Original Article

Polymorphisms of *TLR9* gene are associated with a decreased risk of *H. pylori* infection in a Chinese population

Fang Gao[1#], Jindong Qin[2#], Xingru Wei[3], Xuyang Tian[4], Wenjie Dong[3], Tong Dang[4], Yanbin Jia[3,4*]

[1]School of Medical Technology, [2]Department of Sports Rehabilitation, [3]School of Basic Medicine and Forensic Medicine, Baotou Medical College, Baotou 014060, China; [4]Inner Mongolia Institute of Digestive Diseases, the Second Affiliated Hospital of Baotou Medical College, Baotou 014030, China

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[#]These authors contributed equally to this work.

Correspondence to: Yanbin Jia. School of Basic Medicine and Forensic Medicine, Baotou Medical College, 31 Jianshe Road, Donghe District, Baotou 014060, China. Email: jyb690318@hotmail.com; Tong Dang. Inner Mongolia Institute of Digestive Diseases, The Second Affiliated Hospital of Baotou Medical College, 30 Hudemulin Street, Qingshan District, Baotou 014030, China. Email: dtong999@sina.com.

Background: A series of evidence suggests that genetic variation in toll-like receptor (TLR) 9 might influence the outcome of Helicobacter pylori (*H. pylori*) infection and play an important role in gastric carcinogenesis.

Methods: We conducted a case-control study to evaluate TLR9 polymorphisms on the risk of *H. pylori* infection and non-cardia gastric cancer (GC) in a Chinese population. We genotyped a tagging single-nucleotide polymorphism (SNP), rs164640, and a potentially functional SNP, rs187084, by TaqMan technique among 288 patients with non-cardia GC and 281 controls. Unconditional logistic regression (LR) was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for SNPs in association with *H. pylori* infection and non-cardia GC risk.

Results: Our results indicated that among normal controls, the minor allele homozygotes of both SNPs were significantly associated with a decreased risk of *H. pylori* infection when compared with their major allele homozygotes (for rs164640: OR =0.41, 95% CI, 0.18–0.93; for 187084: OR =0.38, 95% CI, 0.17–0.85). However, neither of the two SNPs demonstrated a significant association with non-cardia GC risk.

Conclusions: Our results revealed that TLR9 polymorphisms might have effects on the risk of *H. pylori* infection, but they do not seem to contribute to the risk of non-cardia GC in our studied population.

Keywords: Helicobacter pylori (*H. pylori*); non-cardia gastric cancer (GC); single nucleotide polymorphism; toll-like receptor 9 (TLR-9)

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Introduction

Gastric cancer (GC) is a serious public health burden as the third leading cause of cancer death worldwide (1). Like many malignancies, it is accepted that gastric oncogenesis is a complex multifactorial process resulting from host genetics, lifestyle, and environmental factors (2). Epidemiological studies suggest that *Helicobacter pylori* (*H. pylori*) infection is the main cause of non-cardia GC, and

*The present address of Yanbin Jia: The Nursing School of Baotou Medical College, Baotou 014060, China.

H. pylori have been identified as a class I carcinogen (3,4). The cases with *H. pylori* infection have an increased 3–20 fold or even higher risk of developing non-cardia GC (5,6). As genetic polymorphisms are related to the diversity and inter-individual variation, they have been recently recognized as the major genetic elements influencing the GC risk.

H. pylori infection can initiate an inflammatory response through its components, which include lipopolysaccharides, DNA, etc., and these components are sensed by transmembrane Toll-like receptors (TLRs) (7). TLRs are critical innate immunity regulators and can be activated by pathogen-associated molecular patterns (PAMPs), which are shared by lots of microorganisms (8). In the human stomach, gastric epithelial cells express TLR2, TLR4, TLR5, and TLR9 (9), play a key role in the host's innate immunity against *H. pylori* (10,11). In particular, TLR9 identifies unmethylated CpG oligonucleotides in bacterial DNA (9). Many studies have shown the role of TLR9 in *H. pylori* infection and GC; thus, compared with the noninflamed gastric mucosa, TLR9 expression tends to be higher in the gastric epithelium in *H. pylori*-related gastritis, and TLR9 polarization seems to be a process dynamically influenced by *H. pylori* (12). TLR9 signaling pathway has anti-inflammatory effects on the early stages of *H. pylori*-related gastritis (13). *H. pylori* DNA can induce TLR9-mediated GC cell invasion (14). Also, TLR9 is aberrantly expressed in GC (15), and *TLR9* promoter polymorphism is correlated with both an increased risk of GC and poor prognosis (16).

