original article

Association of toll-like receptor 4, 5 and 10 polymorphisms with *Helicobacter pylori*-positive peptic ulcer disease in a center in Jordan

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BACKGROUND: *Helicobacter pylori* infection is widespread, affecting about 50% of the global population. Polymorphisms in host genes such as the toll-like receptor 4 (*TLR4*) might affect the susceptibility and severity of infection and treatment success.

OBJECTIVE: Investigate the susceptibility and severity of *H pylori* infection with host *TLR4* (rs11536889, rs4986790, rs200109652, rs10759932), *TLR5* (rs5744174, rs2072493, rs746250566), *TLR10* (rs559182335, rs10004195) polymorphisms.

DESIGN: Analytical, cross-sectional.

SETTING: Endoscopy clinic at tertiary care center.

PATIENTS AND METHODS: Genomic DNA was extracted from formalin-fixed paraffin-embedded tissues collected from *H pylori*-infected patients and healthy individuals. The single nucleotide polymorphisms (SNPs) within the targeted TLR genes were genotyped to assess the genetic association of various SNPs with disease severity.

MAIN OUTCOME MEASURES: Effect of genotype distribution on *H pylori* infection.

SAMPLE SIZE: 250 peptic ulcer patients and 217 controls.

RESULTS: The *TLR10* genotype showed no significant association with *H pylori* infection except for rs10004195 (T>A) (*P*=.002). The genotype frequency of Rs5744174 in *TLR5* had a significant association with the presence of *H pylori* infection (*P*=.046, OR=0.52). Except for gender (P=.022), there were no significant associations between clinical and demographic variables and SNPs relating to the severity of the *H pylori* infections.

CONCLUSIONS: Our findings are consistent with differences in severity of *H pylori* infection due to TLR SNPs in different ethnic groups. Understanding differences in genetic susceptibility could help in classifying patients and matching patients with various treatment options on a genetic basis.

LIMITATIONS: Lack of *H pylori* pathogenicity features assessment.

CONFLICT OF INTEREST: None.

Helicobacter pylori is one of the most common pathogenic bacteria that colonizes the stomach of approximately 50% of the population worldwide.[1-3] *H pylori* infection is a global issue associated with a variety of gastric diseases ranging from chronic gastritis, peptic ulcer disease (PUD), gastric lymphoma, gastric cancer to multiple extraintestinal diseases.[4-11] The risk and severity of *H pylori* infection is influenced by many factors including the host, environmental conditions, strain-specific virulence factors, diet, high salt intake, smoking, host response genes, genetic polymorphisms, and genetic susceptibility.[5,8,12] The epithelial cells of the gastric mucosa are the first line of defense of innate immunity against *H pylori* infection.[13] The pathogen recognition receptors (PRRs), which recognize various bacterial molecular patterns, play a major role in the stimulation of adaptive immunity.[11] The presence of infecting microorganisms such as *H pylori* in the gastrointestinal tract is sensed by toll-like receptors (TLRs) that belong to the PRR family.[15,16] *H pylori* infection is followed by activation of neutrophils and mononuclear cells, which leads to the stimulation of genes involved in the host response. These genes include those encoding antigen-presenting molecules, regulatory cytokines (IL-10), inflammatory cytokines (TNF-α, IL-1, IL-6, and IL-8), and costimulatory molecules that elicit the adaptive immune response.[17,18]

Chronic inflammation can be caused by the long-term presence of *H pylori* colonization of the gastric epithelium.[19] Therefore, an increased immune response, which may eventually lead to an ulcer, can be a precursor of gastric cancer if not controlled.[20] Thus, after weighing the evidence, it is certain that the study of TLR function as an *H pylori* receptor on the surface of gastric epithelial cells is highly important.[16,21] The impaired activity of these receptors could be involved in an impaired response, damage of related-tissue, infectious diseases, and autoimmunity.[14] Previous studies have demonstrated the association of polymorphisms in TLRs genes including TLR1, TLR2, TLR6, and *TLR10* with increased levels of anti-*H pylori* antibodies generated via TLR and associated with the severity of intestinal metaplasia linked to *H pylori* infection.[5,9,22] Therefore, the objective of this study was to examine the association of certain TLR gene polymorphisms with the presence of *H pylori* infection (susceptibility and severity) in the Jordanian population.

