The Role of TLR2, TLR4 and CD14 Genetic Polymorphisms in Gastric Carcinogenesis: A Case-Control Study and Meta-Analysis

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Abstract

Background: In addition to Helicobacter pylori infection, host genetic factors contribute to gastric cancer (GC). Recognition of H. pylori is known to involve Toll-like receptors (TLR), which subsequently leads to activation of NF-κB. Thus, the overall aim of this study was to estimate for the first time the pooled effect size of polymorphisms in TLR2, TLR4 and CD14 on GC development through a meta-analysis.

Methods: A case-control study comprising 284 ethnic Chinese individuals (70 non-cardia GC cases and 214 functional dyspepsia controls) was conducted for the genotyping of TLR2 -196 to -174del, CD14 -260 C/T and TLR4 rs11536889 using PCR, RT-PCR and mass spectrometry. Case-control studies of TLR2, TLR4 and CD14 polymorphisms and GC were searched up to June 2012. Pooled odds ratios and 95% confidence intervals were obtained by means of the random effects model.

Results: In our ethnic Chinese case-control study, the TLR4 rs11536889 C allele increased the risk of GC (OR: 1.89, 95%CI: 1.23–2.92) while the CD14 -260 T allele was protective (OR: 0.62, 95%CI: 0.42–0.91). TLR2 -196 to -174 increased the risk of GC only in H. pylori-infected individuals (OR: 3.10, 95%CI: 1.27–7.60). In the meta-analysis, TLR4 Asp299Gly showed borderline results in the general analysis (pooled OR: 1.58, 95%CI: 0.98–2.60), nevertheless, stratified analysis by ethnicity showed that the mutant allele was a definitive risk factor for GC in Western populations (pooled OR: 1.87, 95%CI: 1.31–2.65). There was a potential association between the TLR2 -196 to -174 deletion allele and GC in Japanese (pooled OR: 1.18, 95%CI: 0.96–1.45). TLR4 Thr399Ile did not provide significant results.

Conclusions: TLR4 rs11536889 and CD14 -260 C/T are associated with non-cardia GC in Chinese. Based on our meta-analysis, the TLR signalling pathway is involved in gastric carcinogenesis, TLR4 Asp299Gly and TLR2 -196 to -174del showing associations with GC in an ethnic-specific manner.

Introduction

Despite a major decline in incidence and mortality rates over several decades, gastric cancer (GC) still remains a major cause of morbidity and mortality worldwide [1]. According to global cancer statistics, 934,000 new cases were diagnosed in 2002, which represents 8.6% of all cancers worldwide [2]. Almost two thirds of GC cases occur in East Asia, Eastern Europe and Central and South America, these regions being classified as high risk-GC populations (age-standardised rates in men >20 per 100,000) [3]. The incidence of GC in Chinese individuals resident in China represents 42% of the above worldwide estimation, with Chinese ethnicity identified as an independent risk factor for the development of GC in multiracial studies [2,3,4]. Chronic inflammation has been associated with an increased risk of developing several human malignancies, including GC [5]. In 1988, Correa proposed a human model of intestinal-type gastric carcinogenesis [6]. The model hypothesised a sequence of events progressing from inflammation to atrophy, to metaplasia, to dysplasia, to carcinoma in situ, and finally to invasive GC. Although Helicobacter pylori has been established as the most important aetiological risk factor for GC, the pathogenesis of GC involves the combined effects of bacterial, host and environmental factors [7,8,9,10,11]. Given that H. pylori is initially targeted by the toll-like receptors (TLR) signalling pathway, it is conceivable that functionally relevant polymorphisms in genes of this arm of the immune system could affect the magnitude and direction of the host response against the infection.
The involvement of the TLR signalling pathway in infectious, autoimmune and inflammatory diseases is well accepted [12]. TLR are pattern recognition receptors (PRR) for the innate immune system that recognise a wide variety of molecules. TLR4 was initially identified as the potential signalling receptor for H. pylori on gastric epithelial cells [13]. After forming a complex with the LPS-binding protein (LBP), H. pylori lipopolysaccharide (LPS) interacts with the monocyte differentiation antigen CD14 (CD14) [14]. Together with TLR4, this complex induces the TLR4-mediated MyD88-dependent signal transduction pathway, which leads to the activation of transcription factors, mainly NF-κB, and cytokines such as TNF-α, IL-1β, IL-6 and IL-12 [12]. Although most studies conclude that TLR4 is the first innate immune response against the microorganism, others argue that TLR2 is the initial barrier against infection [15,16,17]. Furthermore, an interesting publication by Smith et al. showed that H. pylori LPS functions as a classic TLR2 ligand and induces a discrete pattern of chemokine expression in epithelial cells which involves the modulation of the expression of signalling protein tribbles 3 (TRIB3), a molecule that has been implicated in the regulation of NF-κB [18]. Fortunately, a very recent study by Yokota et al. seems to provide a possible explanation for this dilemma [19]. Yokota et al., not only showed that H. pylori LPS is initially targeted by TLR2 as described by others, but, for the first time, showed that this TLR2 activation leads to cell proliferation and TLR4 expression via the ERK1/2-ERK1/2 kinase (MEK1/2) pathway [19]. The final outcome of this signalling pathway is increased proliferation of gastric epithelial cells and the instaura- tion of a strong inflammatory reaction. Interestingly, these authors also proposed that H. pylori can enhance inflammatory reactions mediated by TLR4 agonists such as other bacterial LPS which could contribute to gastric inflammation and carcinogenesis [19].

