Original article

No associations of *Toll-like receptor 2 (TLR2) -196 to -174del* polymorphism with the risk of *Helicobacter pylori* seropositivity, gastric atrophy, and gastric cancer in Japanese

ASAHI HISHIDA¹, KEITARO MATSUO², YASUYUKI GOTO¹, MARIKO NAITO¹, KENJI WAKAI¹, KAZUO TAJIMA²,
and NOBUYUKI HAMAJIMA¹

¹Department of Preventive Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

²Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Nagoya, Japan

Abstract

Background. Recently, the association between gastric cancer risk and a functional polymorphism of *Toll-like receptor 2 (TLR2)*, -196 to -174del, was reported for a Japanese population. This study aimed to confirm the associations of the polymorphism with the risk of gastric cancer, as well as *Helicobacter pylori* seropositivity and the risk of gastric atrophy in Japanese.

Methods. The study subjects were 583 histologically diagnosed gastric cancer patients and 1636 age- and sex-frequency-matched control outpatients who visited Aichi Cancer Center Hospital from the years 2001 to 2005. Serum anti-*H. pylori* IgG antibody and pepsinogens were measured to evaluate *H. pylori* infection and gastric atrophy, respectively. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by a logistic model.

Results. The age- and sex-adjusted ORs of gastric cancer were 1.13 (95% CI: 0.88–1.46) for *ins/del*, 1.17 (95% CI: 0.79–1.73) for *del/del*, and 1.14 (95% CI: 0.89–1.45) for *ins/del* + *del/del*, relative to the *ins/ins* genotype compared with gastric atrophy controls; none of these findings were statistically significant. The *TLR2* -196 to 174del polymorphism was not significantly associated with either *H. pylori* seropositivity or gastric atrophy.

Conclusion. Our study did not reproduce the association between gastric cancer risk and the *TLR2* -196 to -174del polymorphism in Japanese. Further examinations with sufficient numbers of study subjects are required to verify our findings.

Key words *Helicobacter pylori* · *TLR2* · Gastric atrophy · Gastric cancer · Genetic polymorphism

Introduction

Gastric cancer accounts for the greatest proportion of gastrointestinal tract cancers in Japan and other East

Asian countries, and is the second greatest cause of cancer deaths in the world [1, 2]. A number of studies have established that *Helicobacter pylori* infection is a feasible risk factor for gastric cancer through the development of gastric atrophy or subsequent precancerous lesions [3, 4]. Severe gastric atrophy and corpus-predominant gastritis, as well as intestinal metaplasia, are generally recognized as predominant predispositions to gastric cancer [5]. Host proinflammatory genetic factors, in combination with bacterial virulence factors such as CagA, have been shown to determine the severity of gastric damage and the subsequent clinical outcome of *H. pylori* infection [6, 7].

Toll-like receptors (TLRs) are type-I transmembrane proteins with extracellular leucine-rich repeat (LRR) motifs and an intracellular Toll/interleukin-1 receptor (TIR) domain [8]. Toll, the founding member of the TLR family, was initially implicated in the establishment of dorsal-ventral patterning in the early development of the *Drosophila* embryo. Mammalian TLRs have essential roles in the direct recognition of infectious agents, leading to the activation of both innate and adaptive immune responses via nuclear factor-kappa B (NF- κ B) signaling pathways. Ten different human TLRs have been identified [9]. Each TLR recognizes specific pathogen-associated molecular patterns (PAMPs), including the recognition of lipoproteins, lipoteichoic acid, and zymosan by TLR2; double-stranded (ds) RNA by TLR3; lipopolysaccharide (LPS) by TLR4; flagellin by TLR5; single-stranded (ss) RNA by TLR7/8; and C-phosphate-G (CpG) DNA by TLR9 [10]. Among them, TLR2 is reportedly expressed in *H. pylori*-infected gastric epithelial cells, and *H. pylori* induces NF- κ B activation and subsequent chemokine expression by gastric epithelial cells through TLR2. Biological examinations have revealed that the transfection of cells with TLR2 induced augmented expression of interleukin (IL)-8, macrophage inflammatory protein (MIP)-3 α , and growth-regulated oncogene (GRO)- α [11]. Thus, TLR2

is assumed to play essential roles in the host gastric mucosal defense against *H. pylori* infection.

Recently, an association between the *TLR2* -196 to 174 del polymorphism, which has been biologically shown to influence the promoter activity of *TLR2*, and the risk of gastric cancer was reported [12]. Accordingly, we conducted a case-control study, with our gastric cancer case-control dataset, to verify this reported association between the *TLR2* -196 to 174 del polymorphism and gastric cancer risk.

