. Chi-square test was used to compare the descriptive characteristics, endoscopic, and histopathologic findings, presence of *TLR4* gene and *TLR9* gene polymorphisms, Cag-A and Vac-A (presence of S and M1/M2), findings of *H. pylori*-positive and-negative patients. Independent samples t-test was used to compare the mean age of *H. pylori*-positive and-negative patients. Pearson's correlation test was used to examine the relationships between *H. pylori*-positivity, Cag-A and Vac-A (presence of s and M1/M2), endoscopic and histopathologic findings in pediatric patients. The correlation coefficient was evaluated as a a presence of a low-level correlation between 0.00-0.30, a medium correlation between 0.30-0.70 and a high correlation between 0.70-1.00. In the whole study, the levels of statistical significance were set at p=0.05 and 0.01.

RESULTS

The characteristics of patients in both study groups are summarised in Table 1. There was no difference between Groups 1, and 2 patients in terms of age, gender and symptomatology. Only socioeconomic status of Group 1 patients were comparativly lower (p=0.044) (Table 1).

When we compared the endoscopic and histopathological findings of the patients in Groups 1 and 2, the severity and frequency of gastritis, duodenitis, duodenal ulcer and antral nodularity were statistically significantly higher in Group 1. While there was no significant difference between Groups 1 and 2 in terms of the presence of TLR4 Asp299Gly and TLR-9 1237 TC polymorphisms, especially the Homozygous for allele C (CC), was more frequently identified in Group 1 patients (Table 2).

When we compare the positivity rates for Cag-A, Vac-A-S1/S2 region and Vac-A M1/M2 region in *H. pylori* isolates obtained from patients in Group 1 and the endoscopic and histopathological findings of the patients; no difference was observed in terms of the distribution of gastric ulcer, duodenal ulcer, presence of reflux, antral nodularity, atrophy, lymphoid aggregation, and intestinal metaplasia (p>0.05) (Table 3).

However, a low level positive correlation was detected between the presence of Cag-A protein and duodenal ulcer (p<0.05). These results showed that as the expression rate of Cag-A protein in patients

Table 1. Comparison of descriptive characteristics of pediatric patients in terms of the presence of *H. pylori*						
Descriptive characteristics of patients		***H. pylori*-positive (n=100)**		***H. pylori*-negative (n=100)**		**p-value**
		n	**%**	**n**	**%**	
Gender	Female	64	64.0	68	68.0	0.654
	Male	36	36.0	32	32.0	
Age (years)	5-12	40	40.0	40	40.0	1.000
	13-18	60	60.0	60	60.0	
Socio-economic status	Appropriate	2	2.0	6	6.0	0.044*
	Middle	68	68.0	77	77.0	
	Low	30	30.0	17	17.0	
Residence	Urban	61	61.0	52	52.0	0.389
	Rural	39	39.0	48	48	
Drinking water	Clean water	28	28.0	30	30.0	0.102
	Tap water	72	72.0	70	70.0	
Abdominal pain	Yes	80	80.0	86	86.0	0.347
	No	20	20.0	14	14.0	
Heartburn	Yes	55	55.0	64	64.0	0.249
	No	45	45.0	36	36.0	
Nausea	Yes	61	61.0	54	54.0	0.391
	No	39	39.0	46	46.0	
Vomiting	Yes	32	32.0	30	30.0	0.878
	No	68	68.0	70	70.0	
		Mean ± SD, med. (min.-max.)		Mean ± SD, med. (min.-max.)		
Aget		12.88±2.88 13 (5-17)		12.72±3.24 13 (5-18)		0.712

*p<0.5, **p<0.01, χ^2: Chi-square test (categorical data), ^{t}: Independent samples t-test, *H. pylori: Helicobacter pylori,* min.: Minimum, max: Maximum, med.: Median, SD: Standard deviation

increases, the frequency of duodenal ulcer will also increase (Table 4).

Since in the *H. pylori*-positive group, only 6 patients were heterozygous carriers of the TLR4 Asp299Gly (rs4986790) polymorphism, statistical evaluation could not be made. However, in the AG genotype in the *H. pylori*-positive group (n=6), duodenitis (n=2; 33.3%), duodenal ulcer (n=1; 16.6%), antral nodularity (n=1; 16.6%), moderate glandular atrophy (n=2; 33.3%) and intestinal metaplasia (n=1; 16.6%) were observed in respective number of patients.