Given that the TLR9 signaling pathway plays an important role in *H. pylori* infection and GC development, and dysregulation of the TLR9 signaling owing to a single-nucleotide polymorphism (SNP) may alter the ligand binding, we performed a case-control study to investigate the associations of *TLR9* genetic variations with *H. pylori* infection and GC risk, using tag-SNP and selected candidate functional SNPs in a Han Chinese population. Because non-cardia gastric and cardia GCs are different in their etiology, pathology, oncogenesis, and prognosis (17), and *H. pylori* infection is more related to an increased risk of non-cardia GC (5), we limited our cases to non-cardia GC patients in order to have a relatively more homogeneous subject group and enhance the association study power.

Methods

Subjects

We recruited 288 cases at the Cancer Hospital of Baotou between June 2008 and December 2010, as described previously (18,19). All patients were histopathologically diagnosed as incident non-cardia GC. The patients who had secondary cancer, recurrent cancer, radiotherapy, or chemotherapy were excluded. Meanwhile, 281 cancer-free individuals with no identifiable gastric disease or autoimmune disease were randomly collected as controls from a community health examination program. The controls were frequency-matched to the cases for age (±5 years) and sex. All subjects were unrelated ethnic Han Chinese residents in Baotou, Inner Mongolian Autonomous Region, northwest China. At recruitment, each subject was personally interviewed to gather demographic data and lifestyle factors such as smoking and alcohol consumption. Individuals who formerly or currently smoked ≥1 cigarette per day for more than one year were defined as smokers. Subjects that drunk at least twice a week for more than one year were defined as drinkers. No significant differences were found in age, gender, or drinking status between the two groups. There were more smokers in cancer cases than in controls (P=0.03). This study was approved by the institutional review board of Baotou Medical College, and informed consent was obtained from each subject.

Tests for H. pylori infection

H. pylori status was serologically analyzed in controls. Anti-*H. pylori* serum IgG antibody was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kits (Biohit, Helsinki, Finland). According to the manufacturer's recommendation, an anti-*H. pylori* IgG titer ≥30 EIU was defined as positive for *H. pylori* infection.

SNP Selection and genotyping

Using HapMap Phase 2 data of the Han Chinese population (http://hapmap.ncbi.nlm.nih.gov), SNP rs164640 was selected as a tagSNP by Tagger algorithm as implemented in Haploview. The screen area included the whole gene, 20 kb upstream of the first exon, and 10 kb downstream of

the termination of the last exon. Parameters of r^2>0.8 and a minor allele frequency (MAF) ≥0.05 in the Han Chinese population were used for selection. Besides, SNP rs187084 was reported to be associated with an increased risk of GC in a Chinese population (16), so we also analyzed this SNP in the present study.

Genomic DNA was isolated from peripheral blood using proteinase K digestion, followed by phenol-chloroform purification and ethanol precipitation. Both SNPs were genotyped by the TaqMan allelic discrimination using ABI assay-by-designs (Applied Biosystems, Foster City, CA, USA); the genotyping was finished at the Chinese National Human Genome Center, Beijing. For the two SNPs, the success rate of genotyping was higher than 97.9%. In order to achieve the purpose of quality control, about 5% of the samples with high-quality DNA were randomly selected and genotyped twice. The results were 100% consistent.

Table 1 Selected characteristics of study subjects

Variable	Controls, n (%)	Cases, n (%)	P value
Overall	281 [100]	288 [100]	
Gender			
Male	220 (78.3)	224 (77.8)	0.88
Female	61 (21.7)	64 (22.2)	
Age (year)			
Mean ± SD	59.10±11.57	59.48±11.23	0.69
Range	26–85	26–83	
Smoking status			
No	180 (64.1)	159 (55.2)	0.03
Yes	101 (35.9)	129 (44.8)	
Drinking status			
No	145 (51.6)	143 (49.7)	0.64
Yes	136 (48.4)	145 (50.3)	

Statistical analysis

All statistical analyses were performed by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Patients and controls were compared by Student's *t*-test for age and chi-square test (χ^2) for gender, smoking, and drinking status. Hardy-Weinberg equilibrium (HWE) was measured by a goodness-of-fit χ^2 test. The Haplotype frequencies were inferred by Haploview 4.0 program. Unconditional logistic regression (LR) was used to estimate the *H. pylori* infection and non-cardia GC risk in association with tested SNPs.

