

Toll-Like Receptors are Associated with *Helicobacter pylori* Infection and Gastric Mucosa Pathology

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Abstract

Background: Toll-like receptors (TLRs) mediate recognition of *Helicobacter pylori* and initiate the innate immune response to infection. Toll-like receptors polymorphisms are thought to influence susceptibility to *H. pylori* infection and *H. pylori*-related diseases.

Objectives: To investigate the association of TLR polymorphisms with the susceptibility to *H. pylori* infection and various types of gastritis in Thai patients.

Methods: The single nucleotide polymorphisms (SNPs) TLR1 rs4833095, TLR2 rs3804099 and rs3804100, TLR4 rs10759932 and TLR10 rs10004195 were analysed in 400 gastritis patients by real-time PCR using a TaqMan SNP Genotyping Assay. The association between TLR polymorphisms and the risk of *H. pylori* infection was investigated in 196 uninfected and 204 *H. pylori*-infected patients. The associations between TLR genotypes and the risk of gastric pathology changes, including chronic gastritis (N = 312 cases) and precancerous gastric lesions/gastric cancer (GC) (N = 88 cases), were examined. Univariate analysis was used to determine odds ratios (ORs) with confidence intervals (95% CIs).

Results: Patients with the CC-or TT homozygous genotype for TLR1 rs4833095 had a significantly increased risk of *H. pylori* susceptibility (OR = 2.28, 95% CI = 1.27 - 7.60, P = 0.02 and OR = 1.34, 95% CI = 0.97 - 1.86, P = 0.04, respectively). Patients with the AA homozygous genotype for TLR10 rs10004195 had a significantly increased risk of *H. pylori* susceptibility (OR = 1.28, 95% CI = 0.98 - 1.76, P = 0.01) and exhibited a significantly increased risk of precancerous gastric lesions/GC (OR = 8.75, 95% CI = 2.78 - 14.24, P < 0.01).

Conclusions: Our findings suggest that the TLR1 rs4833095 and TLR10 rs10004195 polymorphisms are potential genetic risk factors that modify the *H. pylori* infection susceptibility and influence the clinical manifestation of chronic gastritis, precancerous gastric lesions and GC in Thai patients.

Keywords: Toll-Like Receptors, TLR polymorphisms, Gastritis, Gastric Cancer, *Helicobacter pylori*

1. Background

Helicobacter pylori infection of the gastric mucosa induces acute inflammation that progresses over time to chronic inflammation, gastric atrophy, intestinal metaplasia, dysplasia and intestinal-type gastric cancer (GC) in a subset of patients (1). Several epidemiological studies have detected an association between *H. pylori* infection and the risk of developing GC. The data are consistent among Caucasians, African-Americans and Asians (2-4). The northeast region of Thailand has the highest rate of *H. pylori* infection, although the north has the highest incidence and the south has the lowest incidence of GC (5). *H. pylori* infection is associated with a spectrum of gastric diseases, including peptic ulcer diseases (10%), gastric adenocarcinoma (1% - 3%) and mucosa-associated lymphoid tissue (MALT) lymphoma (< 0.1%) (6, 7). Moreover, *H. pylori* infection is associated with a risk of developing gastric lesions, including gastric atrophy and epithelial metaplasia (8).

In the study of the relationship of Toll-like receptors polymorphisms and GC, the TLR1 I602S variant is the most prevalent functionally relevant SNP in Europeans, with a G allele frequency of 75%; however, the G allele frequency in Africans and Asians is 25% and 1%, respectively (9). There is a strong association between TLR2 polymorphisms and susceptibility to GC, with an increased risk of GC in a Japanese population (10). TLR4, a lipopolysaccharide (LPS) receptor, plays a crucial role in gastric epithelial cell signalling in response to *H. pylori* virulence factors. It is known to enhance inflammatory reactions and contribute to gastric inflammation and carcinogenesis (11-13).