Endoscopic parameters did not differ in the presence of *TLR9* gene 123T/C (rs5743836) polymorphisms in Group 1 (p>0.05). However, in Group 1, the distribution of glandular atrophy, and intestinal metaplasia differed according to the presence or absence of *TLR9* gene polymorphisms (p<0.05). The patients with CC polymorphism had higher rates of glandular atrophy and intestinal metaplasia than patients with homozygous for allele T (TT) and TC polymorphisms (Table 5).

The effect of *TLR9* gene polymorphisms on the presence of intestinal metaplasia was found to be significant chi-square: 26.98, p=0.000, p<0.01. While the presence of the TC allele did not make a significant difference. It was determined that rates of intestinal metaplasia increased significantly in the presence of the CC allele (p<0.05).

The probability of intestinal metaplasia in the presence of *CC* gene polymorphism increased by 46.2-fold when compared with *TT* gene polymorphisms (Table 6).

Similarly, the effect of *TLR9* gene polymorphisms on the presence of glandular atrophy was found to be significant Ki-square=25.29, p=0.000, p<0.01. While the presence of the TC allele did not make a significant difference, the presence of the CC allele significantly increased the rates of glandular atrophy (p<0.05). Considering the odds ratios; the probability of atrophy in the presence of *CC* gene polymorphism was 17.8 times higher than in the presence of *TT* gene polymorphism (Table 6).

Table 2. Comparison of endoscopic and histopathological findings in terms of the presence of *H. pylori* in pediatric patients						
Endoscopic and histopathological findings		***H. pylori*-positivity (n=100)**		***H. pylori*-negativity (n=100)**		
		n	**%**	**n**	**%**	**p-value**
Severity of gastritis	Normal-mild	33	33.0	59	59.0	**<0.001****
	Moderate-severe	67	67.0	41	41.0	
Localization	Antrum-corpus	64	64.0	79	79.0	**0.028***
	Antrum	36	36.0	21	21.0	
Gastritis type	Normal	0	0.0	3	3.0	0.432
	Erythematous-erosive	98	98.0	96	96.0	
	Atrophic	2	2.0	1	1.0	
Duodenitis	No	51	51.0	73	73.0	**0.002****
	Yes	49	49.0	27	27.0	
Gastric ulcer	No	93	93.0	93	93.0	1.000
	Yes	7	7.0	7	7.0	
Duodenal ulcer	No	84	84.0	95	95.0	**0.021***
	Yes	16	16.0	5	5.0	
Esophagitis	No	89	89.0	91	91.0	**0.814**
	Yes	11	11.0	9	9.0	
Antral nodularity	No	13	13.0	40	40.0	**<0.001****
	Mild	16	16.0	31	31.0	
	Moderate	40	40.0	19	19.0	
	Severe	31	31.0	10	10.0	
Chronic inflammation	No	0	0.0	58	58.0	**<0.001****
	Mild	24	24.0	39	39.0	
	Moderate	66	66.0	3	3.0	
	Severe	10	10.0	0	0.0	
Activity	No	19	19.0	97	97.0	**<0.001****
	Mild	29	29.0	3	3.0	
	Moderate	52	52.0	0	0.0	
Glandular atrophy	No	83	83.0	99	99.0	**<0.001****
	Mild	6	6.0	1	1.0	
	Moderate	11	11.0	0	0.0	
Lymphoid aggregation	No-mild	46	46.0	98	98.0	**<0.001****
	Moderate-severe	54	54.0	2	2.0	
Intestinal metaplasia	No	86	86.0	100	100.0	**<0.001****
	Yes	14	14.0	0	0.0	
H. pylori density	No	0	0.0	99	99.0	**<0.001****
	Mild	19	19.0	0	0.0	
	Moderate	70	70.0	1	1.0	
	Severe	11	11.0	0	0.0	
TLR4 Asp299Gly (rs4986790) Polymorphism	AA	94	94.0	94	94.0	**1.000**
	AG	6	6.0	6	6.0	
TLR9 123T/C (rs5743836) Polymorphism	TT	43	43.0	34	34.0	**0.005****
	TC	36	36.0	57	57.0	
	CC	21	21.0	9	9.0	