Results

The selected characteristics of the 288 cases with non-cardia GC and 281 normal controls are described in *Table 1*. The genotype frequencies of both SNPs followed HWE in controls.

In controls, both SNPs showed statistically significant associations with *H. pylori* infection (*Table 2*). Minor allele homozygotes of both SNPs were significantly associated with a decreased risk of *H. pylori* infection when compared with their major allele homozygotes, with adjusted OR = 0.41, 95% CI, 0.18–0.93 for rs164640 AA genotype, and adjusted OR =0.38, 95% CI, 0.17–0.85 for rs187084 GG genotype respectively. However, no significant association between any haplotypes and *H. pylori* infection risk was observed (*Table 3*).

Neither of the two SNPs analyzed demonstrated a

Table 2 Association between tested SNPs and *H. pylori* infection in controls

SNPs	Genotypes	*H. pylori* (+), n (%)[a]	*H. pylori* (–), n (%)[a]	OR (95% CI)[b]	P value
rs164640	GG	39 (32.8)	54 (34.8)	1	
	AG	70 (58.8)	68 (43.9)	1.40 (0.82–2.39)	0.21
	AA	10 (8.4)	33 (21.3)	0.41 (0.18–0.93)	0.03
rs187084	TT	41 (33.9)	52 (34.4)	1	
	TC	70 (57.9)	66 (43.7)	1.34 (0.79–2.27)	0.29
	CC	10 (8.3)	33 (21.9)	0.38 (0.17–0.85)	0.02

[a], sum of column did not add up to total study subjects because of missing data; [b], adjusted for age and sex. SNP, single-nucleotide polymorphism.

Table 3 Associations between haplotypes and *H. pylori* infection

Haplotypes[a]	*H. pylori* (+) (%)	*H. pylori* (–) (%)	OR (95% CI)[b]	P value
GT	62.1	55.7	1	
AC	36.9	42.8	0.77 (0.54–1.08)	0.14
GC	0.5	0.9	0.41 (0.04–4.04)	0.42
AT	0.5	0.6	1.12 (0.16–8.08)	0.87

[a], the SNP order was rs164640, rs187084; [b], adjusted for age and sex.

Table 4 Association between tested SNPs and non-cardia gastric cancer

SNPs	Genotype	Controls, n (%)[a]	Cases, n (%)[a]	OR (95% CI)[b]	P value
rs164640	GG	93 (33.9)	95 (33.7)	1	
	AG	138 (50.4)	134 (47.5)	0.95 (0.65–1.38)	0.78
	AA	43 (15.7)	53 (18.8)	1.21 (0.74–1.99)	0.45
rs187084	TT	93 (34.2)	96 (33.6)	1	
	TC	136 (50.0)	134 (46.9)	0.95 (0.65–1.38)	0.78
	CC	43 (15.8)	56 (19.6)	1.26 (0.77–2.05)	0.37

[a], sum of column did not add up to total study subjects because of missing data; [b], adjusted for age and sex. SNP, single-nucleotide polymorphism.

Table 5 Associations between haplotypes and risk of non-cardia gastric cancer

Haplotypes[a]	Cases (%)	Controls (%)	OR (95% CI)[b]	P value
GT	55.7	58.3	1	
AC	41.5	40.3	1.08 (0.85–1.37)	0.52
GC	1.4	0.7	2.02 (0.60–6.77)	0.25
AT	1.4	0.7	2.08 (0.62–6.98)	0.25

[a], the SNP order was rs164640, rs187084; [b], adjusted for age and sex.

significant association with the risk of non-cardia GC either by single SNP analysis or by haplotype analysis (*Tables 4,5*).

Furthermore, the additional models, adjusted for other factors, including smoking and drinking status, gave qualitatively similar results for the association between *H. pylori* infection or non-cardia GC and the tested SNPs (data not shown).