TLR2 is located in the long arm of chromosome 4 comprising two 5′ non-coding exons followed by a third coding exon. A polymorphism that causes a 22-bp deletion, known as TLR2 -196 to -174del, has been shown to influence the promoter activity of TLR2. This polymorphism has not only been associated with GC but also other inflammation-related cancers including gallbladder, bladder, prostate and cervical cancers [20,21,22,23,24].

TLR4 is located in chromosome 9q32–q33 and contains 4 exons. Polymorphisms in TLR4 have been extensively studied in several populations in an attempt to find associations with diverse pathologies such as cancer, atherosclerosis and infectious diseases [25,26,27]. Among these, TLR4 Asp299Gly (rs986790) and Thr399Ile (rs4986791) are located in the coding sequence resulting in amino acid substitutions that affect the TLR4 extracellular domain. Another functionally relevant polymorphism, identify as TLR4 rs11536889 through the application of matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry, the Sequenom MassARRAY iPLEX™ assay (San Diego, CA, USA [34,35], at the Australian Genome Research Facility Ltd, St Lucia, University of Queensland, Australia. Genotyping of TLR2 -196 to -174del polymorphism was conducted by standard PCR using a pair of primers designed by Tahara et al. [24]. Genotyping of CD14 -260 C/T was performed using real time-PCR. Hairpin primers were designed based on the methodology published by Hazbon et al. and Chan et al. [36,37]. The primer sequences and thermal cycling conditions for genotyping of TLR2 -196 to -174del and CD14 -260 C/T are outlined in Table 1.
We have previously genotyped TLR4 Asp299Gly (TLR4 Asp299Gly and Thr399Ile are in linkage disequilibrium) in a subset of the same ethnic Chinese population. The methodology and results have been published elsewhere [30].

The presence of H. pylori IgG antibodies in GC patients and FD controls was determined by means of an in-house enzyme-linked immunosorbent assay (ELISA), previously described by Mitchell et al. [39], which had high sensitivity and specificity in a Chinese population. In addition to ELISA, serum samples from GC patients shown to be H. pylori negative by ELISA were investigated further by Immunoblot (MPD Helico Blot 2.1, MP Biomedicals, Australia) according to the manufacturer’s instructions. These results have been published in part elsewhere [38].

**Meta-analysis**

**Literature Search Strategy.** Electronic databases (PUBMED, Scopus, Science Direct, Ovid, Biosis Previews, Scirus databases, Ingenta, Proquest, Informaworld, Nature Publishing Group, CINAHL, IMBIOMED, Scielo and LILACS) were searched up to June 2012 for all case-control studies evaluating TLR2, TLR4 and CD14 polymorphisms and risk of GC in humans. The search strategy included Medical Subject Headings (MeSH) terms and text words as described in Table 2. In addition, hand-searching was also conducted. No other limits or special features (e.g., explosion) were employed.

**Study Selection.** Studies were selected if they met the following conditions: diagnosis of primary GC based on histological assessment, the study had a case-control design, the selected polymorphisms in the control subjects satisfied the Hardy Weinberg equilibrium (HWE), it supplied sufficient information to calculate the OR and the manuscript was published as a full paper in a peer-reviewed journal, not as an abstract or similar type of summary.

**Data Extraction.** The following information was collected from each study: author, year of publication, journal, study population, tumour location (cardia and non-cardia GC), molecular technique, H. pylori status (where available), total cases, total controls and number of individuals harbouring each specific allele.

**Statistical Analysis**

In our case-control study, deviation from HWE was tested using the chi-square goodness-of-fit test ($\chi^2$). The direct count method was used to estimate the genotype and allele frequencies. The odds ratios (OR) and 95% confidence intervals (CI) were calculated by means of the Fisher’s exact probability test (two-tailed $p$-values). The data was analysed by means of the programs GraphPad Prism version 5.02 (GraphPad Software Inc; San Diego, USA) and SPSS version 19.0.0 (SPSS Inc; Chicago, USA).

For the meta-analysis, the allele frequencies from individual studies were calculated as the number of cases or controls harbouring at least one allele divided by the total number of chromosomes included in each of the corresponding groups. The pooled ORs were calculated by weighting individual ORs by the inverse of their variance. For each polymorphism, this pooled OR as well as the 95% CI were obtained by means of both the fixed and the random effects model. The random effects model was chosen as it assumes that there is a distribution of true effect sizes rather than one true effect, and it assigns a more balanced weight to each study. Heterogeneity was calculated by means of the Cochrans Q test ($Q$-value $<0.10$ is indicative of heterogeneity). Due to the low power of this statistical test when meta-analysis includes a small number of studies, the Higgins test ($I^2$) was also used to measure the degree of inconsistency in the results of the studies and to describe the percentage of total variation across
studies due to heterogeneity rather than chance. The terms low, moderate and high were assigned to \( I^2 \) values of 25%, 50% and 75%, respectively [40]. Sensitivity (one study removed) and stratified analyses by ethnic group, were conducted to assess the sensitivity of the results and to detect the possible causes of heterogeneity between studies. To assess publication bias, Funnel plots and the Egger’s regression asymmetry tests were used. Data was analysed using the program Comprehensive Meta-Analysis version 2 (Biostat, Englewood, NJ, 2004).