METHODS

Patients recruitment and selection

Participants were all Jordanians older than 18 years who visited the endoscopy clinic for a gastroscopic examination at King Abdullah University Hospital (KAUH), Ramtha, Jordan. A total of 640 formalin-fixed, paraffin-embedded (FFPE) gastric tissue biopsies were collected by the cytology laboratory at KAUH between 2017 and 2018. Of the 468 samples that fulfilled the inclusion criteria, 251 were *H pylori* positive patients with PUD; 217 *H pylori* negative patients were used as control subjects (**Figure 1**). Demographic data was collected based on a structured questionnaire, while the clinical history was collected from medical records. Patients were eligible for inclusion in the study if they were Jordanian and had a confirmed *H pylori* infection using gastric antrum biopsy samples by a specialized physician following gastroscopic examination. Eligible patients had no history of gastric surgery. Patients were classified according to the Updated Sydney Classification on *H pylori* chronic gastritis into two groups (active/acute and inactive/chronic) based on the presence of mononuclear cells in the histopathological analysis.[23] Moreover, the severity of *H pylori* infection was categorized based on the number of mononuclear cells into three categories (mild, moderate, and severe).[23] Ethical approval was obtained from the Institutional Review Board (IRB) committee at the Jordan University of Science and Technology (no. 15/105/2017).

DNA analysis

The genomic DNA was extracted from FFPE tissue collected from the patients who visited the endoscopy clinics using the commercially available kit, DNeasy Blood & Tissue Kit (Qiagen Ltd., West Sussex, UK). The concentration and purity of extracted DNA was assessed using a NanoDrop 1000 spectrophotometer. The pure DNA samples with their concentrations were sent to the Australian Genome Research Facility (AGRF, Melbourne Node, Melbourne, Australia) for genotyping of *TLR4* (rs11536889, rs4986790, rs200109652, and rs10759932), *TLR5* (rs5744174, rs2072493, and rs746250566), and *TLR10* (rs559182335, and rs10004195) using the Sequenom MassARRAY system (iPLEX GOLD) (Sequenom, San Diego, CA, United States).

Statistical analysis

Genotypic, allelic, and clinical data association was performed using IBM SPSS version 25.0 (IBM Corp. Armonk, NY). The distribution of the studied single nucleotide polymorphisms (SNPs) in patients and controls and the association of these polymorphisms with the severity and activity of *H pylori* infection was tested using

the chi-square test. The Hardy-Weinberg equilibrium (HWE) values for genotype distribution and minor allele frequency (MAF) was calculated using the SNPStats Web Tool (*https://www.snpstats.net/start.htm*).

RESULTS

The 468 DNA samples consisted of 217 controls and 250 PUD patients. The median (interquartile range) age of patients and controls were 41 (23.8) and 53 (30), respectively. Overall, there were 98 females (45.2%) and 119 males (54.8%).

About half of the *H pylori*-positive patients (45.2%) had moderate *H pylori* infection, 26.7% had mild infection, 17.1% had severe infection, and 10.7% had inactive *H pylori* infection (**Table 1**). Other clinical and demographic characteristics did not differ except for infection activity between males and females (*P*=.022).

Five SNPs were in Hardy-Weinberg equilibrium and normally distributed (rs11536889, rs4986790, rs2072493, and rs746250566), while the other three SNPs (rs10759932, rs5744174, and rs10004195) were not in HWE (*P*<.05) (**Table 2**). The presence of selection, mutation, migration, and genetic drift mechanisms will affect allele frequencies, indicating that evolution has occurred in a population; this is reflected by violations of HWE assumptions. The last two SNPs, rs200109652 and rs559182335, are monomorphic SNPs.

For the *TLR4* gene, there were no significant differences in the distributions of the polymorphisms between the patients and controls (**Table 3**). For the *TLR10* gene, allele T has emerged as a risk factor for PUD, with the homozygous TT and heterozygous AT genotypes of the rs10004195, which were significantly abundant in the patients over the A allele in all genetic models (*P*=.019, .063, .0056, and .002). If PUD patients carry the AA genotype they have a decreased risk of PUD (OR= 0.34 and *P*=.019). Moreover, the rs5744174/*TLR5* heterozygous GA genotype seems to increase the risk of PUD development. The genotype distribution of the variants based on the activity and severity of *H pylori* infection lack any statistically significant associations (**Tables 4 and 5**, respectively).

