TLR9 Promoter Polymorphism Is Associated with Both an Increased Susceptibility to Gastric Carcinoma and Poor Prognosis

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Abstract

Objective: Genetic polymorphisms of Toll-like receptors (TLRs) may influence the effects of H. pylori infection and play important roles in gastric carcinogenesis. The aim of this study was to determine whether the polymorphisms of TLR4 and TLR9 are associated with susceptibility to gastric carcinoma and its prognosis.

Methods: This study consisted of 314 patients with gastric cancer and 314 healthy controls. The polymorphisms were assessed using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis. Survival was analyzed by Kaplan–Meier survival curves.

Results: No variant genotypes of TLR4+896A/G, TLR4+1196C/T, or TLR9 -1237T/C were detected. For TLR9 -1486 T/C, multiple logistic regression analyses revealed that compared with the TT homozygote, patients with both the TC variant (adjusted odds ratio (OR) = 1.47, 95% confidence interval (CI) = 1.04–2.10) and the CC variant (adjusted OR = 1.63, 95% CI = 1.01–2.64) had higher risks of gastric cancer. Further stratification analyses revealed that an increased risk of gastric cancer associated with C carriers was evident among females (adjusted OR = 1.84, 95% CI = 1.02–3.33), in younger subjects aged less than 60 years old (adjusted OR = 1.86, 95% CI = 1.15–3.00), and subjects with H. pylori infection (adjusted OR = 1.53, 95% CI = 1.03–2.27). We also observed a significant association between C carriers and noncardia gastric cancer (adjusted OR = 1.51, 95% CI = 1.03–2.20). In addition, we demonstrated that the C carrier genotype and H. pylori infection may have a synergistic effect and conferred an OR of 2.44 for developing gastric cancer. TLR9 -1486C was also identified as an independent marker of poor survival of carcinoma.

Conclusions: Our results suggest that TLR9 -1486C carriers are associated with an increased risk and poor prognosis of gastric carcinoma in the Chinese population.

Introduction

Helicobacter pylori is a major cause of gastric cancer [1]. However, only a small percentage of H. pylori-infected patients actually develop gastric cancer. Hence, gastric tumorigenesis is multifactorial; the genetic composition of the infecting strains and the genetic make-up of the host, especially the variations in the host immune responses, are contributing factors [2]. Lichtenstein et al. reported that exposures to exogenous risk factors may contribute to a 72% increased risk of developing gastric cancer, and the host susceptibility may also play an important role in 28% of gastric cancer development [3].

The host immune response has a strong role in defining the outcome of H. pylori infection, and polymorphisms in genes that control this immune response have been shown to influence the risk of gastric cancer[4–6]. Toll-like receptors (TLRs) are important innate immunity regulators that can be activated upon recognition of bacterial and viral ligands, known as pathogen-associated molecular patterns (PAMPs)[7–8], and provide the first line of host defense against several conditions including H. pylori infection [9]. TLRs are characterized by the presence of an extracellular leucine-rich domain and an intracellular Toll/interleukin [IL]-1 receptor (TIR) domain [10]. To date, 13 related TLR genes have been identified and characterized (TLR1–TLR13) [11]. TLR4 and TLR9 are known to be expressed by gastric epithelial cells in the human stomach[12–13], which play an important role in innate immune signaling to H. pylori[14–16]. Some studies have reported controversial findings surrounding the role of TLR4 in activation of the innate immune response to H. pylori[17–18]. However, most studies have concluded that the TLR4/MD-2 system is a potent receptor complex involved in the response to H. pylori lipopolysaccharides in the stomach[14–15,19], with subsequent activation of NF-κB and production of proinflammatory cytokines [8,20]. TLR9 recognizes unmethylated
their association with tumor characteristics of clinical importance. We evaluated whether TLR polymorphisms affect the overall risk of gastric cancer. We also investigated the association between TLR functional polymorphisms and how they relate to the risk of gastric cancer.