The TLR1/TLR10 gene clusters are located in the same region, at a locus on chromosome 4p14. TLR1 is linked to *H. pylori*-associated gastric lesions (14, 15). In a Chinese population, the association between SNPs in the promoter regions of TLR1 rs4833095 and TLR10 rs10004195 and susceptibility to *H. pylori* infection and precancerous gastric lesions or GC was studied (16-18). TLR1 rs4833095 and TLR10

rs10004195 were associated with *H. pylori* infection, increasing the susceptibility to gastroduodenal disease, especially GC, in Malaysian patients (19). Nevertheless, there has been little research on the association between the stage of gastritis and the TLR polymorphisms that contribute to the development of GC among various racial populations.

2. Objectives

The role of TLR polymorphisms in the *H. pylori*-related pathologic process has not been established in the Thai population, especially those living in the northeast region of Thailand, which has the highest rate of *H. pylori* infection. Therefore, we hypothesized that the genetic polymorphisms TLR1 *rs4833095*, TLR2 *rs3804099* and *rs3804100*, TLR4 *rs10759932* and TLR10 *rs1000419* may influence *H. pylori* susceptibility and *H. pylori*-related GC development in patients in Northeast Thailand. These are the TLR genetic variants that are proposed to play crucial roles in the susceptibility to *H. pylori* infection and the process of *H. pylori*-induced gastric carcinogenesis in Asian populations. Thus, the objectives of this study were to examine the associations between TLRs polymorphism and the risk of *H. pylori* infection, precancerous gastric lesions or GC development in Thai patients. This information may help identify individuals at high risk of developing GC, who would need close surveillance.

3. Methods

3.1. Ethics Statement

The study was performed in accordance with good clinical practice and the guidelines of the declaration of Helsinki. All patients provided written, informed consent, and the study protocol was approved by the ethics committee for research involving human subjects, Suranaree University of Technology (EC-58-58 and EC-58-5).

3.2. Patients

In total, 400 patients undergoing esophagogastroduodenoscopy (EGD) for investigation of chronic abdominal pain were enrolled in and participated in this study from December 2014 to March 2016 at the Suranaree University of Technology Hospital in Northeast Thailand. The study exclusion criteria were the following: *H. pylori* eradication treatment 2 months prior to enrolment; significant medical illnesses, including severe sepsis, uncontrolled heart failure, acute myocardial infarction and stroke; a history of previous gastric surgery; and the use of antimicrobials or gastrointestinal medications, such as proton pump inhibitors (PPIs) or bismuth compounds 2 months prior to study enrolment.

3.3. Biopsy Specimens

The esophagogastroduodenoscopy (EGD) procedures were performed using an upper GI video endoscope (Olympus EVIS EXERA III, CV-190, Japan). The whole stomach was examined first with conventional endoscopy, and biopsies were then performed using the "Site-Specific Biopsy" technique (20). Gastric tissue specimens for histological analysis were sent to the pathologist.

3.4. Diagnosis of *H. pylori*-Associated Gastritis

Biopsy samples were taken directly from the observation area and were used for rapid urease testing on site (Prontodyler, GASTREX, France). A diagnosis of *H. pylori*-associated gastritis was made if *H. pylori* were observed during the histopathological examination (supplementary file appendix 1); then, bacterial infection was confirmed by real-time PCR.

3.5. Real-Time PCR Condition

The 16s rRNA gene was examined by qualitative real-time PCR using the QuantiNova® SYBR® Green PCR kit (Qiagen, Hilden, Germany) with the LightCycler® 480 II (Roche diagnostics, Neuilly sur Seine, France). The primer sequences for 16s rRNA used in this study are the following: forward 5'GGA GTA CGG TCG CAA GAT TAA A'3 and reverse 5'CTA GCG GAT TCT CTC AAT GTC AA'3. One microliter of template, 10 µL of the 2 × SYBR Green PCR Master Mix, and 0.7 µmol final concentration of each primer were combined and adjusted with sterile water to a final volume of 20 µL. The PCR conditions were as follows: after an initial denaturation step for 5 minutes at 95°C, the samples underwent 35 - 40 cycles of 10 seconds at 95°C, 15 seconds at 60°C, and 10 seconds at 72°C. The fluorescence was measured at the end of the extension step at 72°C. Subsequently, a melting curve was recorded between 65°C and 97°C with a hold every 2 seconds. The PCR products were examined using melting curve analysis (supplementary file appendix 2).