DISCUSSION

H pylori infection is worldwide spread, especially in developing countries.[11,24] Once acquired, the infection

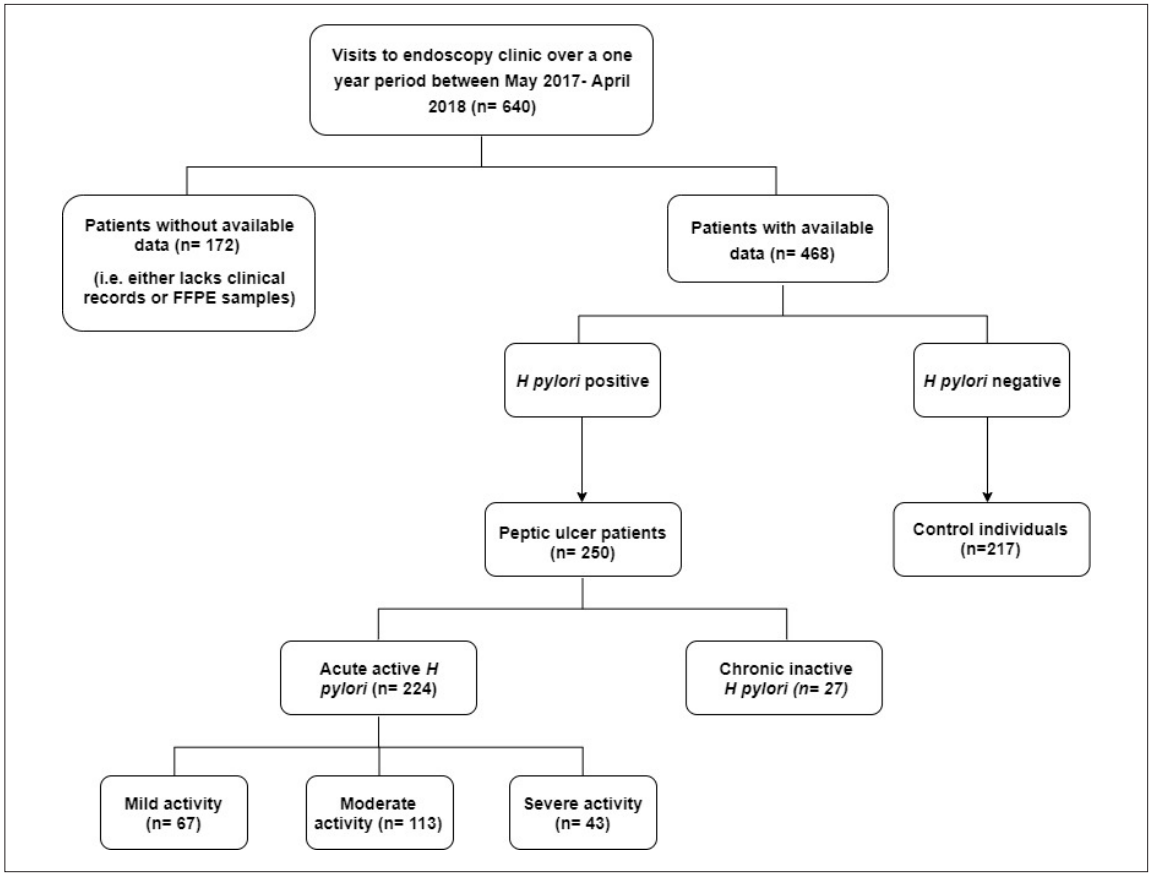

Figure 1. Flow chart of the selection and distribution of the participants. FFPE: formalin-fixed, paraffin-embedded

Table 1. Clinical and demographic characteristics of Jordanian patients with peptic ulcer disease (n=250).