*p<0.05, **p<0.01, χ^2: Chi-square test, *H. pylori: Helicobacter pylori,* TLR: Toll-like receptor, AA: Homozygous for allele A, AG: Heterozygous, TT: Homozygous for allele T, CC: Homozygous for allele C

Table 3. Distribution of endoscopic and histopathological data according to the presence of Cag-A, Vac-A S1/S2 region, Vac-A M1/M2 region in the *H. pylori*-positive Group

Endoscopic and histopathological findings		Yes (n=67)	Cag-A				Vac-A S1/S2 region				Vac-A M1/M2 region				
			No (n=27)				Yes (n=91)		No (n=3)		Yes (n=78)		No (n=16)		
		n	%	Sayı	%	p-value	n	%	Sayı	%	p-value	n	%	n	%	p-value
Gastric ulcer	No	61	91.0	26	96.3	**0.657**	84	92.3	3	100.0	1.000	72	92.3	15	93.8	**1.000**
	Yes	6	9.0	1	3.7		7	7.7	0	0.0		6	7.7	1	6.3	
Duodenal ulcer	No	53	79.1	26	96.3	**0.080**	77	84.6	2	66.7	0.970	67	85.9	12	75.0	**0.478**
	Yes	14	20.9	1	3.7		14	15.4	1	33.3		11	14.1	4	25.0	
Esophagitis	No	60	89.6	26	96.3	**0.515**	83	91.2	3	100.0	1.000	73	93.6	13	81.3	**0.212**
	Yes	7	10.4	1	3.7		8	8.8	0	0.0		5	6.4	3	18.8	
Antral nodularity	No	9	13.4	4	14.8	**0.418**	12	13.2	1	33.3	0.314	11	14.1	2	12.5	**0.717**
	Mild	7	10.4	6	22.2		12	13.2	1	33.3		11	14.1	2	12.5	
	Modarate	30	44.8	8	29.6		38	41.8	0	0.0		33	42.3	5	31.3	
	Severe	21	31.3	9	33.3		29	31.9	1	33.3		23	29.5	7	43.8	
Glandular atrophy	No-mild	53	79.1	24	88.9	**0.300**	74	81.3	3	100.0	1.000	63	80.8	14	87.5	**0.868**
	Moderate-severe	14	20.9	3	11.1		17	18.7	0	0.0		15	19.2	2	12.5	
Lymphoid aggregation	No-mild	30	44.8	12	44.4	**1.000**	41	45.1	1	33.3	1.000	37	47.4	5	31.3	**0.368**
	Moderate-severe	37	55.2	15	55.6		50	54.9	2	66.7		41	52.6	11	68.8	
Intestinal metaplasia	No	56	83.6	24	88.9	**0.739**	77	84.6	3	100.0	1.000	66	84.6	14	87.5	**1.000**
	Yes	11	16.4	3	11.1		14	15.4	0	0.0		12	15.4	2	12.5	
H. pylori density	No-mild	13	19.4	5	18.5	**0.691**	18	19.8	0	0.0	0.501	18	23.1	2	0.0	**0.071**
	Moderate-severe	54	80.6	22	81.5		73	80.2	3	100.0		60	76.9	14	100.0	

*p<0.05, **p<0.01, χ^2: Chi-square test, *H. pylori: Helicobacter pylori, Vac-A: Vacuolating cytotoxin A, Cag-A: Cytotoxin associated gene A*

DISCUSSION

Since *H. pylori* infection is usually acquired during childhood, it can cause chronic inflammation in the gastric mucosa and subsequently increase the risk of stomach cancer[1]. Nearly 50% of the world population is infected with this microorganism, and low socioeconomic status has been found to be one of the main predisposing factors associated with an increased risk of *H. pylori* infection[18-20]. In our study, low socioeconomic level was also a risk factor for contracting *H. pylori* infection (p=0.044). However, we did not find any association between *H. pylori* infection, living in urban/rural areas and choice of drinking water which suggests that living in rural areas is not associated with *H. pylori* infection.

In the pediatric age group, *H. pylori* gastritis is generally asymptomatic[19]. In our study, no statistical difference was found between the two groups in terms of the patients initial complaints (abdominal pain, retrosternal burning sensation and vomiting) (p>0.005).

