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ORIGINAL ARTICLE

Interaction of *H. pylori* with toll-like receptor 2-196 to -174 ins/del polymorphism is associated with gastric cancer susceptibility in southern China

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Abstract

Background Genetic polymorphisms of Toll-like receptors play important roles in gastric carcinogenesis. The aim of this study was to determine the role of *TLR2*-196 to -174 ins/del polymorphism in gastric cancer susceptibility and prognosis.

Methods This study included 520 people from southern China. Samples were genotyped by the allele-specific polymerase chain reaction, among which 10% were randomly selected for sequencing. The serological method was used to determine *Helicobacter pylori*.

Results The *TLR2* genotype was not associated with the risk of *H. pylori* infection. The del/del genotype exhibited significantly higher gastric cancer risk (adjusted OR 2.59, 95% CI 1.33−5.07) than that of the ins/ins genotype. Further stratification analyses demonstrated that the del/del genotype was associated with a risk of intestinal gastric cancer (adjusted OR 2.62, 95% CI 1.34–5.14). In addition, the presence of the del/del genotype and the *H. pylori* infection conferred a synergistic effect (OR 3.04, 95% CI 1.33−6.98) for the development of gastric cancer. The del/del genotype was not associated with a poor prognosis in gastric cancer patients.

Conclusion The del/del genotype is associated with an increased gastric cancer risk in the southern Chinese population. However, TLR2 polymorphism is neither associated with *H. pylori* infection, nor with a poor prognosis.

Keywords *H. pylori* · Gastric cancer · Prognosis · TLR2 · Polymorphism

Introduction

Gastric cancer remains one of the most common malignancies globally and the leading cause of cancer mortality [1]. It ranks as the second highest in incidence rate and the third highest in mortality rate among cancers in China, with an estimated 427,100 new cases in 2013 [2], representing a major problem of public health. Although *Helicobacter pylori* (*H. pylori*) is an important causal factor of gastric cancer [3], only a small proportion of individuals infected with *H. pylori* develop gastric cancer. This suggests that there is a possible involvement of genetic factors in tumorigenesis. The host immune response plays a pivotal role in determining the outcome of *H. pylori* infection. Single nucleotide polymorphisms (SNP) in the genes that are involved in the immune response have been shown to be associated with susceptibility to gastric cancer [4, 5].

Toll-like receptors (TLRs) may modulate host immune responses to invading pathogens *via* selective recognition of pathogen-associated molecules, and mediation of the pathogen–epithelium interactions [6, 7]. In humans, ten different TLRs have been identified [8]. Among them, TLR2 is expressed by the gastric epithelium of the human stomach [9] and participates in gastric mucosal immunity to *H. pylori* infection [10]. Through TLR2, *H. pylori* triggers the activation of NF-κB and subsequently enhances the chemokine expression in gastric epithelial cells. Previous evidence has shown that transfection of *TLR2* in epithelial cells induces the production of interleukin-8, macrophage inflammatory protein-3α, and growth-regulated oncogen-α

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[11]. The human *TLR2* gene, which is located on chromosome 4q32, is composed of two non-coding exons and one coding exon [12]. Several polymorphisms in the *TLR2* gene that participate in host defense and disease progression have been identified [13, 14]. Among them, an insertion/deletion (ins/del) polymorphism of the 22-base pairs (bp) nucleotide (*TLR2*-196 to -174) within the 5′untranslated region leads to abnormalities in the promoter activities. The *TLR2*-196 to -174 del/del genotype is associated with a decreased transcriptional activity [15]. Previous studies have shown that this polymorphism may confer increased susceptibility to breast cancer, hepatocellular cancer and gallbladder cancer [16–18]. However, the results among studies remain inconsistent regarding gastric cancer. Zeng et al., found that a decreased risk of gastric cancer was associated with the -196 to -174 del allele. However, another study found that an increased gastric cancer risk was associated with the -196 to -174 del allele [19, 20].

In this study, we aimed to investigate the influence of the *TLR2*-196 to -174 ins/del polymorphism and *H. pylori* infection, alone or in combination, on the risk of gastric cancer in the southern Chinese population. In addition, the association between *TLR2* polymorphism and the overall survival of gastric cancer patients was evaluated.

Materials and methods

Study population

The study protocol was approved by the Ethics Committee of Changzhou No. 2 People's Hospital and in compliance with the Declaration of Helsinki. All participants provided written informed consent.