Clinical Data	Infection activity				*P* value
	Mild	Moderate	Severe	Inactive infection	
Total	67 (26.7)	113 (45.0)	43 (17.1)	27 (10.7)	
Age (years)	42 (25.5)	39 (18)	47 (28.5)	34 (27.5)	.301
Marital status					
Single	19 (7.6)	32 (13.2)	6 (2.4)	4 (1.6)	
Married	48 (19.2)	79 (31.9)	36 (14.4)	17 (6.8)	.16
Divorced	0	1 (0.4)	0	0	
Widowed	0	0	1 (0.4)	0	
Gender					
Male	33 (13.2)	57 (22.8)	13 (5.2)	7 (2.8)	.022
Female	34 (13.6)	56 (22.4)	30 (12.0)	20 (8.0)	
Other diseases					
No	36 (14.4)	73 (29.2)	2 (8.4)	14 (5.6)	.23
Yes	31 (12.4)	40 (16.0)	22 (8.8)	13 (5.2)	
Duodenal ulcer					
No	58 (23.2)	94 (37.6)	37 (14.8)	25 (10.0)	.67
Yes	9 (3.6)	19 (7.6)	6 (2.4)	2 (0.8)	
Esophagitis					
No	55 (22.0)	90 (36.0)	37 (14.8)	22 (8.8)	.85
Yes	12 (4.8)	23 (9.2)	6 (2.4)	5 (2.0)	
Epigastric pain					
Yes	28 (11.2)	53 (21.2)	17 (6.8)	15 (6.0)	.49
No	39 (15.6)	60 (24.0)	26 (10.4)	12 (4.8)	
Chronicity					
Yes	67 (26.2)	113 (45.2)	43 (17.2)	25 (10.0)	.16
No	0	0	0	2 (0.8)	
Proton-pump inhibitors					
No	0	5 (2.0)	1 (0.4)	2 (0.8)	
Lansoprazole	50 (20.0)	80 (32.0)	31 (12.4)	21 (8.4)	
Esomeprazole	2 (0.8)	0	0	1 (0.4)	.21
Omeprazole	1 (0.7)	1 (0.4)	0	0	
Two or three	14 (0.6)	27 (10.8)	11 (4.4)	3 (1.2)	
Antibiotics					
No	17 (7.6)	26 (10.4)	9 (3.6)	6 (2.4)	
Amoxicillin	3 (1.2)	2 (0.8)	1 (0.4)	1 (0.4)	
Clarithromycin	1 (0.4)	3 (1.2)	1 (0.4)	0	.81
Amox+Clarith	44 (17.6)	80 (32.0)	30 (12.0)	8 (7.2)	
Others	0	2 (0.8)	2 (0.8)	2 (0.8)	

Numbers are n (%), median (interquartile range) for age. Totals may not add to 100% because of missing data on some variables.

persists for life if not treated.[25] To the best of the authors' knowledge, there are a limited number of studies on the association between TLR polymorphisms and PUD in countries of the Middle East, especially in the Jordanian population. The study explores possible interactions of certain genetic polymorphisms in *TLR4*, *TLR5*, and *TLR10* with the risk of *H pylori* infection, in addition to the severity of the disease.

TLR4 was the first TLR identified in humans associated with the recognition of bacterial lipopolysaccharides (LPS, an essential component of the gram-negative bacteria outer membrane).[14] Several studies indicated that TLR variants might decrease the responsiveness to the gram-negative bacteria LPS due to alterations in the binding site, and thus influence the clinical outcome of *H pylori*.[9,26] This could provide a plausible mechanistic explanation for different outcomes in *H pylori* -positive individuals.[16] The results of this study revealed no association with susceptibility to *H pylori* infection (The degree to which individuals are liable to

Table 2. Minor allele frequencies and the Hardy-Weinberg equilibrium *P* values of candidate polymorphisms for patients and controls.