**Results**

**Ethnic Chinese Case-control Study**

Demographics including age, gender and *H. pylori* status of the 284 subjects included in our ethnic Chinese case-control study are outlined in Table 3.

The median age of GC patients was significantly higher than that of FD controls (65.8 vs 54.2, \( P \)-value: <0.0001), despite matching for age (±10 years, mean difference 10 years, \( P \)-value: <0.0001).

Male gender was found to be more predominant in GC patients than in FD controls, showing a positive association with the development of GC in this ethnic Chinese population (OR: 1.61, 95% CI: 1.06–2.44).

*H. pylori* infection was found to be present in (67%) of individuals in this study (56/70 GC patients and 134/214 FD controls) (Table 3). Comparison of the prevalence of *H. pylori* infection in GC patients and FD controls showed that *H. pylori* infection was a risk factor for the development of GC (OR: 2.14, 95% CI: 1.06–2.44).

All polymorphisms genotyped in our case-control study were found to be in HWE in the control group showing non-significant \( \chi^2 \) values (less than 3.84). Allele and genotype frequencies as well as ORs and 95% CIs are shown in Table 4. The *TLR4* rs11536889 C allele and CC genotype markedly increased the risk of GC in these ethnic Chinese individuals showing ORs of 5.29 (95% CI: 1.71–16.34), respectively. Individuals harbouring the *CD14* -260 T allele (OR: 0.62, 95% CI: 0.42–0.91) and TT genotype (OR: 0.37, 95% CI: 0.16–0.82) were found to be significantly protected against the development of GC. Although the *CD14* -260 CT genotype showed a tendency towards protection against GC, this was not statistically significant (OR: 0.66, 95% CI: 0.34–1.31). No significant association was found between *TLR2* -196 to 174del and GC in our case-control study.

Given that *H. pylori* is known to be a major risk factor associated with GC, further analyses were performed to assess the joint effect of *TLR2* -196 to -174del, *TLR4* rs11536889 and *CD14* -260 C/T and *H. pylori* infection on risk of GC. First, to confirm that no association existed between *TLR2* -196 to -174del, *TLR4* rs11536889 and *CD14* -260 C/T and risk of *H. pylori* infection, univariate statistical analyses were conducted, showing non significant results (Table 5). Second, statistical analyses were performed to assess not only the presence or absence of the selected polymorphisms but also *H. pylori* status in relation to the risk of GC, in an attempt to determine the existence of biological interaction in the form of synergism or antagonism (Table 6). Individuals harbouring the *TLR4* rs11536889 T allele and infected with *H. pylori* were found to be at most risk of developing GC (OR: 9.75, 95% CI: 2.77–34.34). In addition, the *TLR2* -196 to -174 deletion allele increased the risk of GC only in individuals infected with *H. pylori* (OR: 3.10, 95% CI: 1.27–7.60). In contrast, *CD14* -260 C/T analyses did not provide significant results (OR: 1.78, 95% CI: 0.37–8.56).

Data on *TLR4* Asp299Gly and Thr399Ile in our ethnic Chinese case-control study has been published previously [38], therefore the results of that study were included in our meta-analysis.

**Studies Included in the Meta-analysis**

Based on our literature search strategy, 33 studies investigating the association between *TLR2*, *TLR4* and *CD14* polymorphisms and risk of GC were identified for potential inclusion in the meta-analysis (Figure 1). Of these, 18 studies, including our case-control study, fulfilled the inclusion criteria after detailed evaluation. Five of these studies were included in the *TLR2* -196 to -174del meta-analysis [24,41,42,43], six in the *TLR4* Asp299Gly meta-analysis [38,41,44,45,46], three in the *TLR4* Thr399Ile meta-analysis [41,44,46], three in the *TLR4* rs11536889 meta-analysis [47,48] and six in the *CD14* -260 C/T meta-analysis [49,50,51,52]. Two of the articles by Hold et al. [45,49] included in the current meta-analyses were each divided into two separate studies according to the geographical location of the subjects. Therefore, Hold et al.-1 and -2 refer to Polish subjects and to subjects from the United States, respectively. The general characteristics of our ethnic Chinese case-control study and the other studies included in the meta-analysis are outlined in Table 7. The present meta-analysis met the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) statement requirements (Checklist S1).

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**Table 2.** Medical subject headings and text words used in the literature search strategy.

| Gene | MeSH terms and text words |
|------|---------------------------|
| CD14 | “Antigens, CD14” [Majr] OR “CD14” AND “Polymorphism, Genetic” [Mesh] AND “Stomach Neoplasm” [Mesh] OR “gastric cancer” |
| TLR4 | “Toll-Like Receptor 4/genetic” [Mesh] OR “TLR4” AND “Stomach Neoplasm” [Mesh] OR “gastric cancer” |
| TLR2 | “Toll-Like Receptor 2” [Mesh] OR “TLR2” AND “Stomach Neoplasm” [Mesh] OR “gastric cancer” |

MeSH: medical subject headings, NA: not available.