Discussion

In light of the extensive epidemiological evidence, *H. pylori* is an important risk factor for non-cardia GC development (3,4). Previous studies have shown that TLR9 is responsible for initiating innate immune responses to *H. pylori* CpG DNA (13), while TLR9 expression has been shown to be altered upon the *H. pylori* infection and GC (12,15). Because genetic variation, particularly in the polymorphic sites of the *TLR9* gene, may change the transcription and expression process and potentially influence the outcome of *H. pylori* infection, we performed a case-control study to evaluate the associations of *TLR9* polymorphisms with susceptibility to *H. pylori* infection and non-cardia GC risk. Our results revealed that *TLR9* SNP rs164640 and rs187084 were associated with a decreased risk of *H. pylori* infection, but not associated with non-cardia GC risk, suggesting that common genetic variations in the *TLR9* gene only play an important role at the early stage of the gastric carcinogenesis. Interestingly, some studies reported that TLR9 expression was upregulated in *H. pylori*-induced gastritis (12), but was not detectable in intestinal metaplasia or dysplasia, the important precursor lesions of GC, and only focally detectable in limited gastric tumor cells (15). Furthermore, variants in other genes that play a key role in *H. pylori*-related GC have also been recognized as risk factors in the precursor stages of the disease process but not at the cancer stage (20). The evidence mentioned above

indicates that our results might not be random.

It is generally accepted that host genetic factors could influence the susceptibility to *H. pylori* infection. Previous studies have shown that about 5% to 10% of a population remain uninfected with *H. pylori* under a higher exposure condition (21). A significantly higher concordance for *H. pylori* infection was found in monozygotic than in dizygotic twins, while host genetic variation could contribute 57% of the variation in acquiring *H. pylori* infection (22). The heterozygous variant of *TLR9* rs352140 favors the persistence of the *H. pylori* infection (23). Recently, a few studies have focused on SNP rs5743836 in the *TLR9* gene. One study has shown that rs5743836 is not associated with *H. pylori* infection in Caucasians (11). However, the minor allele of rs5743836 is very rare in the Chinese population (16). Because tagSNPs, which represent SNPs in a region of the genome with high linkage disequilibrium (LD), can identify genetic variations without genotyping every SNP in a chromosomal region, we genotyped tagSNP rs164640 as a surrogate to estimate the association between *TLR9* genetic variation and *H. pylori* infection in the present study, and found this SNP was associated with a decreased risk of *H. pylori* infection. The discrepancy between the two studies may be due to racial differences and a difference in the selection of study subjects. In our study, we analyzed the relationship between SNP and *H. pylori* infection only in normal controls with no identifiable gastric disease, but in Ng's study, the analyzed subjects including all infected patients with or without precancerous abnormalities. In addition, in silico analysis has shown that *TLR9* promoter SNP rs187084 creates a putative Sp1 binding site, which may be functionally relevant (24). Thus, we also analyzed this SNP in the present study and found it was also associated with the susceptibility to *H. pylori* infection. Based our data, rs164640 and rs187084 were in high LD with D'=0.954 and r^2=0.911. SNP rs187084 may be the real causal variant in *H. pylori* infection. The precise mechanism of both tested SNPs in the risk of *H. pylori* infection has to be elucidated in further studies.

Currently, a very limited number of studies have analyzed the association between *TLR9* polymorphisms and GC risk. For example, Hold *et al.* (25) reported that *TLR9* rs5743836 was not associated with GC in Caucasian populations. Trejo-de la *et al.* (26) found that rs5843836 and rs352140 in the *TLR9* gene were not associated with GC in a Mexican population. Another two studies suggested that rs187480 was not associated with the risk of GC in a northern Chinese population (27,28). However, Wang's study showed that rs187084 was associated with increased susceptibility to non-cardia GC, but was not associated with *H. pylori* infection in controls among an eastern Chinese population (16), which is inconsistent with our study. The reason for the discrepancy is unclear, but it could be attributable to other environmental risk factors or the heterogeneous genetic backgrounds between different subpopulations.

Some limitations of our study need to be addressed. First, we did not obtain information on *H. pylori* infection in some cases, which restricted us to adjust this potential confounding bias in the analysis. However, it is difficult to estimate *H. pylori* infection in GC patients because the loss of *H. pylori* from the stomach and decreased immune response always occurs during gastric tumorigenesis (29). Furthermore, patients could have possibly received long-term *H. pylori* eradication therapy, and significant serological changes would probably have occurred over the therapy process. Second, our sample size was relatively small, so our results were only considered to be exploratory screening in nature.

In conclusion, this preliminary study suggests that SNP rs164640 and rs187084 in the *TLR9* gene are not associated with non-cardia GC risk, but our findings offer the first evidence of the association between polymorphisms of the *TLR9* gene and a decreased risk for *H. pylori* infection in a Chinese population. Functional and future large-scale studies are needed to elucidate the role of genetic variations in the *TLR9* gene in the carcinogenesis of non-cardia GC.

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Footnote

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.11.45). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional review board of Baotou Medical College (No. 2012003). Informed consent was obtained from each subject.

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