**Study Population**

This study consisted of 314 patients with gastric cancer and 314 healthy controls. All subjects were unrelated ethnic Han Chinese and residents in Jiangsu Province, People’s Republic of China. Patients were consecutively recruited between July 2007 and July 2009, from Changzhou No. 2 Hospital, Affiliated with Nanjing Medical University and the First Affiliated Hospital of Nanjing Medical University. All cases were histologically confirmed as having adenocarcinoma of the stomach, and were classified into diffuse type, intestinal type and mixed type according to the Lauren classification by pathologists in participating hospitals. These patients were divided into two subgroups according to the location of the tumor: cardia cancer and noncardia cancers. We defined cardia cancer as a lesion primarily involving the area between 1.0 cm proximal and 2.0 cm distal to the gastroesophageal junction, and we classified lesions primarily involving the fundus, body, or antrum as noncardia cancers. The population controls were selected from cancer-free individuals living in the same residential areas as the cases and were frequency matched to the cases based on age (±5 years) and sex. The median duration of follow-up was 35 months (ranging from 3 to 56 months).

Clinical data of patients were collected from medical records and structured patient interviews using a questionnaire. Fifty-three of the patients were transferred to other hospitals with their medical history records or were unwilling to complete the questionnaires; in addition, some clinical data of patients were unable to be traced, leaving a total of 261 patients with sufficient clinical information available. After the interview, 2 mL of venous blood was collected from each subject.

**TLR Activation**

TLR activation plays a role in initiating the inflammatory response. Consequently, dysregulation of TLR signaling may contribute to an unbalanced ratio between pro-inflammatory cytokines and thus lead to a higher risk of developing chronic inflammatory diseases and cancer [9]. It is well known that chronic inflammation plays an important role in promoting gastric cancer [23–24]. Furthermore, the association between an IL-1 receptor (IL-1R) gene polymorphism and enhanced gastric cancer risk has been demonstrated [25–26]. Of note, TLRs are characterized by a TIR intracellular domain with high homology to IL-1R. For all these reasons, TLR polymorphisms should be studied as possible susceptibility factors in the development of gastric cancer.

Polymorphisms in TLR4 have already been studied. Among them are +896A/G (SNP ID: rs 4986790) and +1196G/C (SNP ID: rs 4986791) in exon 3. These two nonsynonymous single nucleotide polymorphisms (SNPs) affect the TLR4 extracellular domain and result in amino acid exchanges: an aspartic acid for a glycine at position 299 (Asp299Gly) and a threonine for an isoleucine at position 399 (Thr399Ile) [27]. Worldwide, studies have shown inconsistent results regarding the association between TLR4 polymorphisms and gastric cancer [6,24,28].

Several SNPs have been identified within the TLR9 gene. The -1237T/C SNP (rs187084), located in the promoter region, alter the functional ability of TLR9 [29]. The variant alleles of these polymorphisms can possibly modify the response to *H. pylori* infection, thereby increasing the risk of gastric cancer.

The aims of the present work were to investigate the potential functional polymorphisms and how they relate to the risk of gastric cancer. We also investigated the association between TLR polymorphisms and the risk of *H. pylori* infection. In addition, we evaluated whether TLR polymorphisms affect the overall survival of gastric cancer patients, either independently or through their association with tumor characteristics of clinical importance.

**Materials and Methods**

**Ethics Statement**

This study was approved by the Ethics Committee of Nanjing Medical University. Written informed consents were obtained from all subjects before participating in the study.

**Study Population**

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**Serological Determination of *H. pylori* Infection**

An indirect solid-phase immunochromatographic assay was used to investigate the presence of immunoglobulin G (IgG) antibodies to *H. pylori* (Genelabs Diagnostics Pty Ltd., Singapore). This method was validated in our lab previously with an accuracy of 92.3% [30]. The test was performed in accordance with the manufacturer’s instructions. The absence of the “A” band, which is a control line, indicated an invalid result. The presence of an “A B” band or an “A C” band was categorized as positive for *H. pylori* infection.

**Genotype Analysis**

Genomic DNA was extracted from a leukocyte pellet by proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. The TLR4+896A/G, TLR4+1196G/C, and TLR9 -1237T/C polymorphisms were assessed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis as described previously [6,31–32]. TLR9 -1486T/C was also genotyped by PCR-RFLP. In brief, the primers of TLR9 -1486T/C were 5’-TCCGACCAGCAA-CAATTCATTA-3’ (forward) and 5’-CTGCTTCGAGTT- GACTGTGT-3’ (reverse), which generated a 499-bp fragment. The 20-μL PCR mixture contained 50 ng of genomic DNA, 12.5 pmol of each primer, 0.1 mM each deoxynucleotide triphosphate, 1× PCR buffer, 1.5 mM MgCl₂, and 1.0 U of Taq polymerase. The PCR profile for detecting the TLR9 -1486T/C polymorphism consisted of an initial melting step of 95°C for 4 min; 35 cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 30 s; and a final extension step of 72°C for 5 min. The PCR product was then digested with AflII (New England Biolabs, Beverly, MA, USA). The digested products were visualized on a 3.0% agarose gel containing ethidium bromide. The products that could be cleaved showed two bands at 172 and 327 bp, while those that could not be cleaved were indicated by the presence of an “A” band, whereas those that could not be cleaved had a single 499-bp band representing allele T, leaving a total of 261 patients with sufficient clinical information available. After the interview, 2 mL of venous blood was collected from each subject.