3.6. DNA Preparation

Genomic DNA from 400 specimens was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue using the QIAamp DNA FFPE tissue kit (Qiagen, Düsseldorf, Germany). The genomic DNA extraction was performed according to the manufacturer's instructions. Briefly, the paraffin-embedded tissues were deparaffinized in xylene, hydrated in 100% ethanol and digested by lysis buffer and proteinase K. The genomic DNA was purified from the tissue lysates using QIAamp spin columns and eluted and stored at -20°C.

3.7. SNP Selection and Genotyping

Five SNPs [TLR1 *rs4833095* (C > T), TLR2 *rs3804099* (T > C) and *rs3804100* (T > C), TLR 4 *rs10759932* (T > C) and TLR10 *rs10004195* (T > A)], which have been associated with functional effects or GC risk, were selected from the SNP database of the national center for biotechnology information. The genotype for each TLR polymorphism was determined by TaqMan allelic discrimination using a pre-designed Custom TaqMan SNP Genotyping Assay and real-time PCR. Forward and reverse primers were used along with a wild-type VIC probe and a FAM probe for the variant allele. Primers and probes were supplied by Applied Biosystems (Foster City, CA, USA). The real-time PCR system was run according to the manufacturer's instructions (LightCycler® 480 II instrument, Roche diagnostics, Neuilly sur Seine, France). Briefly, the PCR conditions were as follows: 95°C for 10 minutes, followed by 55 cycles of 95°C for 15 seconds and 60°C for 1 minute. The genotyping success rate for each SNP was more than 94%. Negative controls and duplicate samples were used to confirm the accuracy of genotyping, and the data were analysed with LightCycler® 480 Software 1.5 (Roche Applied Science, Mannheim, Germany).

3.8. Statistical Analyses

The associations between TLR polymorphisms and the risk of *H. pylori* infection, as well as the risk of precancerous gastric lesions or GC, were evaluated using a two-tailed Fisher's exact test. The associations were expressed as odds ratios (OR) with 95% confidence intervals (CI). SNPs with P values < 0.05 were considered potentially associated with disease risk and statistically significant. All statistical analyses were performed using the SPSS 20.0 software package for Windows (IBM SPSS Statistics for Windows, version 20, Armonk, NY, USA).

4. Results

4.1. Association of TLR Polymorphisms with *H. pylori* Infection

In total, 400 patients (204 *H. pylori*-positive and 196 *H. pylori*-negative patients) were genotyped. The associations were examined between the risk of *H. pylori* infection and the following TLR polymorphisms: TLR1 *rs4833095*, TLR2 *rs3804099* and *rs3804100*, TLR4 *rs10759932* and TLR10 *rs10004195*. As shown, 60% of patients with *H. pylori*-positive status carried the TLR1 *rs4833095* CC homozygous wild type (Table 1). Of the *H. pylori*-positive patients, 38% carried the TLR1 *rs4833095* TT homozygous variant. The TLR1 *rs4833095* CC and TT genotypes were associated with a significantly increased risk of *H. pylori* infection (OR = 2.28, 95% CI: 1.27 - 7.60, P = 0.02 and OR = 1.34, 95% CIs: 0.97-1.86,

P = 0.04, respectively). Of the *H. pylori*-positive patients, 66% carried the TLR10 *rs10004195* TT homozygous variant. Moreover, the TT homozygous variant was associated with a significantly increased risk of *H. pylori* infection (OR=1.28, 95% CI = 0.98 - 1.76, P = 0.01). These results suggest that TLR1 *rs4833095* and TLR10 *rs10004195* are associated with the effect of *H. pylori*-mediated infection on the susceptibility to gastritis. Regarding the TLR2 *rs3804099* and *rs3804100* and TLR4 *rs10759932* polymorphisms, the TT, TC or CC genotypes were associated with a decreased risk of *H. pylori* infection. The decreased risk of infection associated with TLR2 *rs3804099* and *rs3804100* and TLR4 *rs10759932* suggests that these polymorphisms may confer a protective effect for *H. pylori* infection, although the data were not statistically significant.