A total of 260 patients with histologically diagnosed gastric adenocarcinoma were recruited from the Changzhou No. 2 People's Hospital between July 2007 and July 2009. None of the included patients had a previous history of tumors, chemotherapy or radiotherapy. 192 patients underwent radical surgery (73.8%), and 19 patients underwent palliative surgery (7.3%). Among the patients, 108 (41.5%) received adjuvant chemotherapy, while 152 received no adjuvant chemotherapy for various reasons. 260 age-matched (± 5 years) cancer-free controls (162 men and 98 women) who had no clinical history of gastroduodenal disease were also included. Cancer-free controls were randomly selected from health checkup clinics. The controls underwent endoscopic examination and excluded cancer lesions. The endoscopic findings of the controls showed normal appearance or gastritis only. None of the included subjects had received treatment for *H. pylori* infection before enrollment. All of the subjects were unrelated ethnic Han Chinese residents of the Jiangsu Province, China.

According to the Lauren classification, gastric cancer was classified into diffuse, intestinal or mixed type. Gastric cancer samples were also divided into cardia and non-cardia, according to the anatomical location. Patients were graded into stages I + II or stages III + IV based on the tumor, lymph node, and metastasis (TNM) classification.

Demographic and clinical data were collected from medical records. All of the patients were followed up for 3–56 months (median 35 months), and 51 patients were lost to follow-up. During the follow-up period, 98 patients died. The majority of the deaths were due to gastric cancer itself, with only seven patients dying due to causes that were unrelated to gastric cancer during the study period.

Serological determination of *H. pylori* infection

2 ml of venous blood samples were collected from each subject. The presence of the serum immunoglobulin G (IgG) antibody to *H. pylori* was determined by using an indirect solid-phase immunochromatographic assay (Genelabs Diagnostics Pty Ltd., Singapore), according to the instructions of the manufacturer. The absence of a colored control line (A band) indicated that the test was invalid. Appearance of colored bands of ''A B C'' or ''A C'' on the strip represented a *H. pylori* antibody-positive test result. This method had an accuracy of 92.3% in examining *H. pylori* infection as validated in our lab previously [21].

Genotype analysis

Genomic DNA was obtained from leukocytes by the proteinase K digestion method. The DNA profile was purified with phenol–chloroform followed by ethanol precipitation. The *TLR2*-196 to -174 ins/del polymorphism was analyzed by the allele-specific polymerase chain reaction (PCR) method. In brief, the primers of the *TLR2*-196 to -174 ins/del were 5′-CACGGAGGCAGCGAGAAA-3′ (forward) and 5′-CTG GGCCGTGCAAAGAAG-3′ (reverse). The 25-μL PCR mixture was used for PCR analysis. The mixture contained: 200 ng of genomic DNA, 12.5 pmol of each primer, 200 ng of each deoxynucleotide triphosphate and 0.6 U Taq polymerase. Thermal cycling conditions were as follows: an initial melting step of 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 40 s; and a final extension step for 7 min. The PCR products were separated by the 3.5% agarose gel and visualized by ethidium bromide. Wild type (ins/ins) and homozygous type (del/del) were presented as a single band at 286-bp and 264-bp, respectively; while the heterozygous type (ins/del) was two bands at 286-bp and 264-bp. Genotyping was performed without knowledge of the disease status of the subjects. 10% of the samples were randomly chosen for DNA sequencing, allowing the

validation of the genotyping results. The results of the two methods were in agreement.

Statistical analysis

The Hardy–Weinberg equilibrium of the *TLR2* gene allele in the healthy controls was assessed by Chi squared (χ^2) analysis. The frequencies of the *TLR2* genotype between the gastric cancer patients and the healthy controls were compared with χ^2 statistics. The association between the *TLR2* genotype and the gastric cancer risk was analyzed using logistic regression, as presented as odds ratios (OR) and 95% confidence intervals (CIs), with adjustment for age, sex and *H. pylori* infection. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. The multivariate Cox regression model was used to test the association between *TLR2* polymorphism and prognosis, expressed as hazard ratios (HRs) and 95% CIs, with adjustments for age, gender, *H. pylori* serology, and TNM stage. A p value < 0.05 was considered statistically significant. All data analyses were performed using SPSS software (version 16.0; Chicago, IL, USA).