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**Table 3.** Clinical characteristics of gastric cancer patients and functional dyspepsia controls in our ethnic Chinese case-control study.

| Characteristic | GC cases (N = 70) | FD controls (N = 214) |
|----------------|-------------------|----------------------|
| Median age ± SD (years) | 65.8±13.8 | 54.2±13.0 |
| Male/Female N (%) | 42/28 (60/40) | 95/119 (44/56) |
| *H. pylori* positive N (%) | 56 (80) | 134 (63) |

GC: gastric cancer, FD: functional dyspepsia, N: total number, SD: standard deviation.

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Publication Bias

Funnel plots (not shown) and Eggers regression tests showed that no publication bias existed in the following meta-analyses: 1) TLR2 -196 to -174del risk of GC (t-value: 0.91, degrees of freedom (df): 3, P-value: 0.429), 2) TLR4 Thr399Ile and risk of GC (t-value: 0.294), and 3) TLR4 rs11536889 and risk of GC (t-value: 0.427). In contrast, potential publication bias was observed in the meta-analyses of CD14 -260 C/T and TLR2 Asp299Gly and risk of GC, respectively.

Association between TLR2 -196 to -174del and Risk of Gastric Cancer

For the meta-analysis of TLR2 -196 to -174del and risk of GC, 1364 GC patients and 2487 controls were included. The general analysis showed a potential association to exist between TLR2 -196 to -174del and risk of GC (pooled OR: 1.23, 95% CI: 0.89–1.70). After performing a stratified analysis by ethnicity an increased risk was observed in Japanese individuals (pooled OR: 1.18, 95% CI: 0.96–1.45) although did not reach significance (Figure 2A). Interestingly, when a sensitivity analysis (one study removed) was conducted, in which the study by Zeng et al. [43] was excluded, a significant association was observed between TLR2 -196 to -174del and risk of GC (pooled OR: 1.41, 95% CI: 1.05–1.90). Heterogeneity was high in the general analysis (Q: 32.28, P-value: <0.001, I²: 87.61%) and moderate in the stratified analysis (Q: 2.43, P-value: 0.119, I²: 58.77%).

Table 4. Association between TLR2, TLR4 and CD14 polymorphisms and risk of gastric cancer in ethnic Chinese individuals.

| Polymorphism | Genotype/Allele | Cases (N = 70) | Controls (N = 214) | OR (95% CI) | P-value*
|---------------|-----------------|---------------|-------------------|-------------|----------
| TLR2 -196 to 174del | ins/ins | 26 | 101 | 1 | 0.190
| | ins/del | 36 | 93 | 1.50 (0.84–2.68) | 0.430
| | del/del | 7 | 18 | 1.51 (0.57–4.00) | 0.208
| | ins | 88 | 295 | 1 | 0.028
| | del | 50 | 129 | 1.30 (0.87–1.95) | 0.131
| TLR4 rs11536889 | GG | 32 | 127 | 1 | 0.028
| | GC | 28 | 70 | 1.23 (0.98–1.55) | 0.131
| | CC | 8 | 6 | 5.29 (1.71–16.34) | 0.005
| | G | 92 | 324 | 1 | 0.028
| | C | 44 | 82 | 1.89 (1.23–2.92) | 0.005
| CD14 -260 C/T | CC | 18 | 34 | 1 | 0.028
| | CT | 38 | 108 | 1.66 (0.34–8.15) | 0.282
| | TT | 14 | 72 | 0.37 (0.16–0.82) | 0.021
| | C | 74 | 176 | 1 | 0.028
| | T | 66 | 252 | 0.62 (0.42–0.91) | 0.028

OR: odds ratio, CI: confidence intervals, ins: insertion, del: deletion. *Fisher’s exact test two-tailed P-value. This polymorphism was genotyped only in 69 GC patients and 212 FD controls.

Association between TLR4 Polymorphisms and Risk of Gastric Cancer

Meta-analysis of the 6 case-control studies (979 GC patients and 1404 controls) included in the TLR4 Asp299Gly analysis showed a borderline association with GC (pooled OR: 1.58, 95% CI: 0.98–2.63).

Table 5. Association between TLR2, TLR4 and CD14 polymorphisms and risk of Helicobacter pylori infection in ethnic Chinese individuals.