4.2. Association of TLR Polymorphisms with Changes in Gastric Pathology

A total of 400 patients were divided into a chronic gastritis group (N = 312) and a precancerous gastric lesions group that included gastric atrophy, internal metaplasia and GC (N = 88). We evaluated the association between each of the five TLR polymorphisms and the risks for changes in gastric pathology, from chronic gastritis to precancerous lesions and/or GC (Table 2). Of the five SNPs, 3% of chronic gastritis patients carried the TLR10 *rs10004195* AA homozygous wild type, while 84% of the precancerous gastric lesions/GC patients carried the same SNP. Interestingly, we found an association between TLR10 *rs10004195* and changes in gastric pathology. Patients with the TLR10 *rs10004195* AA homozygous wild type exhibited a significantly increased risk of precancerous gastric lesions/GC (OR = 8.75, 95% CI = 2.78 - 14.24, P < 0.01). Patients carrying the TT or CC genotypes in TLR2 *rs3804099*, as well as the TT, TC and CC genotypes in TLR2 *rs3804100* and TLR4 *rs10759932*, had a decreased risk of precancerous gastric lesions/GC, but these associations were not statistically significant. These results indicate that TLR10 *rs10004195* confers an increased risk for changes in gastric pathology and may contribute to GC development.

5. Discussion

In Northeast Thailand, *H. pylori* infection is associated with an increased risk of colorectal polyps. Additionally, approximately 32% of patients with *H. pylori*-associated gastritis develop gastric ulcers, while only 2% - 3% progress to GC (21). With our studies host genetic factors, the Mdm2 SNP309 GG homozygous genotype may be a risk factor for GC in Thai patients (22). Moreover, a specific genetic polymorphism of Mdm2, SNP309 GG, was associated with more

Table 1. Association Between Toll-Like Receptor Polymorphisms and *H. pylori* Infection^a

Polymorphisms	Genotype	<i>H. pylori</i> -Negative	<i>H. pylori</i> -Positive	OR (95% CIs)	P Value ^b
TLR1 rs4833095	CC	14 (7)	122 (60)	2.28 (1.27 - 7.60)	0.02
	CT	182 (93)	4 (2)	1.21 (0.87 - 1.91)	0.34
	TT	-	78 (38)	1.34 (0.97 - 1.86)	0.04
TLR2 rs3804099	TT	131 (67)	126 (62)	0.96 (0.63 - 1.43)	0.41
	TC	41 (21)	27 (13)	1.52 (0.40 - 5.75)	0.63
	CC	24 (12)	51 (25)	0.77 (0.47 - 1.23)	0.72
TLR2 rs3804100	TT	149 (76)	147 (72)	0.52 (0.26 - 1.22)	0.62
	TC	43 (22)	49 (24)	0.47 (0.23 - 1.07)	0.53
	CC	4 (2)	8 (4)	0.68 (0.31 - 1.14)	0.48
TLR4 rs10759932	TT	139 (71)	153 (75)	0.47 (0.26 - 1.02)	0.82
	TC	53 (27)	37 (18)	0.57 (0.32 - 1.03)	0.43
	CC	4 (2)	14 (7)	0.46 (0.37 - 1.06)	0.61
TLR10 rs10004195	AA	22 (11)	59 (29)	0.57 (0.32 - 1.03)	0.42
	AT	123 (63)	10 (5)	1.21 (0.87 - 1.91)	0.68
	TT	51 (26)	135 (66)	1.28 (0.98 - 1.76)	0.01

Abbreviations: CIs, confidence intervals; OR, odds ratio; TLR, Toll-like receptor.