**Statistical Analysis**

Hardy–Weinberg equilibrium of alleles was assessed using chi-squared (χ²) analysis. For each characteristic including genotype frequencies, the differences in the distribution between cases and controls were tested using the chi-squared test. The association between the polymorphisms and gastric cancer risk was estimated using an odds ratio (OR) and a 95% confidence interval (CI). Logistic regression was used to control for potential confounders (sex, age, and *H. pylori* serology) and to estimate crude and adjusted ORs and 95% CIs. Kaplan–Meier survival curves and the log–rank test for trend were used to evaluate the relationship between TLR polymorphisms and the prognosis from the date of primary
diagnosis to the end of follow-up. Multivariate Cox regression analysis was performed to assess the prognostic value of the TLR polymorphism with adjustments for age, gender, H. pylori serology, and tumor, lymph node, and metastasis (TNM) stage. Differences were regarded significant at \( P<0.05 \). All data analyses were performed using SPSS software (version 13.0; Chicago, IL, USA).

Results

Study Population Characteristics

The cancer cases and controls seemed to be adequately matched in terms of sex and age. The median age was 60 years old (range, 21–84 years) for the patients and 59 years old (range, 22–85 years) for the controls (\( P=0.44 \)). There was no difference between patients and controls in terms of sex distribution (67.2% men in cases vs. 67.2% in controls; \( P=1.00 \)). The percentage of subjects who tested positive for \( H. \) pylori infection was significantly higher in the case group than in the control group (\( P=0.02 \)). In addition, \( H. \) pylori infection increased the risk of developing gastric cancer (OR = 1.51, 95% CI = 1.07–2.13). The characteristics of the subjects are summarized in Table 1.

Prevalence of TLR4-896A/G, TLR4-1196C/T, and TLR9-1237T/C in the Chinese Population

Table 1. Baseline characteristics of cases and controls.

| Characteristics       | Cases (n = 314) | Controls (n = 314) | \( P \) value |
|-----------------------|----------------|-------------------|--------------|
| Age (years)           |                |                   | 0.44         |
| Median                | 60             | 59                |              |
| Standard deviation    | 12.61          | 12.01             |              |
| Range                 | 21–85          | 22–85             |              |
| Sex                   |                |                   | 1.00         |
| Male                  | 211 (67.20%)   | 211 (67.20%)      |              |
| Female                | 103 (32.80%)   | 103 (32.80%)      |              |
| \( H. \) pylori       |                |                   | 0.02         |
| Seronegative          |                |                   |              |
| 81 (25.80%)           | 108 (34.39%)   |                  |              |
| Seropositive          |                |                   |              |
| 233 (74.20%)          | 206 (65.61%)   |                  |              |
| Location              |                |                   |              |
| Cardia                | 87 (27.71%)    |                   |              |
| Noncardia             | 208 (66.24%)   |                   |              |
| TNM stage             |                |                   |              |
| I                     | 53 (20.31%)    |                   |              |
| II                    | 37 (14.18%)    |                   |              |
| III                   | 111 (42.53%)   |                   |              |
| IV                    | 60 (22.99%)    |                   |              |
| Follow-up, months     |                |                   |              |
| Median                | 35             |                   |              |
| Range                 | 3–56           |                   |              |

TNM: Tumor, Lymph Node, Metastasis. Among 314 patients, 53 have missing TNM stage data.

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The genotype distributions of TLR9-1486 T/C in the cases and the controls are shown in Table 2. Compared with the TT homozygote, the C heterozygote (TC) (adjusted OR = 1.47, \( P=0.03 \), 95% CI = 1.04–2.10) and homozygote (CC) (adjusted OR = 1.63, \( P=0.04 \), 95% CI = 1.01–2.64) both had significantly higher gastric cancer risks (Table 2), suggesting a dominant effect of the C allele. Overall, C carriers (either TC or CC) were associated with a higher gastric cancer risk when compared with TT carriers (adjusted OR = 1.51, \( P=0.02 \), 95% CI = 1.08–2.11).