| Polymorphism | Genotype/Allele | H. pylori positive (N) | H. pylori negative (N) | OR (95%CI) | P-value*
|---------------|-----------------|------------------------|-----------------------|-------------|----------
| TLR2 -196 to 174del | ins/ins | 81 | 46 | 1 | 0.3534
| | ins/del | 90 | 39 | 1.31 (0.78–2.21) | 0.3562
| | del/del | 19 | 6 | 1.80 (0.67–4.82) | 0.208
| | ins | 252 | 131 | 1 | 0.3562
| | del | 128 | 51 | 1.31 (0.89–1.92) | 0.208
| TLR4 rs11536889 | GG | 110 | 50 | 1 | 0.4147
| | GC | 62 | 36 | 1.00 (0.46–1.33) | 0.7566
| | CC | 10 | 3 | 1.52 (0.40–5.75) | 0.8278
| | G | 282 | 136 | 1 | 0.8278
| | C | 82 | 42 | 0.94 (0.62–1.44) | 0.5312
| CD14 -260 C/T | CC | 41 | 11 | 1 | 0.1145
| | CT | 97 | 49 | 0.53 (0.25–1.12) | 0.0857
| | TT | 55 | 31 | 0.48 (0.21–1.06) | 0.1038
| | C | 179 | 71 | 1 | 0.1038
| | T | 207 | 62 | 0.74 (0.52–1.06) | 0.0857

OR: odds ratio, CI: confidence intervals, ins: insertion, del: deletion. *Fisher’s exact test two-tailed P-value. Genotyping information available for only 281 individuals.
2.56 (Figure 2B). In addition, stratified analysis by ethnicity showed the TLR4 Asp299Gly G allele to be a significant risk factor for GC in Western populations (pooled OR: 1.87, 95% CI: 1.31–2.65) (Figure 2C). Low to moderate heterogeneity was detected in the TLR4 Asp299Gly meta-analyses (Q: 11.44, P-value: 0.043, I²: 56.29% and Q: 5.48, P-value: 0.241, I²: 27.04% for the general and stratified by ethnicity analyses, respectively).

The meta-analysis of TLR4 Thr399Ile included three studies comprising 423 GC patients and 612 controls. Both the general (pooled OR: 1.56, 95% CI: 1.05–2.30) and stratified by ethnicity (pooled OR: 0.81, 95% CI: 0.50–1.34 in Mestizos) analyses showed to have a statistically significant association between this polymorphism and the risk of GC. In contrast, sensitivity analysis showed significant results after excluding the study by Zhao et al. [52] was excluded (pooled OR: 0.87 95% CI: 0.64–1.13) or when stratified by ethnicity (pooled OR: 0.89 95% CI: 0.64–1.22 and pooled OR: 0.98 95% CI: 0.77–1.25 in Asians and Caucasians, respectively). However, borderline results were obtained after performing sensitivity analysis, in which the study by Zhao et al. [52] was excluded (pooled OR: 0.87 95% CI: 0.72–1.05). High heterogeneity was observed in the CD14 -260 C/T meta-analysis (Q: 16.77, P-value: 0.005, I²: 70.18% for the general analysis).

**Association between TLR2, TLR4 and CD14 Polymorphisms and Risk of Helicobacter pylori Infection**

In order to determine the association between TLR2 -196 to -174del, TLR4 Asp299Gly, TLR4 Thr399Ile, TLR4 rs11536889 and CD14 -260 C/T and H. pylori infection, we performed a meta-analysis with only those case-control studies that provided H. pylori status in relation to the genotype. Six studies, including our ethnic Chinese case-control study, provided sufficient data to calculate the OR and 95% CIs [38,42,44,48,52]. No statistically significant associations were found (Figure 3). Diverse heterogeneity was observed (Q: 1.93, P-value: 0.163, I²: 48.15%; Q: 2.03, P-value: 0.154, I²: 50.83%; Q: 0.32, P-value: 0.854, I²: 0% and Q: 4.42, P-value: 0.036, I²: 77.35% for TLR2 -196 to -174del, TLR4 Asp299Gly, TLR4 rs11536889 and CD14 -260 C/T, respectively).

**Discussion**

According to extensive epidemiological evidence, H. pylori is a risk factor for the development of non-cardia GC [8,10,11]. Current evidence would suggest that H. pylori is initially recognised by TLR2 and 4, members of the TLR signalling pathway, which initiate an innate immune response in the host. Thus, we postulated that polymorphisms in TLR2, TLR4 and CD14, genes that encode key components of the TLR signalling pathway, might affect the magnitude of the host response against H. pylori infection leading to severe long-term outcomes such as GC. Given that available studies assessing the association between genetic polymorphisms of molecules involved in the TLR signalling pathway and risk of GC were limited and conflicting, we conducted a global meta-analysis to increase the statistical power of our case-control study, to detect the pooled effect size and its magnitude, and to identify potential causes for the previous conflicting findings in other studies. General, sensitivity and stratified analyses were conducted in the current meta-analysis of TLR2 -196 to -174del, TLR4 Asp299Gly, TLR4 Thr399Ile, TLR4 rs11536889 and CD14 -260 C/T polymorphisms and the risk of GC.

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**Table 6. Effect of Helicobacter pylori infection and TLR2, TLR4 and CD14 polymorphisms on gastric cancer risk.**

| HP status | CD14 -260 C/T | Cases | Controls | OR  | 95% CI  | P-value* |
|-----------|--------------|-------|----------|-----|--------|----------|
| HP (+)    | C carrier    | 8     | 31       | 4.04| 0.99–16.44 | 0.053    |
| HP (+)    | GG           | 28    | 81       | 5.42| 1.56–18.79 | 0.0043   |
| HP (-)    | deletion carrier | 4 | 41       | 0.54| 0.15–2.00 | 0.5223   |
| HP (+)    | ins/ins      | 19    | 62       | 1.71| 0.66–4.44  | 0.3612   |
| HP (+)    | deletion carrier | 39 | 70       | 3.10| 1.27–7.60  | 0.0121   |

HP: Helicobacter pylori, OR: odds ratio, CI: confidence intervals. * Fisher’s exact test two-tailed P-value.