As is known, *H. pylori* infection is associated with antral nodularity, erythematous and erosive gastritis, duodenal inflammation and ulcer in children, and can also be seen in the presence of mild gastritis or normal endoscopic findings[21,22]. Antral nodularity, which is the most important indicator of *H. pylori* infection in children has been reported at a rates of 69-91% in different countries, and the presence of antral nodularity has a sensitivity of

Table 4. Correlations between Cag-A and Vac-A S and M1/M2 region positivity, and endoscopic and histopathological findings in the *H. pylori*-positive Group

Variable	Coefficient	Cag-A protein positivity	Vac-A S region positivity	Vac-A M1/M2 region positivity
Cag-A protein	r	1	0.018	0.15
	p		0.86	0.148
Vac-A S region positivity	r	0.018	1	-0.082
	p	0.86		0.431
Vac-A M1/M2 region positivity	r	0.15	-0.082	1
	p	0.148	0.431	
Severity of gastritis	r	0.105	0.001	0.104
	p	0.315	0.99	0.32
Localisation	r	0.184	0.137	-0.071
	p	0.0.075	0.189	0.494
Gastritis type	r	0.094	0.027	0.067
	p	0.37	0.798	0.523
Duodenitis	r	0.071	-0.061	-0.057
	p	0.499	0.562	0.588
Gastric ulcer	r	0.091	0.052	0.021
	p	0.386	0.622	0.843
Duodenal ulcer	r	0.212*	-0.086	-0.112
	p	0.04	0.409	0.283
Presence of reflux	r	0.109	0.055	-0.166
	p	0.294	0.596	0.109
Antral nodularity	r	0.057	0.104	-0.072
	p	0.587	0.32	0.493
Chronic inflammation	r	0.202	-0.145	-0.044
	p	0.05	0.162	0.674
Activity	r	0.148	-0.001	-0.099
	p	0.154	0.994	0.342
Atrophy	r	0.089	0.084	0.072
	p	0.395	0.423	0.488
Lymphoid aggregation	r	-0.003	-0.041	-0.122
	p	0.977	0.692	0.24
Intestinal metaplasia	r	0.067	0.076	0.03
	p	0.518	0.467	0.771
H. pylori density	r	0.042	0.025	0.216*
	p	0.686	0.814	0.037

*p<0.05, **p<0.01, r: Correlation coefficient, *H. pylori: Helicobacter pylori, Vac-A: Vacuolating cytotoxin A, Cag-A: Cytotoxin associated gene A*

91.6% and a specificity of 91% for *H.pylori* infection[23-25]. In our study, we think that antral nodularity, with a rate of 87% in Group 1 and 60% in Group 2 detected during endoscopies performed by the same endoscopist, is an important indicator of *H. pylori*-positivity in children (p<0.001).

Studies have revealed that duodenal ulcers develop in 20% of people infected with *H. pylori* and can be asymptomatic even in children under 10 years of age. Incidence of peptic ulcer disease in children vary between 1.8% and 19.5%[26]. According to our results, the incidence rates of duodenal ulcer were 16% in the *H.*

Table 5. Comparison of endoscopic and histopathological findings according to *TLR9* gene 123T/C (rs5743836) polymorphisms in the *H. pylori*-positive group

Endoscopic and histopathological findings		TT (n=43)		TC (n=36)		CC (n=21)		
		n	%	n	%	n	%	p-value
Severity of gastritis	Normal-mild	12	27.9	12	33.3	9	42.9	0.495
	Moderate-severe	31	72.1	24	66.7	12	57.1	
Localization	Antrum-corpus	25	58.1	24	66.7	15	71.4	0.527
	Antrum	18	41.9	12	33.3	6	28.6	
Gastritis type	Normal	41	95.3	36	100.00	21	100.0	0.352
	Erythematous-erosive	2	4.7	0	0.0	0	0.0	
Duodenitis	No	24	55.8	13	36.1	14	66.7	0.059
	Yes	19	44.2	23	63.9	7	33.3	
Gastric ulcer	No	41	95.3	33	91.7	19	90.5	0.680
	Yes	2	4.7	3	8.3	2	9.5	
Duodenal ulcer	No	35	81.4	29	80.6	20	95.2	0.285
	Yes	8	18.6	7	19.4	1	4.8	
Esophagitis	No	36	83.7	35	97.2	18	85.7	0.160
	Yes	7	16.3	1	2.8	3	14.3	
Antral nodularity	No	2	4.7	7	19.4	4	19.0	0.391
	Mild	8	18.6	4	11.1	4	19.0	
	Moderate	17	39.5	16	44.4	7	33.3	
	Severe	16	37.2	9	25.0	6	28.6	
Chronic inflammation	Mild	11	25.6	9	25.0	4	19.0	0.493
	Modarate	29	67.4	21	58.3	16	76.2	
	Severe	3	7.0	6	16.7	1	4.8	
Activity	No	6	14.0	8	22.2	5	23.8	0.500
	Mild	11	25.6	13	36.1	5	23.8	
	Moderate	26	60.5	15	41.7	11	52.4	
Glandular atrophy	No	40	93.0	34	94.4	9	42.9	**p<0.001****
	Mild	0	0.0	1	2.8	5	23.8	
	Moderate	3	7.0	1	2.8	7	33.3	
Lymphoid aggregation	No-mild	21	48.80	19	52.80	6	28.60	0.199
	Moderate-severe	22	51.20	17	47.20	15	71.40	
Intestinal metaplasia	No	42	97.70	34	94.40	10	47.60	**p<0.001****
	Yes	1	2.30	2	5.60	11	52.40	
H. pylori density	Mild	7	16.30	6	16.70	6	28.60	0.413
	Moderate	32	74.40	27	75.00	11	52.40	
	Severe	4	9.30	3	8.30	4	19.00	