Stratification Analyses of TLR9-1486 T/C and the Risk of Gastric Cancer

Stratified analyses based on the dominant models are shown in Table 3. A significantly increased risk of gastric cancer associated with C carriers was evident among females (adjusted OR = 1.84, 95% CI = 1.02–3.33), subjects with \( H. \) pylori infection (adjusted OR = 1.53, 95% CI = 1.03–2.27), and noncardia gastric cancer patients (adjusted OR = 1.51, 95% CI = 1.03–2.20), compared with the TLR9-1486 TT genotypes. When stratified by the histologic subtype and tumor depth, the C carriers were found to be positively associated with intestinal gastric cancer (adjusted OR = 1.79, 95% CI = 1.93–2.69) and advanced gastric cancer (adjusted OR = 2.33, 95% CI = 1.43–3.80), compared with the TT genotype (Table 3). We evaluated the role of the TLR9-1486 T/C polymorphism in gastric cancer in different age groups. In those less than 60 years old, carriers of the C allele were significantly associated with gastric cancer with an OR of 1.86 (95% CI = 1.15–3.00), compared with those with the TT genotype. As shown in Table 4, patients who were C carriers with \( H. \) pylori infection (adjusted OR = 2.44, 95% CI = 1.36–4.36) were at a significantly increased risk of gastric carcinoma, compared with those with the TT genotype without \( H. \) pylori infection. However, because of the limited study sample size, all the results from stratified analyses were preliminary.

TLR9 Genotype and Disease Progression

The overall survival of gastric cancer patients was analyzed for dependence on TLR9-1486 genotypes using Kaplan-Meier survival curves. Kaplan–Meier survival curves showed that overall individuals had the same genotype: TLR4+896AA, TLR4+1196CC, and TLR9-1237TT. Thus, there were no TLR4+896A/G, TLR4+1196C/T, or TLR9-1237T/C polymorphisms among the ethnic Chinese examined.

Association of the TLR9-1486 T/C Polymorphism with Risks of \( H. \) pylori Infection

In our study population, the alleles at TLR9-1486 loci were in Hardy–Weinberg equilibrium (\( P=0.95 \)). We investigated the association between the TLR9-1486 T/C polymorphism and \( H. \) pylori infection risk in gastric cancer patients as well as in the control group. In the gastric cancer patients, the TLR9 genotype distribution was 31.33% TT, 51.50% TC, and 17.17% CC in \( H. \) pylori-positive patients; and 25.93% TT, 54.32% TC, and 19.75% CC in \( H. \) pylori-negative patients. Meanwhile, in the control group, the TLR9 genotype distribution was 40.29% TT, 44.66% TC, and 15.05% CC in \( H. \) pylori-positive subjects; and 36.11% TT, 51.85% TC, and 12.04% CC in \( H. \) pylori-negative subjects. No association was observed between the TLR9 genotype and the risk of \( H. \) pylori infection (\( P=0.64 \) and 0.46, respectively, by \( 3 \times 2 \) tables using the \( \chi^2 \)-test).

TLR9-1486 C Carriers were Associated with an Increased Risk of Gastric Cancer

The genotype distributions of TLR9-1486 T/C in the cases and the controls are shown in Table 2. Compared with the TT homozygote, the C heterozygote (TC) (adjusted OR = 1.47, \( P=0.03 \), 95% CI = 1.04–2.10) and homozygote (CC) (adjusted OR = 1.63, \( P=0.04 \), 95% CI = 1.01–2.64) both had significantly higher gastric cancer risks (Table 2), suggesting a dominant effect of the C allele. Overall, C carriers (either TC or CC) were associated with a higher gastric cancer risk when compared with TT carriers (adjusted OR = 1.51, \( P=0.02 \), 95% CI = 1.08–2.11).
survival of gastric cancer patients was associated with the TLR9 -1486 genotypes. There was a statistical difference among different TLR9 -1486 genotypes. Cox proportional hazards analysis with adjustments for age, sex, H. pylori infection, and TNM stage showed that TC (HR = 2.49, CI = 1.48–4.17, \( P \), 0.001) and CC (HR = 4.95, CI = 2.87–8.55, \( P \), 0.001) genotypes were significantly associated with poor survival, compared with the TT genotype. High TNM stage including III (HR = 3.91, CI = 1.86–8.23, \( P \), 0.001) and IV (HR = 10.04, CI = 4.70–21.45, \( P \), 0.001) certainly showed a strong association of poor overall survival, compared with stage I.