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Our ethnic Chinese case-control study failed to show an association with TLR2 -196 to -174del. Consistent with these findings, meta-analysis of TLR2 -196 to -174del and risk of GC showed that only in Japanese individuals TLR2 -196 to -174del might be a risk factor for GC. Further case-controls studies are required to establish if this association is present in other ethnic groups including Caucasians. Given that TLR2 is known to stimulate the release of IL-8 in response to H. pylori infection [53,54,55], it is possible that in subjects carrying the TLR2 -196 to -174 del/del genotype, which has been associated with decreased transcriptional activity [56,57], IL-8 production might be reduced. Further functional studies are required to assess this question.

TLR4 polymorphisms provided interesting findings. Although the general analysis of TLR4 Asp299Gly, which included data from all studies, only showed borderline results, stratified analysis by ethnicity indicated that the TLR4 Asp299Gly G allele noticeably increased the risk of developing GC in the subgroup of individuals who belonged to Western populations. The fact that TLR4 Asp299Gly has been shown to disrupt the normal structure of the extracellular domain of TLR4, which has the potential to reduce responsiveness to H. pylori by diminishing the binding affinity of the bacterial ligands, increases the importance of our findings [58]. It has been suggested that this alteration might be responsible for prolonged infection and the subsequent chronic inflammation of the gastric mucosa, which has been related to an imbalance of pro- and anti-inflammatory cytokines [58]. Interestingly, Higgins et al. have shown that in TLR4-defective mice severe tissue destruction occurs due to an inadequate production of IL-10 [59]. This finding is consistent with the fact that IL-10 inhibits the production of IL-12 by activated macrophages and dendritic cells, a cytokine known to induce local production of IFN-γ from NK cells and T lymphocytes and to stimulate the subsequent adaptive immune system directing it towards a Th1 response and subsequent inflammation [60].

While TLR4 Thr399Ile failed to show a significant association with GC in the general analysis, after performing sensitivity analysis, in which the study by Garza-Gonzalez et al. [44] was excluded, significant results were found. Sensitivity analysis is an important tool in meta-analysis when studies of doubtful eligibility, as well as poor quality or outlier studies (studies showing very different results compared to the rest of the studies), may be present. Nevertheless, given the results obtained in our meta-analysis, which only included studies conducted in Western populations, and the low frequency of this polymorphism in Asian populations [61,62], it may be concluded that TLR4 Thr399Ile is not involved in gastric carcinogenesis.

Figure 1. Flow diagram of included/excluded studies.
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Table 7. Individual studies included in the meta-analysis of TLR2, TLR4 and CD14 polymorphisms and gastric cancer.

| Author                        | Year | Reference  | Population | Location* | Cases | Controls | Total | Polymorphism | OR (95% CI)  |
|-------------------------------|------|------------|------------|-----------|-------|----------|-------|--------------|--------------|
| Castaño-Rodríguez et al.      | 2012 | Current study | Chinese    | Non-cardia | 692   | 2121    | 281   | TLR2 -196 to -174 del | 1.30 (0.87–1.95) |
| de Oliveira and Silva         | 2012 | 41         | Brazilian  | NA        | 174   | 225      | 399   | TLR2 -196 to -174 del | 1.95 (1.38–2.74) |
| Zeng et al.                   | 2011 | 43         | Chinese    | NA        | 248   | 4961    | 744   | TLR2 -196 to -174 del | 0.71 (0.56–0.89) |
| Hishida et al.                | 2010 | 42         | Japanese   | NA        | 583   | 10971   | 1680  | TLR2 -196 to -174 del | 1.08 (0.93–1.26) |
| Tahara et al.                 | 2007 | 24         | Japanese   | Non-cardia | 289   | 4551    | 744   | TLR2 -196 to -174 del | 1.34 (1.07–1.67) |
| Total                         |      |            |            |           | 1364  | 2487     | 3851  |              |              |
| de Oliveira and Silva         | 2012 | 41         | Brazilian  | NA        | 174   | 225      | 399   | TLR2 -196 to -174 del |              |
| Schmidt et al.                | 2011 | 38         | Chinese    | Non-cardia | 60    | 162      | 222   | TLR4 Asp299Gly | 2.68 (1.24–5.81) |
| Santini et al.                | 2008 | 46         | Italian    | NA        | 171   | 151      | 322   | TLR4 Asp299Gly | 1.05 (0.46–2.37) |
| Hold et al.-1                 | 2007 | 45         | Caucasian* | Non-cardia | 312   | 419      | 731   | TLR4 Thr399Ile | 3.90 (1.30–11.72) |
| Hold et al.-2                 | 2007 | 45         | Caucasian5 | Non-cardia | 184   | 211      | 395   | TLR4 Asp299Gly | 1.85 (1.01–3.40) |
| Garza-Gonzalez et al.         | 2007 | 44         | Mexican    | Non-cardia | 78    | 2361     | 314   | TLR4 Asp299Gly | 1.07 (0.41–2.77) |
| Total                         |      |            |            |           | 423   | 612      | 1035  |              |              |
| Castaño-Rodríguez et al.      | 2012 | Current study | Chinese    | Non-cardia | 682   | 2032     | 271   | TLR4 rs11536889 | 1.89 (1.23–2.92) |
| Kupcinskas et al.             | 2011 | 48         | Caucasians | NA        | 113   | 236      | 349   | TLR4 rs11536889 | 1.03 (0.62–1.71) |
| Hishida et al.                | 2009 | 47         | Japanese   | NA        | 583   | 10561    | 1639  | TLR4 rs11536889 | 0.99 (0.84–1.16) |
| Total                         |      |            |            |           | 979   | 1404     | 2383  |              |              |
| Castaño-Rodríguez et al.      | 2012 | Current study | Chinese    | Non-cardia | 70    | 214      | 284   | CD14 -260 C/T | 0.62 (0.42–0.91) |
| Hold et al.-1                 | 2009 | 49         | Caucasian6 | Non-cardia | 327   | 389      | 716   | CD14 -260 C/T | 1.09 (0.89–1.35) |
| Hold et al.-2                 | 2009 | 49         | Caucasian7 | Non-cardia | 184   | 211      | 395   | CD14 -260 C/T | 0.85 (0.64–1.12) |
| Tahara et al.                 | 2007 | 50         | Japanese   | NA        | 143   | 94       | 237   | CD14 -260 C/T | 0.71 (0.49–1.02) |
| Zhao et al.                   | 2007 | 52         | Chinese    | NA        | 470   | 470      | 940   | CD14 -260 C/T | 1.26 (1.04–1.52) |
| Wu et al.                     | 2006 | 51         | Chinese    | Non-cardia | 204   | 210      | 414   | CD14 -260 C/T | 0.98 (0.75–1.29) |
| Total                         |      |            |            |           | 1398  | 1588     | 2986  |              |              |