Results

Study population

This study included 260 gastric cancer cases and 260 cancer-free controls. The sex and age of the participants were not significantly different between groups ($p = 0.93$ and $p = 0.13$, respectively). The characteristics of the participants are summarized in Table 1. The rate of *H. pylori* infection in patients with gastric cancer was significantly higher than that of the control group (72.3% vs. 63.8%, $p = 0.04$).

Table 1 Baseline characteristics of participants

Characteristics	Cases ($n = 260$)	Controls ($n = 260$)	p value
Age, years			
Median	59.5	59.0	0.13
SD	12.51	12.17	
Range	21–85	22–82	
Sex, *n* (%)			
Male	163 (62.7)	162 (62.3)	0.93
Female	97 (37.3)	98 (37.7)	
H. pylori, n (%)			
Seronegative	72 (27.7)	94 (36.2)	0.04*
Seropositive	188 (72.3)	166 (63.8)	
Location, n (%)			
Cardia	77 (29.6)		
Non-cardia	183 (70.4)		
TNM stage [a], *n* (%)			
I	46 (19.1)		
II	39 (16.2)		
III	107 (44.4)		
IV	49 (20.3)		
Follow-up, months			
Median	35		
Range	3–56		

SD standard deviation, *TNM* tumor, lymph node, metastasis

*Statistical significance ($p < 0.05$)

[a]Data from 19 patients are missing

Association of the *TLR2*-196 to -174 ins/del polymorphism with risk of gastric cancer

The frequency of *TLR2* polymorphism in the healthy controls was within Hardy–Weinberg equilibrium ($\chi^2 = 2.10$, $p = 0.15$). The genotype distributions of *TLR2*-196 to -174 ins/del in gastric cancer patients and controls are shown in Table 2.

Using the χ^2 test (3×2 tables), no association was observed between the *TLR2* genotype and the risk of *H. pylori* infection in the gastric cancer patients or the controls ($p = 0.25$ and 0.99, respectively). The del/del homozygote exhibited significantly higher risk of gastric cancer than the ins/ins homozygote (adjusted OR 2.59, 95% CI 1.33–5.07; $p = 0.01$); while the ins/del heterozygote did not increase the gastric cancer risk (adjusted OR = 1.36, 95% CI 0.95–1.96; $p = 0.10$), suggesting a recessive model. Overall, the del/del genotype was associated with a higher risk of gastric cancer as compared with the risk observed with the ins carriers (adjusted OR 2.21, 95% CI 1.16–4.22; $p = 0.02$). Multivariate analysis revealed that del/del genotype (OR 2.59, 95% CI 1.33–5.07; $p = 0.01$) and *H. pylori* infection (OR 1.49, 95% CI 1.02–2.17; $p = 0.04$) were risk factors of gastric cancer (Table 3).

Association between *TLR2* polymorphism and gastric cancer risk through stratification analysis

In the stratified analysis, a significantly increased risk of gastric cancer associated with the del/del genotype was evident among males (adjusted OR 2.35, 95% CI 1.03–5.34) and those less than 60 years old (adjusted OR 3.19, 95% CI 1.20–8.50), than the risks observed in other groups (Table 4). Compared with the risks observed in the healthy controls, the del/del genotype increased the risks of both cardia (adjusted OR 2.61, 95% CI 1.13–6.07) and non-cardia gastric cancers (adjusted OR 2.09, 95% CI 1.03–4.25). With regard to tumor stage, the del/del genotype increased the risks of both TNM I–II (adjusted

Table 2 The genotype distributions of TLR2 in GC cases and controls

Genotype	Controls ($n=260$)		Cases ($n=260$)		GC					
	No.	%	No.	%	Crude OR	95% CI	*p* value	Adjusted OR [a]	95% CI	*p* value
Ins/ins	132	50.8	105	40.4	1.00					
Ins/del	113	43.4	124	47.7	1.38	0.96−1.98	0.08	1.36	0.95−1.96	0.10
Del/del	15	5.8	31	11.9	2.60	1.33−5.07	0.01*	2.59	1.33−5.07	0.01*
Recessive model										
Others	245	94.2	229	88.1	1.00					
Del/del	15	5.8	31	11.9	2.21	1.16−4.20	0.02*	2.21	1.16−4.22	0.02*