Subjects and methods

Subjects

The study subjects were participants in the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) study, in which first-visit outpatients were consecutively invited to provide lifestyle data and blood samples after giving their informed consent [13]. Among the participants who visited Aichi Cancer Center Hospital from the years 2001 to 2005, 583 patients diagnosed with gastric cancer (cases) and 1636 age- and sex-frequency-matched cancer-free outpatients (controls) were recruited. This gastric cancer case-control dataset is the same as the one previously described [14, 15]. Informed consent was obtained from all the subjects and the study protocol was approved by the Ethics Committees of Aichi Cancer Center and Nagoya University Graduate School of Medicine.

Laboratory tests

The serum samples of the participants were immediately stored at -20°C until analysis. Anti-*H. pylori* IgG antibody was measured with a direct enzyme-linked immunosorbent assay (ELISA) kit, E Plate "Eiken" *H. pylori* Antibody (Eiken Kagaku, Tokyo, Japan). According to the instructions for this kit, a finding of 10.0 units or higher was regarded as seropositive. Serum pepsinogens (PGs) were measured by chemiluminescence enzyme immunoassay (CLEIA). Gastric mucosal atrophy was grouped into categories of "none" (PG I > 70 ng/ml or PG I/PG II > 3), "mild" (PG I $\leq$ 70 ng/ml and PG I/PG II $\leq$ 3, excluding "severe" cases), or "severe" (PG I $\leq$ 30 ng/ml and PG I/PG II $\leq$ 2). Because it was planned to use the serum samples of the gastric cancer cases for a study with higher priority, the *H. pylori* antibody and PG levels of the cases were not measured.

Genotyping

DNA was extracted from the buffy coat with a DNeasy mini-kit (QIAGEN, Tokyo, Japan). The *TLR2* -196 to -174del polymorphism was genotyped with a poly-

Fig. 1. Agarose gel (2%) showing the different genotypes for the Toll-like receptor 2 (*TLR2*) -196 to 174del polymorphism. Lane M, 100-bp marker; lane 1, *ins/ins* genotype (286-bp band); lane 2, *ins/del* genotype (286- and 263-bp bands); lane 3, *del/del* genotype (263-bp band); lane 4, newly found genotype with the long insertion allele and the *del* allele

merase chain reaction (PCR). The primers were: F1, CAC GGA GGC AGC GAG AAA; R1, CTG GGC CGT GCA AAG AAG, as described previously [12]. Amplification conditions were 10 min of initial denaturation at 95°C , followed by 35 cycles of 1 min at 95°C , 1 min at 62°C , and 1 min at 72°C , then a 5-min final extension at 72°C . The amplified DNA was visualized on a 2% agarose gel with ethidium bromide staining. The amplified DNA was 286 bp for the *ins* allele and 263 bp for the *del* allele. We found a new form of *TLR2* variant allele with a longer insertion than that of the common *ins* allele in one control subject, who was excluded from the analyses in this study because the function of this newly found allele is still unknown. Representative gel for genotyping is shown in Fig. 1.

Statistical analysis

Differences in percentages were examined with Fisher's exact test. The 95% confidence intervals (CIs) for percentages were calculated based on binomial distributions. Logistic regression analysis was performed for estimating odds ratios (ORs) and 95% CIs. Age adjustment in

Table 1. Characteristics of the subjects and the *Toll-like receptor 2 (TLR2)* -196 to -174del polymorphism

	Controls			Cases <i>n</i> = 583 (%)
	<i>Helicobacter pylori</i> (−)	<i>Helicobacter pylori</i> (+)		
	<i>n</i> = 699 (%)	GA (−) <i>n</i> = 443 (%)	GA (+) <i>n</i> = 494 (%)	
Sex				
Male	479 (68.5)	364 (82.2)	360 (72.9)	429 (73.6)
Female	220 (31.5)	79 (17.8)	134 (27.1)	154 (26.4)
Age (years)				
<30	6 (0.9)	1 (0.2)	1 (0.2)	2 (0.3)
30–39	67 (9.6)	11 (2.5)	3 (0.6)	31 (5.3)
40–49	138 (19.7)	54 (12.2)	31 (6.3)	64 (11.0)
50–59	195 (27.9)	152 (34.3)	140 (28.3)	214 (36.7)
60–69	211 (30.2)	153 (34.5)	221 (44.7)	166 (28.5)
70–	82 (11.7)	72 (16.3)	98 (19.8)	106 (18.2)
Genotype				
<i>ins/ins</i>	304 (43.5)	194 (43.8)	224 (45.3)	243 (41.7)
<i>ins/del</i>	316 (45.2)	202 (45.6)	212 (42.9)	267 (45.8)
<i>del/del</i>	79 (11.3)	47 (10.6)	58 (11.7)	73 (12.5)