NA: not available, OR: odds ratio, CI: confidence intervals. * GC location according to human stomach anatomy. 1 OR and 95% CI correspond to TLR2 -196 to -174del, TLR4 Asp299Gly, TLR4 Thr399Ile, TLR4 rs11536889 or CD14 -260 C/T allele analysis. This polymorphism was genotyped only in 69 gastric cancer patients and 212 functional dyspepsia controls. 5 539 subjects were excluded from the control group since they presented atrophic gastritis. 6 Non-cancer patients (individuals with normal gastric appearance, gastritis, gastric ulcers and peptic ulcers) and healthy individuals were included in the control group. 7 The study population is from Poland. 8 The study population is from the United States. 9 Twenty three individuals were excluded from the initial control group (N = 259) since they had atrophic gastritis and intestinal metaplasia. 10 This polymorphism was genotyped only in 68 gastric cancer patients and 203 functional dyspepsia controls. 11 536 subjects were excluded from the control group since they presented atrophic gastritis.

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Although our meta-analysis did not provide significant results for TLR4 rs11536889, our ethnic Chinese case-control study showed that individuals harbouring the TLR4 rs11536889 C allele and CC genotype were at an increased risk of developing GC. The finding that this specific polymorphism is only a risk factor for GC in Chinese individuals is consistent with other studies where a number of polymorphisms investigated in Asian and Caucasian individuals have shown different risk associations with GC according to ethnicity [63], which potentially could explain the variability in GC incidence between races.

The TLR4 rs11536889 is located in the centre of the 2818-bp TLR4 3'UTR and therefore, may affect mRNA stability [64]. Interestingly, this polymorphism has been shown not only to be relevant to gastric pathologies but also to other inflammation-related cancers. For example, two studies have shown a statistically significant association between TLR4 rs11536889 and the risk of prostate cancer [64,65].

Although our meta-analysis did not provide significant results for TLR4 rs11536889, our ethnic Chinese case-control study showed that individuals harbouring the TLR4 rs11536889 C allele and CC genotype were at an increased risk of developing GC. The finding that this specific polymorphism is only a risk factor for GC in Chinese individuals is consistent with other studies where a number of polymorphisms investigated in Asian and Caucasian individuals have shown different risk associations with GC according to ethnicity [63], which potentially could explain the variability in GC incidence between races. TLR4 rs11536889 is located in the centre of the 2818-bp TLR4 3'UTR and therefore, may affect mRNA stability [64]. Interestingly, this polymorphism has been shown not only to be relevant to gastric pathologies but also to other inflammation-related cancers. For example, two studies have shown a statistically significant association between TLR4 rs11536889 and the risk of prostate cancer [64,65].