GC gastric cancer, *OR* odds ratio, *CI* confidence interval, *ins* inserted, *del* deleted

*Statistical significance ($p<0.05$)

[a]Adjusted for age, sex, and *Helicobacter pylori* infection

Table 3 Multivariable logistic regression analysis of the risk factors of gastric cancer

Parameters	Odds ratio	95% CI	*p* value
Sex			
Female	1		
Male	0.99	0.69–1.42	0.96
Age, years old			
<60	1		
≥60	1.13	0.80–1.61	0.49
H. pylori			
Seronegative	1		
Seropositive	1.49	1.02–2.17	0.04*
Genotype			
Ins/ins	1		
Ins/del	1.36	0.95−1.96	0.10
Del/del	2.59	1.33−5.07	0.01*

*Statistical significance ($p<0.05$)

OR 2.38, 95% CI 1.07–5.29) and TNM III–IV (adjusted OR 2.04, 95% CI 1.01–4.13) gastric cancers, compared to the risks observed in the other groups. When stratified by histologic subtype, the del/del genotype was associated with a 2.62-fold increased risk of intestinal gastric cancer (adjusted OR = 2.62, 95% CI 1.34–5.14).

The joint effect of *TLR2* polymorphism and *H. pylori* infection on gastric cancer risk was evaluated. As shown in Table 5, the OR of developing gastric cancer in subjects with the del/del genotype or *H. pylori* infection alone was 2.91 (95% CI 0.93–9.13) or 1.55 (95% CI 1.05–2.30), respectively. However, further analysis indicated a significantly elevated risk of gastric cancer in subjects with concurrent del/del genotype and *H. pylori* infection (adjusted OR 3.04, 95% CI 1.33−6.98), as compared with those with the ins/ins + ins/del genotype without *H. pylori* infection.

Association between the TLR2 genotype and overall survival

The overall survival of patients with gastric cancer was analyzed using the Kaplan–Meier survival curve. High TNM stages, including stage III (HR 4.77, 95% CI 2.02–11.23) and stage IV (HR 15.48, 95% CI 6.53–36.67), were strongly associated with poor survival, as compared with the survival observed in stage I cases. Kaplan–Meier survival curve analysis showed that the overall survival of patients with gastric cancer was not different among subjects carrying different genotypes (Fig. 1). Cox proportional hazards analysis showed that ins/del genotype (HR 1.23, 95% CI 0.82–1.87) and del/del genotype (HR 1.60, 95% CI 0.76–3.40) were not associated with poor survival, compared with the survival observed in the ins/ins genotype group.

Discussion

TLR2 plays a crucial role in various biological processes of inflammation and carcinogenesis that are initiated by *H. pylori* infection. Thus, genetic variation within this gene may have a great impact on the host defense against *H. pylori* or gastric cancer susceptibility. The results of previous studies regarding the association between *TLR2* ins/del polymorphism and gastric cancer risk have been inconsistent [19, 20, 22]. Thus, further investigation was required. In the present study, we assessed the influence of *TLR2* -196 to -174 del polymorphism on the risk of developing gastric cancer and *H. pylori* infection in the southern Chinese population. We found that the *TLR2* -196 to -174 del/del genotype was significantly associated with an increased risk of gastric cancer. However, these findings were inconsistent with the results of another Chinese study conducted by Zeng et al., which revealed a decreased risk of gastric cancer among -196 to -174 del carriers [20]. This discrepancy

Table 4 Stratified analyses between TLR2 polymorphisms and gastric cancer risk

Variables	(Case/control)		OR (95% CI) [a]		*p* value
	Ins/ins + ins/del	Del/del	Ins/ins + ins/del	Del/del	
Age, years old					
<60	113/129	17/6	1.00	3.19 (1.20–8.50)	0.02*
≥60	116/116	14/9	1.00	1.53 (0.63–3.69)	0.35
Sex					
Male	143/153	20/9	1.00	2.35 (1.03–5.34)	0.04*
Female	86/92	11/6	1.00	2.01 (0.69–5.81)	0.20
Location site					
Cardia	66/245	11/15	1.00	2.61 (1.13–6.07)	0.03*
Non-cardia	163/245	20/15	1.00	2.09 (1.03–4.25)	0.04*
Histologic type[a]					
Intestinal	162/245	26/15	1.00	2.62 (1.34–5.14)	0.01*
Diffuse	64/245	5/15	1.00	1.27 (0.44–3.65)	0.66
TNM					
I–II	74/245	11/15	1.00	2.38 (1.07–5.29)	0.03*
III–IV	138/245	18/15	1.00	2.04 (1.01–4.13)	0.04*