Discussion

In this study, we did not detect TLR4 +896G, TLR4 +1196T, or TLR9 -1237C alleles in gastric cancer patients and healthy controls in the Chinese population. All 628 subjects had the same genotype: TLR4 +896AA, TLR4 +1196CC, and TLR9 -1237TT. These results were similar to those found by Hang et al. who screened 491 Chinese textile workers in Shanghai and did not detect alleles +896G or +1196T of TLR4 [33]. Similarly, Ng et al. screened 799 healthy Chinese people and 467 systemic lupus erythematosus (SLE) patients in Hong Kong for TLR9 -1237T/C polymorphisms, and they found that only 12 people were C carriers [34]. Similar results have also been reported in other Japanese and Korean studies[28,35–36]. TLR4 +896G, TLR4 +1196T, and TLR9 -1237C alleles may be very rare, at least in Chinese, Korean, and Japanese populations; thus, this gene may not be a candidate for determining gastric cancer susceptibility in this area. Our results are in contrast with those previously reported in several Caucasian populations that identified TLR4+896G and TLR4+1196T alleles increased the risk of gastric carcinoma [6,37]. The reason for the differences between these reported results are probably due to racial differences.

In the present study, we found that the TLR9 -1486T/C polymorphism is associated with an increased risk of gastric cancer in the Chinese population. The precise mechanism of this phenomenon is unclear, although in silico analysis has shown that this SNP creates a putative Sp1 binding site, which may be functionally relevant [38]. In addition, an association between the C allele of TLR9- 1486T/C and an increased risk of SLE in the Japanese population has been found; and a reporter gene assay showed that this allele downregulates TLR9 expression [39]. Because regulation of TLR9 expression and signaling play important roles with respect to the immune response against H. pylori [40,41], this altered function may be relevant in carcinogenesis of the stomach. To date, there are no functional data available for the TLR9 -1486T/C polymorphism with regard to gastric cancer. We intend to assess TLR9 expression in gastric epithelial cells by immunohistochemistry and real-time quantita-

### Table 2. The genotype distributions of TLR9-1486 T/C in cases and controls.

| Genotype | Controls (314) | Patients (314) | Crude OR | Gastric Carcinoma |
|----------|----------------|----------------|----------|------------------|
|          | No. | %      | No. | %      | 95% CI | \( P \) | Adjusted OR* | 95% CI | \( P \) |
| TT       | 122 | 38.9% | 94  | 29.9% | 1.00  |        |        |        |        |
| TC       | 148 | 47.1% | 164 | 52.2% | 1.44  | 0.04   | 1.47   | 0.03  | 1.04–2.10 | 0.03   |
| CC       | 44  | 14.1% | 56  | 17.8% | 1.65  | 0.04   | 1.63   | 0.04  | 1.01–2.64 | 0.04   |
| Recessive model | Others | 270 | 85.9% | 258 | 82.2% | 1.00  |        |        |        |
|          | CC  | 44  | 14.1% | 56  | 17.8% | 1.33  | 0.19   | 1.33   | 0.20  | 0.86–2.05 | 0.20   |
| Dominant model | TT | 122 | 38.9% | 94  | 29.9% | 1.00  | 0.02   | 1.51   | 0.02  | 1.08–2.11 | 0.02   |
|          | Others | 192 | 61.1% | 220 | 70.1% | 1.49  |        |        |        |

*Adjusted for age, sex, and H. pylori infection.

OR, odds ratio; CI, confidence interval.

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### Table 3. Stratified analyses between TLR9 -1486 T/C polymorphisms and gastric cancer risk.

| variable | T-1486C (case/control) | OR (95% CI)* | \( P \) |
|----------|-------------------------|--------------|--------|
| TT       | TC+CC                   |              |        |
| Age      | <60                     | 1.86 (1.15–3.00) | 0.01  |
|          | \( \geq 60 \)          | 1.36 (0.84–2.19) | 0.22  |
| Sex      | Male                    | 1.44 (0.95–2.16) | 0.08  |
|          | Female                  | 1.84 (1.02–3.33) | 0.04  |
| Hp infection | Positive | 1.53 (1.03–2.27) | 0.03  |
| Location site  | Cardia | 1.15 (0.70–1.90) | 0.58  |
|          | Non-cardia             | 1.51 (1.03–2.20) | 0.03  |
| Lauren's | Intestinal             | 1.79 (1.93–2.69) | 0.01  |
|          | Diffuse                | 1.59 (0.94–2.71) | 0.09  |
| Tumor depth | Early  | 1.15 (0.61–2.17) | 0.68  |
|          | Advanced               | 2.33 (1.43–3.80) | 0.01  |

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

OR, odds ratio; CI, confidence interval.