Gene	Chromosomal location	rs numbers	Minor allele	Patients (n=223)		Controls (n=217)	
				Minor allele frequency	HWE *P* value	Minor allele frequency	HWE *P* value
TLR4	9q33.1	rs11536889	C	.1	.48	.09	.061
		rs4986790	G	.01	.999	.02	.999
		rs200109652	C	1	NA	1	NA
		rs10759932	C	.16	.0005	.16	.02
TLR5	1q41	rs5744174	G	.38	<.0001	.4	.011
		rs2072493	C	.1	.61	.08	.999
		rs746250566	A	0	.999	1	.999
TLR10	4p14	rs559182335	T	1	NA	1	NA
		rs10004195	A	.07	<.0001	.15	<.0001

HWE: Hardy-Weinberg equilibrium

Table 3. Distribution of the genotypes and alleles among cases and controls.

Gene/SNP/Alleles	Patients (n=223)	Controls (n=217)	Odds ratio (95% CI)	*P* value
TLR4 (rs11536889)				
GG/GC/CC	79.8/19.8/0.4	84.2/13.9/1.9	1/1.5 /.23	.083
GG/GC + CC	79.8/20.2	84.2/15.8	1/1.35	.23
GG + GC/CC	99.6/0.4	98.1/1.9	1 0.21	.12
GG + CC/GC	80.2/19.8	86.1/ 13.9	1/1.53	.096
C	10	9	0.874	.516
G	90	91	1.014	
TLR4 (rs4986790)				
AA/AG	98.3/1.7	96.4/3.4	1/0.5	.26
A	99	98	0.992	.264
G	1	2	1.973	
TLR4 (rs20010965)				
CC	100	100	NA	NA
C	100	100	NA	NA

Table 3 (cont.). Distribution of the genotypes and alleles among cases and controls.

Gene/SNP/Alleles	Patients (n=223)	Controls (n=217)	Odds ratio (95% CI)	*P* value
TLR4 (rs10759932)				
TT/ TC/CC	76.1/18.6/5.3	73.6/21.2/5.2	1/0.85/0.99	.79
TT/TC + CC	76.1/23.9	73.6/26.4	1/0.87	.55
TT + TC/CC	94.7/5.3	94.8/5.2	1/1.03	.95
TT + CC /TC	81.4/18.6	78.8/21.2	1/0.85	.5
C	15	16	1.082	.629
T	85	84	0.986	
TLR5 (rs5744174)				
GG/ GA/AA	30.5/63.2/6.3	32.9 /54.1/12.9	1/1.26/0.52	**.046**
GG /GA + AA	30.5/69.5	32.9/67.1	1/1.12	.61
GG + GA/AA	93.7/6.3	87.1/12.9	1/0.45	**.024**
GG + AA/GA	36.8/63.2	45.9/54.1	1/1.46	.069
A	62	60	0.969	.582
G	38	40	1.051	
TLR5 (rs2072493)				
TT/TC/CC	81.1/18/0.9	84.3/15.7/0	1/1.19/NA	.28
TT/TC + CC	81.1/18.9	84.3/15.7	1/ 1.25	.39
TT + TC/CC	99.1/0.9	100/0.0	1/NA	.11
TT + CC/TC	82/18	84.3/15.7	1/1.18	.53
C	10	8	0.795	.302
T	90	92	1.023	
TLR5 (rs74625056)				
GG/GA	100/0.0	99.5/0.5	1/0.0	.21
A	0	0.5	NA	.28
G	100	99.5	0.998	
TLR10 (rs55918233)				
TT	100	100	NA	NA
T	100	100	NA	NA
TLR10 (rs10004195)				
TT/AT/AA	90.5/4.7/4.7	84/3.1/13	1/1.42/0.34	**.019**
TT/AT + AA	90.5/9.5	83.9/16.1	1/0.55	.063
TT + AT/AA	95.3/4.7	87/13	1/0.33	**.0056**
TT + AA/AT	95.3/4.7	96.9/3.1	1/1.56	.43
A	7	15	2.0242	**.002**
T	93	85	0.92	

Table 4. The effect of genotype distribution of single nucleotide polymorphisms on the activity of infection in patients.