^aValues are expressed as No. (%).^bFisher's exact test two-tailed. Statistic significant (P < 0.05).**Table 2.** Association of Toll-Like Receptor Genotypes and Risk of Precancerous Gastric Lesions or Gastric Cancer^a

Polymorphisms	Genotype	Gastritis	Precancerous Gastric Lesions ^b /GC	OR (95% CIs)	P Value ^c
TLR1 rs4833095	CC	68 (22)	72 (82)	1.18 (1.07 - 6.60)	0.63
	CT	172 (55)	8 (9)	1.31 (0.97 - 1.81)	0.35
	TT	72 (23)	8 (9)	1.14 (0.98 - 1.76)	0.42
TLR2 rs3804099	TT	194 (62)	62 (70)	0.87 (0.57 - 1.24)	0.73
	TC	56 (18)	13 (15)	1.42 (0.48 - 4.75)	0.83
	CC	62 (20)	13 (15)	0.97 (0.64 - 1.33)	0.42
TLR2 rs3804100	TT	230 (74)	66 (75)	0.53 (0.27 - 1.32)	0.72
	TC	70 (22)	22 (25)	0.46 (0.24 - 1.17)	0.54
	CC	12 (4)	-	0.69 (0.32 - 1.24)	0.58
TLR4 rs10759932	TT	216 (69)	76 (86)	0.57 (0.28 - 1.12)	0.92
	TC	90 (29)	-	0.67 (0.37 - 1.23)	0.53
	CC	6 (2)	12 (14)	0.56 (0.38 - 1.16)	0.62
TLR10 rs10004195	AA	8 (3)	74 (84)	8.75 (2.78 - 14.24)	< 0.01
	AT	132 (42)	5 (6)	0.93 (0.87 - 1.19)	0.69
	TT	172 (55)	9 (10)	1.18 (0.98 - 1.56)	0.71

Abbreviations: CIs, confidence intervals; OR, odds ratio; TLR, Toll-like receptor.

^aValues are expressed as No. (%).^bPrecancerous gastric lesions: gastric atrophy and intestinal metaplasia.^cTwo-tailed Fisher's exact test; P < 0.05 indicates statistical significance.

severe inflammation in *H. pylori*-associated gastritis (23). Toll-like receptors 1 was associated with *H. pylori* seroprevalence in a European population and is essential for protective immunity against *H. pylori* infection (24). Therefore, it is possible that host genetic factors promote *H. pylori*-mediated gastric diseases.

Recently, the TLR1 rs4833095 CC homozygous wild type and the TLR10 rs10004195 AA homozygous wild type were associated with *H. pylori* susceptibility and increased risk of

H. pylori infection in chronic gastritis patients (25). Moreover, TLR10 polymorphism was associated with *H. pylori*-induced inflammation with gastric mucosal patterns, suggesting that the AA homozygous wild type contributes to severe inflammation in *H. pylori*-associated gastritis (26). Therefore, in this study, precancerous gastric lesions and GC were extensively investigated. Our study found that TLR1 rs4833095 was associated with *H. pylori* susceptibility, and the various genotypes showed different risk associa-

tions for *H. pylori* infection.

The results indicated that the TLR1 *rs4833095* CC and TT homozygous variants were significantly associated with an increased risk of *H. pylori* infection while there was no association with the CT heterozygous variant in Thai patients. In contrast, in a Chinese population, the TLR1 *rs4833095* CT heterozygous variant was associated with a decreased risk of *H. pylori* infection (27). Moreover, the TLR1 TT homozygous variant was associated with reduced risks of chronic atrophic gastritis and intestinal metaplasia in a Chinese population (27) but significant risk associations for precancerous gastric lesions and/or GC were not found in Thai patients. These results indicate that TLR1 *rs4833095* may be an ethnicity-dependent risk factor for GC.

The TLR10 *rs10004195* T alleles were not protective against GC development in Malaysian patients, while the AA homozygous variant was associated with increased GC irrespective of *H. pylori* infection (19). Similarly, this study showed that the TLR10 *rs10004195* T alleles confer a protective effect on GC development, while the AA homozygous allele confers an 8-fold increased risk of precancerous gastric lesions or/and GC. Interestingly, the TLR10 *rs10004195* TT homozygous variant was significantly associated with an increased risk of *H. pylori* infection. The TT homozygous variant was the most abundant in gastritis patients (46.5% of the 400 gastritis patients) and was highest for chronic gastritis (55%). Therefore, the high prevalence of *H. pylori* infection in the Northeast Thailand population may be because the TT homozygous variant is commonly found and is associated with an increased risk of *H. pylori* infection.