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action between suggesting a potential gene–environment interaction. The inter- and has been classified by the World Health Organization (WHO) follows.

H. pylori important in recognizing receptor [40]. TLR9 expression and signaling is quite
critical for the protection and recovery from gut injury [44]. A
modulation of human gastric epithelial cells in primary cultures by incubation with CpG oligodeoxynucleotides to investigate whether differential signaling and cellular responses occur in patients expressing different TLR9 SNPs.

Our results are inconsistent with the study conducted in another Chinese population that demonstrated that the TLR9 -1486T/C polymorphism was not associated with an increased gastric cancer risk [42]. The reason for these different findings remains unclear, which might due to heterogeneous genetic backgrounds. Our study conducted in Nanjing and Changzhous county, two areas in Jiangsu province, southern China, however, Zeng HM et al. conducted the study in Linqu county, Shangdong province, northern China. Heterogeneous genetic backgrounds in different populations is likely to explain the discrepancy in the results.

Considering the potential functional relevance between TLR9 and H. pylori infection, we were also interested in evaluating the possible interaction between the TLR9 polymorphism and H. pylori infection. In our study, we observed significant joint effects of the TLR9 -1486T/C polymorphism and H. pylori infection, suggesting a potential gene–environment interaction. The interaction between H. pylori infection and the presence of the C allele conferred an OR of $>2.0$ for developing gastric cancer. The molecular mechanisms underlying this phenomenon may be as follows. H. pylori is an established etiologic factor for gastric cancer and has been classified by the World Health Organization (WHO) as a Class I human carcinogen. TLR9 is an important H pylori-recognizing receptor [40]. TLR9 expression and signaling is quite important in H. pylori-associated gastric cancer [43]. In addition, appropriate activation of Toll-like receptors has been shown to be critical for the protection and recovery from gut injury [44]. A study has shown that the C allele of TLR9 -1486T/C downregulates TLR9 expression [39], which may affect H. pylori recognition. Thus, this polymorphism may lead to persistent infection. Therefore, C carriers increase the risk for the development of intestinal-type gastric cancer. When stratified by sex, the association was restricted to females, but there is the possibility that this finding may be attributed to chance due to the limited numbers of patients.

The TNM stage, which is a widely accepted prognostic factor, significantly correlated with the survival of gastric cancer patients in our study. We also found that the TLR9 -1486C allele was an independent prognostic factor by Cox proportional hazard analysis of survival in this study. The molecular mechanism of this phenomenon is unclear, and further studies concerning the TLR9 -1486C allele and poor prognosis are needed.

Some limitations in our study need to be addressed. First, inherent selection bias cannot be completely excluded. The cancer cases are from two hospitals and may not be representative of the entire target population. However, we reduced the selection bias to the lowest possible level by matching the age, residential areas, and gender between the cases and controls. Second, alcohol and tobacco smoking are well-known causes of gastric cancer. Genetic polymorphisms may interact with these factors to influence gastric cancer risk. Unfortunately, we did not have available information on these factors in our study, and therefore we were unable to perform further analysis.

We investigated the TLR9 -1486T/C polymorphism in a limited region of China. Because the polymorphism may show variations in different ethnic groups, further studies are needed in a larger and ethnically diverse population to confirm the impact of this gene on the susceptibility of gastric cancer.

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Author Contributions
Conceived and designed the experiments: L. Xue GZ. Performed the experiments: XW L. Xu. Analyzed the data: XW YY. Contributed reagents/materials/analysis tools: GZ L. Xu. Wrote the paper: XW L. Xue.