OR odds ratio, *CI* confidence interval, *ins* inserted, *del* deleted, *TNM* tumor, lymph node, and metastasis

*Statistical significance ($p < 0.05$)

[a]Adjusted for age, sex and *H. pylori* infection in a logistic dominant model. Lauren classification

Table 5 Association between TLR2 polymorphisms and gastric cancer risk in the presence or absence of *H. pylori* infection

TLR2	*H. pylori* infection	OR[a] (95% CI)	*p* value
Ins/ins + ins/del	Negative	1.00	
Ins/ins + ins/del	Positive	1.55 (1.05–2.30)	0.03*
Del/del	Negative	2.91 (0.93–9.13)	0.07
Del/del	Positive	3.04 (1.33–6.98)	0.01*

OR odds ratio, *CI* confidence interval, *ins* inserted, *del* deleted

*Statistical significance ($p < 0.05$)

[a]Adjusted for age and sex

may be caused by the diversity of the studied populations. These studies included participants with different genetic backgrounds. Furthermore, the sample sizes varied between studies. China was geographically divided into northern and southern China based on the demarcation line of the Qinling-Huaihe. Our study was conducted in Changzhou, southern China, while the study of Zeng et al. was conducted in Linqu county, northern China. The hereditary backgrounds of these two regions differ [23]. For instance, *IL-1β-511C/T* polymorphism has been shown to increase the susceptibility of GC in a southern Chinese population. In contrast, no significant correlation between this polymorphism and GC susceptibility was found in a northern Chinese population [23]. Additionally, our study included a total of 260 gastric cancer patients. This is a larger case sample size than that of the study of Zeng et al. We also found that the *TLR2* del/del genotype was not related to a poor survival status. To the best of our knowledge, this is the first study to investigate the associations between *TLR2*-196 to -174 ins/del polymorphism and gastric cancer survival.

Although the *TLR2* del/del genotype was found to be associated with gastric cancer risk in the Chinese population, the exact mechanism of this association remains to be elucidated. We assessed the association between *TLR2* polymorphism and risk of *H. pylori* infection. The results revealed no impact of this polymorphism on susceptibility to *H. pylori*, eliminating the possibility that the *TLR2* del/del genotype conferred an increased risk of gastric cancer through an enhancement of the susceptibility to *H. pylori* infection. The human *TLR2* gene is located on the long arm of chromosome 4, and it is composed of two 5′non-coding exons and a third coding exon [12]. The ins/del polymorphism is located in the 5′ untranslated region (5′UTR), which is essential for transcriptional and translational regulation of genes [24, 25]. Noguchi E et al. performed a reporter assay and found that constructs with del allele exhibited reduced luciferase activity in comparison with that of the wildtype constructs, suggesting a decreased transcriptional activity of the deletion allele [15]. During *H. pylori* infection, the expression of TLR2 is up-regulated in gastric epithelial cells to directly respond to *H. pylori*. Thus, TLR2 deficiency may be detrimental and implicated in carcinogenesis of the stomach [10, 26]. Currently, there are no functional data available for the relationship between TLR2 ins/del polymorphism and gastric cancer. This unsolved issue requires further investigation, including the determination of the correlation between genetic polymorphisms and TLR2 expression