In particular, the TLR10 *rs10004195* AA homozygous wild type was associated with an increased risk of precancerous gastric lesions and/or GC, suggesting that the AA homozygous wild type was associated with the development of pathological changes and likely one of the factors contributing to GC in this population. The AA homozygous wild type was the least abundant (20.5%) among patients with chronic gastritis, precancerous gastric lesions and GC. These polymorphisms could, therefore, provide evidence to uncover the factors underlying the low incidence of GC in Thailand or provide evidence of a Thailand enigma.

The involvement of TLRs in infection, autoimmune processes and other inflammatory diseases is well established. For *H. pylori* infection, TLRs on gastric epithelia and immune cells recognize diverse pathogen-associated molecular patterns (PAMPs) such as flagellin, unmethylated CpG motifs and lipopolysaccharides (LPS) (28). For example, in addition to monocyte differentiation antigen (CD14) and myeloid differentiation protein (MD-2), TLR4 can interact with LPS, and this interaction induces signal transduction pathways that activate NF- κ B and cause the

expression of several cytokines, including TNF- α , IL-1 β , IL-6 and IL-12 (29). Additionally, TLR2 induces cell proliferation and TLR4 activation expression via the MEK1/2-ERK/2 pathway (13, 30).

As a critical part of the innate immune response, the varying functional roles of TLRs is based on genetic polymorphisms, in human diseases. However, conflicting data concerning their role in GC development have been reported. For example, a 22-bp nucleotide deletion in the promoter region of TLR2 (196 to 174) has been shown to either increase or decrease the risk of GC, depending on racial differences, increasing the GC risk approximately 6.06-fold in a Japanese population but decreasing the GC risk approximately 0.66-fold in a Chinese population (29). Additionally, TLR2 *rs3804099* and *rs3804100* were not significantly associated with decreased risk of *H. pylori* susceptibility, precancerous gastric lesions and/or GC development in this study. Furthermore, TLR4 *rs4986790* has been shown to promote and protect against GC development in a Brazilian population (31).

The homozygous wild-type alleles also displayed an increased risk for peptic ulcers but did not show any association with *H. pylori* positivity or the features of gastric inflammation in a Finnish population (32). TLR4 *rs4986790* polymorphism conferred a significant risk of chronic *H. pylori* infection and peptic ulcer disease in Indian Tamils (33). We found that TLR4 *rs10759932* decreases both *H. pylori* susceptibility and the risk of precancerous gastric lesions and/or GC development, even though the association was not statistically significant.

6. Conclusions

In conclusion, the TLR1 *rs4833095* and TLR10 *rs10004195* polymorphisms play roles in susceptibility to *H. pylori* infection, indicating that TLR10 *rs10004195* may be an ethnicity-dependent risk factor for GC development. Thus, our findings suggest that the TLR1 *rs4833095* and TLR10 *rs10004195* polymorphisms are potential genetic risk factors influencing *H. pylori* infection susceptibility and the clinical manifestation of chronic gastritis, precancerous gastric lesions and GC in Thai patients. However, multi-centre studies in Thailand are needed for larger population sizes, and this hypothesis should be extensively evaluated by studying the distribution of TLR polymorphisms in the Thai population. Our results support the notion that the various TLR polymorphisms have different effects on cancer development and that these effects are dependent on patient ethnicity. Further studies are needed in a larger GC population to confirm the impact of these genes on susceptibility to GC.

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Footnotes

Authors' Contribution: Study concept and designed: Taweesak Tongtawee; acquisition of data: Taweesak Tongtawee and Theeraya Simawaranon; analysis and interpretation of data: Theeraya Simawaranon; drafting of the manuscript: Theeraya Simawaranon; critical revision of the manuscript for important intellectual content: Taweesak Tongtawee and Theeraya Simawaranon; statistical analysis: Taweesak Tongtawee; administrative, technical, and material support: Taweesak Tongtawee, Theeraya Simawaranon and Wareeporn Wattanawongdon; study supervision: Theeraya Simawaranon.

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