CD14 -260 C/T showed a statistically significant association with GC in our ethnic Chinese case-control study. The results showed that the CD14 -260 T allele and TT genotype conferred protection against the disease in these subjects. In the meta-analysis, while the general and stratified by ethnicity analyses showed that the association between CD14 -260 C/T and the development of GC was not statistically significant, sensitivity analysis excluding the study by Zhao et al. [52], the only study that did not specify tumour location, provided borderline results inferring that the CD14 -260 T allele might be a protective factor against the development of non-cardia GC mainly, the main GC subtype in East Asian populations including our ethnic Chinese population. CD14 -260 C/T involves a C-T substitution at base-pair 260 of the 5'flanking region where the promoter, other enhancers and protein binding sites are likely to be located. Currently, there is some controversy regarding the influence of CD14 -260 C/T on the expression of soluble CD14 (sCD14). According to a number of studies, the CD14 -260 T allele is proposed to increase sCD14 production, which leads to increased serum levels of sCD14 [29,30,66,67]. In contrast, in subjects with H. pylori infection elevated sCD14 levels have been associated with the CD14 -260 CC genotype [52]. Furthermore, others argue that this polymorphism has no effect on transcription [68]. If the CD14 -260 CC genotype indeed increases sCD14 serum levels, it could explain why the T allele seems to be a protective factor as shown in our case-control study and meta-analysis. Since the current
evidence regarding the influence of CD14 -260 C/T on the expression of soluble CD14 (sCD14) is conflicting, more functional studies are required to clarify this issue.

As H. pylori is known to be the main risk factor for GC, we also examined the potential interaction between H. pylori and TLR2, TLR4 and CD14 polymorphisms in the development of GC. Our ethnic Chinese case-control study and meta-analysis showed that selected polymorphisms do not increase the risk of H. pylori infection. Furthermore, additional analyses in our ethnic Chinese population showed that the presence of these polymorphisms and H. pylori infection increases dramatically the risk of GC in these individuals. Not only H. pylori-infected subjects harbouring the TLR4 rs11536889 C allele showed the highest risk of all but also H. pylori-infected individuals harbouring the TLR2 -196 to -174 deletion allele were shown to be more susceptible to GC, a phenomenon that is not observed if the bacterium is absent. In contrast, the risk of GC in H. pylori-infected individuals harbouring the CD14 -260 T allele, which was shown to be a protective factor in our initial analyses, was not magnified or reduced in the presence of H. pylori. Overall, these findings are consistent with our hypothesis that given that H. pylori is initially targeted by the TLR signalling pathway, polymorphisms in this branch of the innate immune system could modulate the direction and magnitude of the host response against the infection, explaining why only approximately 1% of H. pylori-infected individuals develop GC.

The results obtained in our meta-analysis differ from those presented by the other two available meta-analyses addressing the association between TLR4 polymorphisms and risk of cancer [27,31]. From a more generalized perspective, Jing et al. found that TLR4 Asp299Gly and Thr399Ile increase the risk of gastrointestinal cancers (combining oesophageal, gastric and colorectal cancers) showing ORs of 1.64 (95% CI: 1.02–2.64) and 2.01 (95% CI: 1.40–2.89), respectively [31]. In the context of GC alone, Zhang et al. [27], who attempted to address the association between TLR4 Asp299Gly and this pathology, showed an OR of 1.85 (95% CI: 1.06–3.22). However, the authors included a study in their meta-analysis that did not comply with the inclusion criteria of our meta-analysis since the subjects recruited in that case-control study did not suffer from GC but a ‘’GC phenotype’’ characterized by corpus-predominant gastritis or pangastritis [69]. Comparable to the meta-analysis by Jing et al. [31], Zhang et al. showed that TLR4 Thr399Ile is associated with cancer in general (combining gastric, cervical, colorectal, gallbladder and prostate cancers) showing an OR of 1.81 (95% CI: 1.18–2.77) [27]. In addition, Zhang et al. failed to show an association between TLR4 rs11536889 and risk of cancer after analysing prostate, gastric and hepatocellular cancers as a group [27]. Thus, the differences between our meta-analysis and the mentioned meta-analyses are due to the types of cancer analysed and the studies included in the analyses.

Our meta-analysis has some limitations. First, publication bias assessment suggested the possibility that studies addressing the association between CD14 -260 C/T and TLR4 Asp299Gly polymorphisms and risk of GC that showed negative results, were not published. Nevertheless, asymmetry tests have limited power when the number of included studies in a meta-analysis is small [70]. Although an extensive literature search was performed for this meta-analysis, it is always difficult to measure the extent of unpublished data. Second, the control groups were not homogeneous since both healthy individuals and non-cancer patients (e.g. individuals presenting gastritis, gastric ulcers and peptic ulcers) were included in some of the studies incorporated in the GC meta-
analysis. Third, since sensitivity analysis of TLR2 to -196 to -174del, TLR4 Thr399Ile and CD14 -260 C/T provided different results compared to the general analysis, these results must be taken with discretion.

In conclusion, based on our ethnic Chinese case-control study and meta-analysis, TLR2 -196 to -174del, TLR4 Thr299Gly and CD14 -260 C/T polymorphisms appear to play an important role in the development of GC inferring that polymorphisms in the TLR signalling pathway are clearly involved in gastric carcinogenesis. Continued research efforts directed towards the identification of major associations between molecules involved in innate immunity and H. pylori-related GC, and subsequent detection of their functional implications, have the potential to provide novel insights into targeted treatment in genetically-susceptible individuals, and thus, improve primary and secondary prevention of H. pylori-related GC, a disease that remains the second-leading cause of cancer-related deaths worldwide.

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