Table 4. Association between TLR9 -1486 T/C and gastric carcinoma in relation to H. pylori infection.

| TLR9 -1486 T/C | H. pylori infection | Odds ratio* (95% Confidence interval) | P value |
|----------------|---------------------|-------------------------------------|---------|
| TT             | Negative            | 1.00                                |         |
| TT             | Positive            | 1.61 (0.86–2.99)                    | 0.13    |
| TC+CC          | Negative            | 1.67 (0.87–3.22)                    | 0.12    |
| TC+CC          | Positive            | 2.44 (1.36–4.36)                    | <0.001  |

*Adjusted for age and sex.
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tive PCR experiments and determine its correlation with the genetic polymorphisms. We also intend to assess the functional modulation of human gastric epithelial cells in primary cultures by incubation with CpG oligodeoxynucleotides to investigate whether differential signaling and cellular responses occur in patients expressing different TLR9 SNPs.

is the most important risk factor for cancer. Therefore, the increased risk associated with being a C carrier in older subjects may be overwhelmed by the presence of more genomic aberrations and the deleterious alterations of the immune system in them, which may partly contribute to the age difference we observed. We also found that the C carriers were positively associated with intestinal gastric cancer. The intestinal gastric cancer develops in stomachs affected by chronic inflammation with passing through the intermediate steps of atrophic gastritis or intestinal metaplasia. The C allele of TLR9 -1486T/C downregulates TLR9 expression [39], which may affect H. pylori recognition. Thus, this polymorphism may lead to persistent infection. Therefore, C carriers increase the risk for the development of intestinal-type gastric cancer. When stratified by sex, the association was restricted to females, but there is the possibility that this finding may be attributed to chance due to the limited numbers of patients.

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| TLR9 -1486 T/C | H. pylori infection | Odds ratio* (95% Confidence interval) | P value |
|----------------|---------------------|-------------------------------------|---------|
| TT             | Negative            | 1.00                                |         |
| TT             | Positive            | 1.61 (0.86–2.99)                    | 0.13    |
| TC+CC          | Negative            | 1.67 (0.87–3.22)                    | 0.12    |
| TC+CC          | Positive            | 2.44 (1.36–4.36)                    | <0.001  |

*Adjusted for age and sex.
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tive PCR experiments and determine its correlation with the genetic polymorphisms. We also intend to assess the functional modulation of human gastric epithelial cells in primary cultures by incubation with CpG oligodeoxynucleotides to investigate whether differential signaling and cellular responses occur in patients expressing different TLR9 SNPs.

is the most important risk factor for cancer. Therefore, the increased risk associated with being a C carrier in older subjects may be overwhelmed by the presence of more genomic aberrations and the deleterious alterations of the immune system in them, which may partly contribute to the age difference we observed. We also found that the C carriers were positively associated with intestinal gastric cancer. The intestinal gastric cancer develops in stomachs affected by chronic inflammation with passing through the intermediate steps of atrophic gastritis or intestinal metaplasia. The C allele of TLR9 -1486T/C downregulates TLR9 expression [39], which may affect H. pylori recognition. Thus, this polymorphism may lead to persistent infection. Therefore, C carriers increase the risk for the development of intestinal-type gastric cancer. When stratified by sex, the association was restricted to females, but there is the possibility that this finding may be attributed to chance due to the limited numbers of patients.

The TNM stage, which is a widely accepted prognostic factor, significantly correlated with the survival of gastric cancer patients in our study. We also found that the TLR9 -1486C allele was an independent prognostic factor by Cox proportional hazard analysis of survival in this study. The molecular mechanism of this phenomenon is unclear, and further studies concerning the TLR9 -1486C allele and poor prognosis are needed.

Some limitations in our study need to be addressed. First, inherent selection bias cannot be completely excluded. The cancer cases are from two hospitals and may not be representative of the entire target population. However, we reduced the selection bias to the lowest possible level by matching the age, residential areas, and gender between the cases and controls. Second, alcohol and tobacco smoking are well-known causes of gastric cancer. Genetic polymorphisms may interact with these factors to influence gastric cancer risk. Unfortunately, we did not have available information on these factors in our study, and therefore we were unable to perform further analysis.

We investigated the TLR9 -1486T/C polymorphism in a limited region of China. Because the polymorphism may show variations in different ethnic groups, further studies are needed in a larger and ethnically diverse population to confirm the impact of this gene on the susceptibility of gastric cancer.

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Author Contributions
Conceived and designed the experiments: L. Xue GZ. Performed the experiments: XW L. Xu. Analyzed the data: XW YY. Contributed reagents/materials/analysis tools: GZ L. Xu. Wrote the paper: XW L. Xue.

Table 4. Association between TLR9 -1486 T/C and gastric carcinoma in relation to H. pylori infection.
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