Gene/SNP/Alleles	Activity		Odds ratio (95% CI)	*P* value
	Yes (n=223)	No (n=27)		
TLR4 (rs11536889)				
GG/GC/CC	79.9/19.6/0.5	85.2/14.8/0	1/1.41/NA	.73
GG/GC + CC	79.9/20.1	85.2/14.8	1/1.45	.5
GG + GC/CC	99.5/.5	100/0	1/NA	.63
GG + CC/GC	80.4/19.6	85.2/14.8	1/1.4	.54
TLR4 (rs4986790)				
AA/AG	98.1/1.9	100/0	1/NA	.3
TLR 4 (rs200109652)				
CC	100	100	NA	NA
TLR4 (rs10759932)				
TT/TC/CC	76.5/18/5.5	72/24/4	1/.71/1.29	.76
TT/TC + CC	76.5/23.5	72/28	1/.79	.63
TT + TC/CC	94.5/5.5	96/4	1/1.4	.74
TT + CC/TC	82/18	76/24	1/.7	.48
TLR5 (rs5744174)				
GG/GA/AA	30.5/62.4/7.1	25/75 /0	1/.68/NA	.14
GG/GA + AA	30.5/69.5	25/75	1/.79	.58
GG + GA/AA	92.9/7.1	NA	1/NA	.068
GG + AA/GA	37.6/62.4	25/75	1/.55	.21
TLR5 (rs2072493)				
TT/TC/CC	80 /18.3/1	84/16/0	1/1.19/NA	.75
TT/TC + CC	80.7/19.3	84/16	1/1.25	.669
TT + TC/CC	99/1	100/0	1/NA	.48
TT + CC/TC	81.7/18.3	84/16	1/1.17	.79
TLR5 (rs746250566)				
GG	100	100	NA	NA
TLR10 (rs559182335)				
TT	100	100	NA	NA
TLR10 (rs10004195)				
TT/AT/AA	91.5/4.8/3.6	81.8/4.5/13.6	1/.94/.23	.2
TT/AT + AA	91.8/8.3	81.8/18.2	1/0.41	.18
TT + AT/AA	96.4/3.6	86.4/13.6	1/0.23	.075
TT + AA/AT	95.2/4.7	95.5/4.5	1/1.05	.96

Table 5. The effect of genotype distribution of single nucleotide polymorphisms on the severity of *H pylori* infection in patients.

Gene/SNP/Alleles	Severity			*P* value
	Mild (n=67)	Moderate (n=113)	Severe (n=43)	
TLR4 (rs11536889)				
GG	54 (25.2)	83 (38.8)	34 (15.9)	
GC	7 (3.3)	29 (13.6)	6 (2.8)	.053
CC	1 (0.5)	0 (0)	0 (0)	
G	115 (26.9)	195 (45.6)	80 (18.7)	.001
C	9 (2.1)	29 (12.9)	0 (0)	
TLR4 (rs4986790)				
AG	1 (0.5)	1 (0.5)	2 (1.0)	.31
GG	58 (28.2)	107 (51.9)	37 (18)	
G	1 (0.2)	1 (0.2)	2 (0.4)	.31
A	117 (28.4)	215 (52.2)	76 (18.4)	
TLR4 (rs200109652)				
CC	67 (30.0)	113 (50.7)	43 (19.3)	NA
C	134 (30.0)	226 (50.7)	86 (19.3)	NA
TLR4 (rs10759932)				
TT	41 (20.5)	84 (42.0)	28 (14)	
TC	13 (6.5)	16 (8.0)	7 (3.5)	.63
CC	4 (2.0)	4 (2.0)	3 (1.5)	
T	95 (23.8)	184 (46.0)	63 (2.0)	.22
C	21 (5.3)	24 (6.0)	13 (3.3)	
TLR5 (rs5744174)				
GG	3 (1.5)	6 (3.0)	5 (2.5)	
AG	43 (21.8)	58 (29.4)	22 (11.2)	.09
AA	12 (6.1)	38 (19.3)	10 (5.1)	
A	67 (17.0)	134 (34.0)	42 (10.7)	.24
G	49 (12.4)	70 (17.8)	32 (8.1)	
TLR5 (rs2072493)				
TT	46 (23.4)	86 (43.7)	27 (13.7)	.07
TC	9 (4.6)	14 (7.1)	13 (6.6)	
CC	0 (0)	2 (1.0)	0 (0)	
T	101 (25.6)	186 (47.2)	67 (17)	.13
C	9 (2.3)	18 (4.6)	13 (3.3)	

Table 5 (cont.). The effect of genotype distribution of single nucleotide polymorphisms on the severity of *H pylori* infection in patients.