*p<0.05, **p<0.01, χ^2: Chi-square test, *H. pylori: Helicobacter pylori,* TLR: Toll-like receptor, TT: Homozygous for allele T, CC: Homozygous for allele C

pylori-positive and 5% in the *H. pylori*-negative group (p=0.021). Duodenal hyperemia and nodularity were observed in 49% of our patients (p = 0.002). In a study, *H. pylori* infection was reported in 92% of children with duodenal ulcer and 25% of children with peptic ulcer[27]. In our study, *H. pylori* was positive in 76.1% of children with duodenal ulcer and 50% of those with stomach ulcer. It is also known that the density of *H. pylori* in the antrum in children with *H. pylori* infection is significantly lower than in adults, and this is one of the reasons why gastric ulcers are less common than duodenal ulcers in children[27].

Table 6. Correlations between *TLR9* gene 123T/C (rs5743836) polymorphisms, glandular atrophy and intestinal metaplasia in *H. pylori*-positive group

Model	Estimated variable	B	S.E.	p-value	Exp(B)/odds ratio	CIs 95% CI for Exp(B)	
						Lower	Upper
Intestinal metaplasia	*TLR9* gene			**0.000**			
	TLR9 (TC)	0.90	1.25	0.468	2.47	0.22	28.42
	TLR9 (CC)	3.83	1.10	**0.001**	46.20	5.33	400.67
	Constant	-3.74	1.01	**0.000**	0.02		
Glandular atrophy	*TLR9* gene			**0.000**			
	TLR9 (TC)	-0.24	0.94	0.797	0.78	0.12	4.97
	TLR9 (CC)	2.88	0.74	**0.000**	17.78	4.14	76.34
	Constant	-2.59	0.60	**0.000**	0.08		

Dependent variable: Intestinal metaplasia and atrophy, Exp(B): Odds ratio, CI: Confidence interval, S.E.: Standard error, TLR: Toll-like receptor, AA: Homozygous for allele A, AG: Heterozygous, CC: Homozygous for allele C

H. pylori infection is the most common cause of chronic superficial gastritis. Atrophic gastritis, intestinal metaplasia and dysplasia, and finally gastric adenocarcinoma may develop after chronic gastritis in cases with *H. pylori* infection. These disease stages evolve very slowly and can stop at any stage[26,27]. Gastric inflammation in children may not demonstrate obvious pathological changes as in adults, or the transition between stages may be very slow. Therefore, different publications report different rates of chronic inflammation, atrophy or intestinal metaplasia in cases with *H. pylori* infection. While moderate to severe chronic inflammation was reported in 65.8-68.2% of these cases, we found its incidence as 76% in our study[28,29].