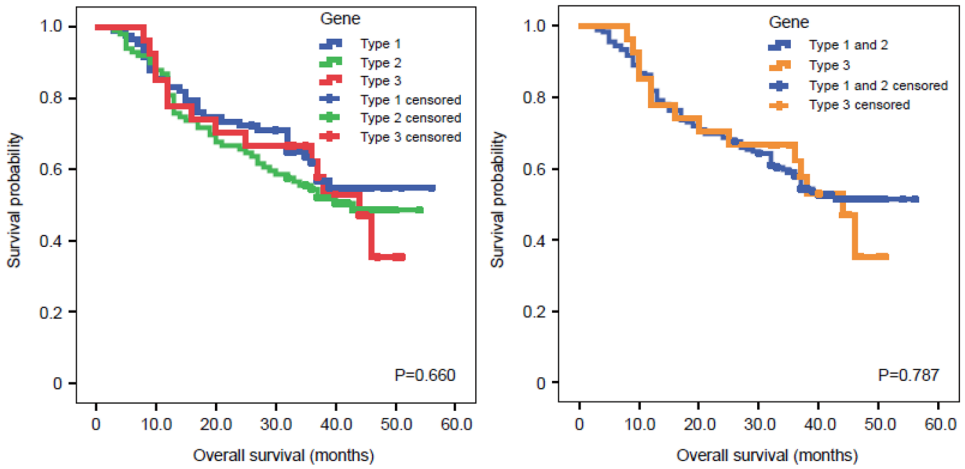

Fig. 1 Kaplan–Meier survival curves for gastric cancer patients by the genotypes of *TLR2*-196 to -174 ins/del

Type 1: ins/ins genotype
Type 2: ins/del genotype
Type 3: del/del genotype

in gastric cancer tissues using immunohistochemistry and real-time quantitative PCR. Additionally, cytokine expression in gastric epithelial cells expressing different TLR2 SNPs should be measured when incubated with *H. pylori*.

We observed a synergistic effect between *TLR2*-196 to -174 ins/del polymorphism and *H. pylori* infection, which conferred a > threefold increased risk of developing gastric cancer, suggesting a potential gene–environment interaction. This correlation may be explained as follows: *H. pylori* is a class I human carcinogen [27]. TLR2 is a critical receptor for the recognition of *H. pylori* [10]. TLR2 with the del allele causes reduced transcriptional activity of TLR2 [15]. As a result, the decreased expression of TLR2 may weaken the immune response against *H. pylori* and favor a persistent infection [10]. Furthermore, chronic inflammation, triggered by *H. pylori*, plays a critical role in the initiation and promotion of gastric cancer [28]. Therefore, it is reasonable to believe that the concurrent presence of the *TLR2* del/del genotype and *H. pylori* infection is linked to the highest risk of developing gastric cancer.

We also conducted stratification analysis to explore the impact of the *TLR2* del/del genotype on the characteristics of gastric cancer. The results showed that the del/del genotype was associated with an increased risk of both cardia and non-cardia gastric cancers, as well as both TNM stage I–II and III–IV gastric cancers. However, with regards to histologic type, the del/del genotype was only associated with the risk of intestinal gastric cancer. *H. pylori* infection has been considered to be the main cause of nearly 60% of the intestinal-type gastric cancer [29]. The *TLR2* del/del genotype downregulates TLR2 expression [15], which impairs the immune responses against *H. pylori*. The persistence of gastric inflammation resulting from chronic *H. pylori* infection leads to epithelial damage and resultant gastric atrophy and intestinal metaplasia, and eventually progression towards intestinal gastric cancer [29].

The TNM classification has been widely accepted as a guide for estimating tumor prognosis. As expected, this study demonstrated that advanced TNM stage predicted poor survival outcome in patients with gastric cancer. In contrast, the ins/del polymorphism was not identified to be an independent prognostic factor for gastric cancer patients. These data suggested that, although it plays a critical role in carcinogenesis, the *TLR2* ins/del polymorphism it is not a prognostic factor for gastric cancer.

The present study has some strengths as well as limitations. *TLR2* gene polymorphisms in control subjects were in accordance with the Hardy–Weinberg equilibrium. All gastric cancer cases were histopathologically confirmed, and cases and controls were individually matched for potential confounding variables. With regard to the limitations, first, the exact mechanism of *TLR2* polymorphisms on the *H. pylori*-associated carcinogenesis requires further elucidation. Second, the subjects of this study were restricted to the southern Chinese population. Therefore, caution should be taken when generalizing the results of this study to other racial and ethnic groups. Third, this study had a limited sample size, which limits the statistical power of the results. Fourth, this study had a relatively short follow-up period. Thus, further investigation is needed to confirm our findings.

In conclusion, the *TLR2*-196 to -174 del/del genotype is associated with an increased risk of gastric cancer, which facilitates the gastric cancer risk stratification in individuals. However, *TLR2* polymorphism is neither associated with *H. pylori* infection, nor with a poor prognosis. Further investigation is required to confirm the impact of the *TLR2* gene

on the susceptibility of gastric cancer in larger studies with ethnically diverse populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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