Gene/SNP/Alleles	Severity			*P* value*
	Mild (n=67)	Moderate (n=113)	Severe (n=43)	
TLR5 (rs746250566)				
GG	67 (30.0)	113 (50.7)	43 (19.3)	NA
G	134 (30.0)	226 (50.7)	86 (19.3)	NA
TLR5 (rs559182335)				
TT	65 (26.3)	113 (45.7)	42 (17.0)	NA
T	130 (26.3)	226 (45.7)	84 (19.1)	NA
TLR10 (rs10004195)				
TT	49 (29.2)	78 (46.4)	27 (16.1)	
AT	1 (0.6)	7 (4.2)	2 (1.2)	.062
AA	1 (0.6)	2 (1.2)	3 (1.5)	
T	99 (29.5)	163 (48.5)	56 (16.1)	.18
A	3 (0.9)	11 (3.3)	6 (1.8)	

be harmed by *H pylori*) with the *TLR4* polymorphisms. In agreement with Meliț et al,[27] we found no association between *TLR4* variants (rs4986790 and rs4986791) and *H pylori* infection, which was inconsistent with many previous studies. Additionally, a northern Indian study illustrated that *TLR4* rs4986791 is associated with a higher risk for plasma cell infiltration, which leads to atrophy and intestinal metaplasia.[28] The rs4986790 GG genotype may functionally reduce the *TLR4* binding affinity to *H pylori* LPS, resulting in a weakened adaptive immune response compared to the wild *TLR4* type, which may vary by population.[18]

The *TLR10* showed considerable association in the rs10004195 (T>A) variant. The A allele frequency in the patients (7%) is around half of controls (15%). As a result, the A allele could have protective characteristics against the *H pylori* infection. The heterodimers formed from *TLR10* that are expressed on the surface of gastric epithelial cells with TLR2 and/or TLR6 recognize multiple distinct patterns of *H pylori* LPS.[18] *TLR10* (rs10004195) is linked with infection susceptibility, where the frequency of the TT genotype is reported to be 66% in *H pylori*-positive cases from a Chinese population.[29] Therefore, the T allele of *TLR10* rs10004195 polymorphism showed an increased risk of chronic atrophic gastritis.

Significant linkage of the *TLR5*/rs5744174 implies that the AA genotype could decrease the risk of PUD development due to *H pylori* infection, a finding consistent with a case-control study that examined *TLR5* variants with *H pylori* infection in Chinese patients who have gastric cancer. Among the studied variants, rs5744174 (*P*=.001) is associated with gastric cancer susceptibility, indicating that *TLR5* variants can impact the role of *H pylori* infection in gastric cancer formation.[30] On the other hand, rs5744174 was not a risk factor for chronic *H pylori* in the population of Indian Tamils, where rs2072493 conferred resistance to the infection.[31]

In summary, different host cytokine responses to the gastric mucosal inflammation induced by *H pylori* appear to produce a significant role in the clinical outcome, such as the development of gastric diseases and gastric cancer.[9,32,33] However, the correlation between polymorphisms of host cytokine genes and the susceptibility to *H pylori* infection and severity of the clinical outcome has not been investigated thoroughly.[25,32] Genetic heterogeneity as a result of ethnic diversity is a vital factor in the variation of allele frequency for several markers; hence, genetic heterogeneity affects the susceptibility and severity of the infection.[34,35]

Ultimately, rs10004195/*TLR10* and rs5744174/*TLR5* variants appear to be genetic risk factors for *H pylori* infection and severity in the Jordanian population. Our findings further support the evidence of TLR polymorphisms as physiopathological actors in susceptibility to *H pylori* infection and related gastric problems. This type of genetic susceptibility could help in classifying patients based on their genetic profile and matching patients with various treatment options on a genetic basis.

Author contributions

LNA-E, FAA designed the study. LNA-E, FAA AND SMA-K were responsible for sample, demographic and clinical data collection. LNA-E, FAA, SMA-K, RAA and MAA analyzed the sample and interpreted the data. LNA-E prepared the manuscript. All authors helped in reviewing the manuscript.

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