Although gastric atrophy, also defined as glandular tissue loss, is not as common in children as in adults, it can develop secondary to *H. pylori* infection. Chronic gastritis in adults is often accompanied by intestinal metaplasia, and its incidence increases with the duration of the disease. In studies conducted in different countries, various incidence rates have been reported for gastric atrophy (Tunisia: 9.3%; USA: 52.6%; Japan: 51.7%, and Taiwan: 30.4) and intestinal metaplasia (USA: 15.7%; and Japan: 4.6%)[30-33]. In studies conducted in Turkey, the rates of gastric atrophy and intestinal metaplasia were reported as 2.2% and 1.1% by Usta et al.[34] and 2.5% and 0% by Tutar et al.[35]. In summary, based on our results, consistent with the literature data, in *H. pylori*-positive cases gastric atrophy, and intestinal metaplasia were detected in 17% and 14% of the cases, respectively. Gastric atrophy in children is often found in the antrum or antrum corpus region[36,37]. For this reason, when performing endoscopic biopsies, care was taken to take two biopsy specimens from the antral region in all patients.

The prevalence rates of Cag-A positivity in isolated *H. pylori* strains, and their relationship with GI diseases varied widely in different parts of the world[38]. While the Cag-A positivity rates in Europe and the USA generally vary between 60-70%, almost all *H. pylori* strains in East Asian countries are Cag-A (+)[39,40]. In studies conducted in Asian countries, different prevalence rates of Cag-A in isolated *H. pylori* strains have been reported (India: 96%; China: 86%; Bangladesh: 95%, and Iran: 77%)[41-44]. Ghasemi et al.[45] found a Cag-A positivity rate of 85% in their study performed in Iran and reported that presence of Cag-A positivity was associated with peptic ulcer. Podzorski et al.[46] found that 66% of 61 strains isolated in their study performed in the USA were Cag-A(+). Similarly, Gatti et al.[47] reported that 73.4% of 95 *H. pylori* strains isolated in their study carried out in Brazil were Cag-A (+). The prevalence of Cag-A in Europe shows a profile similar to that reported for the USA. Cag-A positivity rates were reported as 66%, and 68% in studies carried out in Spain and in England, respectively[48,49]. In this study, 67 (71.2%) of 94 *H. pylori* strains demonstrated Cag-A (+). There was a low positive correlation between Cag-A positivity rates and the presence of duodenal ulcer (p=0.04). These results have shown that the rates of duodenal ulcers would increase in parallel with an increse in the levels of Cag-A protein. In various studies conducted in Turkey, Cag-A positivity and prevalence have been reported to vary between 65, and 80%, and it has been suggested that the presence of Cag-A is associated with peptic ulcer and duodenal ulcer[50,51]. There are publications in the literature showing that prepyloric duct ulcers are

more prominent in many single-center pediatric patient groups, especially in cases of duodenal ulcer and severe antral gastritis, and in cases infected with Cag-A positive bacteria[45,50-52].

Although all *H. pylori* strains have the *Vac-A* gene, only 50% of them produce active Vac-A toxin[53]. The *Vac-A* gene contains a signal (s) and a middle (m) region, which show significant sequence diversities among strains. It has been reported that S1/M1 genotypes show greater cytotoxic activity *in vitro* and are more frequently associated with peptic ulcer disease[53]. There are also differences in Vac-A genotypes between countries or regions. In a study conducted on 119 children in Portugal, it was stated that only Vac-A S2 caused less severe inflammation in clinical and histopathological terms[54]. None of the Vac-A genotypes extracted from *H. pylori* strains obtained from 33 Korean children were associated with neutrophil activity or chronic inflammation[55]. In a study conducted in Slovakia, a statistically significant relationship was detected between high bacterial infiltration and chronic inflammation in Vac-A S1-positive samples, but no relationship could be established with precancerous lesions such as antral atrophy and intestinal metaplasia[56]. One limitation of this study is that we were not able to examine both the s- and m-domain subclasses of Vac-A. However, a low positive correlation was found between the presence of Vac-A M1 in the *H. pylori*-positive group ($p<0.05$). It was observed that the density of *H. pylori* in the tissue would increase with the presence of the Vac-A M1/M2 region.

H. pylori infection increases the expressions of TLR 2, 4, 5, and 9 in the gastric mucosa and the number of epithelial cells expressing IL-8, IL-10, and tumor necrosis factor-alpha[57]. Twelve different mutations in TLR4 have been described in the literature. It has been demonstrated that TLR4 Asp299Gly polymorphism disrupts the normal structure of the extracellular domain of TLR4 with the potential of reducing susceptibility to *H. pylori* by weakening the binding affinity of bacterial ligands to the TLR4 receptor A>G. *H. pylori* passes through the extracellular space and causes an exaggerated inflammatory response with serious tissue damage[58-60]. As a result, *H. pylori* colonization accelerates the development of severe inflammation, hypochlorhydria and gastric atrophy[59]. It is stated that the presence of G allele is the responsible risk factor in this process of mutation[60]. However, conflicting results have been reported by researchers in clinical studies. Studies conducted on adult patients do not associate TLR4 Asp299Gly polymorphism with gastritis, duodenal ulcer and stomach cancer[61-65]. Some studies suggest that TLR4 Asp299Gly carriage significantly affects the occurrence of chronic gastritis and peptic ulcer disease, causing atrophy and intestinal metaplasia[66-69]. According to our results, only 6 patients in the *H-pylori*-positive group were heterozygous carriers of the TLR4Asp299Gly (rs4986790) polymorphism and no relationship was found between this mutation and *H. pylori*-positivity. Therefore, statistical evaluation could not be made. Similarly, in a study conducted in pediatric cases, it was found that TLR4 Asp299Gly polymorphism was not associated with *H. pylori*-positivity[70].

Expression of TLR9, an endosomal sensor of unmethylated CpG-rich DNA motifs, increases when the gastric epithelium is infected with *H. pylori*, leading to stimulation of T helper 1 monocytes and increased activation of macrophages[71-73]. In an *in vitro* mouse experiment, TLR 9 expression was found to be 4 times higher in tissues infected with *H. pylori*[74]. TLR91237 TC (rs5743836) polymorphism further worsens this inflammation and predisposes the patient to neoplastic complications with chronic infection in the presence of the C allele[75]. The TLR9-1237 TC (rs5743836) SNP is located in the promoter region. An in silico study found that the C variant allele creates an alternative NF-κB binding site, which may be functionally relevant. Presumably this process increases transcriptional activation by TLR9 and potentially exacerbates the inflammatory reaction by affecting CpG DNA activation of pro-inflammatory cytokines[17]. In addition, functional studies have shown that individuals carrying the C variant allele have significantly higher luciferase activity, by demonstrating modulation of TLR9 transcriptional activity by rs5743836[17].

The TLR9 1237, TC+CC or CC genotype has been associated with a higher risk of gastric cancer than the C genotype [recessive odds ratio (OR) =5.01, 95% confidence interval (CI): 2.52 to 9.94, $p<0.0001$] in chronic gastritis (recessive OR =4.63; 95% CI: 2.44 to 8.79, $p<0.0001$) groups[76]. Another study conducted in an Asian population found no link between *H. pylori* infection and the risk of developing gastric cancer[77]. A study performed in a Mexican population found that the 1237C allele of TLR9 was more commonly detected in patients with metaplasia (19.35%) than in patients with gastritis (15.63%), cancer (15.93%), or duodenal ulcer (12.82%). However, the differences in incidence rates were not statistically significant[78].

CONCLUSION

Based on litearture data the TLR9 1237T/C polymorphism has not been reported in the pediatric age group. According to our results, patients with CC polymorphism had moderate/severe glandular atrophy (57.1%) and intestinal metaplasia (52.4%). The higher rates of glandular atrophy and intestinal metaplasia in patients with the CC polymorphism compared to patients with the TT and TC polymorphisms suggest that the likelihood of cancer in patients carrying this allele increases, especially in developing countries where exposure to *H. pylori* is higher starting from a young age. We attributed the high incidence of intestinal metaplasia and glandular atrophy in children carrying the CC polymorphism to the fact that this study was conducted in only one province of Turkey with a small group of cases. Large case scans and even the identification of the rs5743836 TLR9 minor C allele in different ethnic populations may provide a better identification of the individuals who are more susceptible to critically serious complications of chronic *H. pylori* infection and therefore may require strict endoscopic surveillance more frequently.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of the Manisa Celal Bayar University (approval number: 27, date: 17.03.2012).

Informed Consent: When the patients came for their routine polyclinic check-ups after their treatment was achieved based on their established diagnoses, written information, and consent forms were given to their parents to be read, and signed by them voluntarily if they approved the conduction of the study.

Author Contributions

Surgical and Medical Practices: A.C.T., H.E.K., Concept: H.E.K., Design: H.E.K., Data Collection and Processing: A.C.T., Analysis and Interpretation: A.C.T., H.G., H.O., F.Ö., S.A., Literature Search: A.C.T., Writing: A.C.T.

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