GA (-), subjects with no gastric atrophy; GA (+), subjects with gastric atrophy (pepsinogen [PG] I ≤ 70 ng/ml and PG I/PG II ≤ 3)

the logistic analysis was done as a continuous variable. The calculations were done using STATA version 10 (Stata, College Station, TX, USA).

Results

Characteristics of the subjects and allele frequency of the TLR2 polymorphism

The characteristics of the subjects are summarized in Table 1. The mean age ± SD was 58.7 ± 10.6 years for the controls and 58.8 ± 10.5 years for the cases. The percentages of females were 26.5% in the controls and 26.4% in the cases. About three-quarters of the controls were infected with *H. pylori*, while about one-third of the controls had gastric atrophy. The genotype frequency of the *TLR2* polymorphism among the controls was 44.1% for *ins/ins*, 44.6% for *ins/del*, and 11.2% for *del/del*, which was in Hardy-Weinberg equilibrium ($\chi^2 = 0.001$; $P = 0.9796$). The allele frequency was 66.4% for the *ins* allele and 33.6% for the *del* allele. Among the cases the genotype frequency was 41.7% for *ins/ins*, 45.8% for *ins/del*, and 12.5% for *del/del*. The allele frequency was 64.6% for the *ins* allele and 35.4% for the *del* allele.

TLR2 polymorphism, H. pylori seropositivity, gastric atrophy, and gastric cancer

H. pylori seropositivity was not associated with sex, but increased with age. There was no significant association

between the *TLR2* polymorphism and *H. pylori* seropositivity, although the OR of the *del/del* genotype was 0.90 (95% CI: 0.64–1.26) and that of the *ins/del* + *del/del* genotype was 0.92 relative to the *ins/ins* genotype (Table 2).

There were 937 *H. pylori*-seropositive subjects, of whom 494 (52.7%) subjects had atrophy. On the other hand, there were 45 (6.4%) subjects with atrophy among the 699 seronegative subjects. The difference in the prevalence was statistically significant ($P < 0.001$). The genotype distribution according to seropositivity and atrophy is shown in Table 3.

The age- and sex-adjusted ORs of gastric atrophy among *H. pylori* seropositive subjects were not statistically significant: 0.89 (95% CI: 0.68–1.18) for *ins/del*, 1.08 (95% CI: 0.70–1.67) for *del/del*, and 0.93 (95% CI: 0.71–1.21) for *ins/del* + *del/del*, compared with the *ins/ins* genotype. The age- and sex-adjusted ORs of severe gastric atrophy among *H. pylori* seropositive subjects were also not significant: 0.90 (95% CI: 0.63–1.30) for *ins/del*, 0.99 (95% CI: 0.57–1.74) for *del/del*, and 0.92 (95% CI: 0.65–1.30) for *ins/del* + *del/del* (Table 4).

To investigate how this polymorphism of *TLR2* contributes to gastric carcinogenesis from gastric atrophy, we next compared the genotype frequency between gastric atrophy subjects and gastric cancer cases. The age- and sex-adjusted ORs of gastric cancer were 1.13 (95% CI: 0.88–1.46) for *ins/del*, 1.17 (95% CI: 0.79–1.73) for *del/del*, and 1.14 (95% CI: 0.89–1.45) for *ins/del* + *del/del*, relative to the *ins/ins* genotype, and none of these were statistically significant (Table 5).

Table 2. Age- and sex-adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) of *TLR2* -196 to -174del polymorphism for *H. pylori* seropositivity

Genotype	<i>n</i>	<i>H. pylori</i> +	Percentage	OR ^a	95% CI
<i>ins/ins</i>	722	418	57.9	1	Reference
<i>ins/del</i>	730	414	56.7	0.93	0.75–1.15
<i>del/del</i>	184	105	57.1	0.90	0.64–1.26
<i>ins/del</i> + <i>del/del</i>	914	519	56.8	0.92	0.75–1.13

^aAdjusted for age and sex**Table 3.** *TLR2* -196 to -174del genotype distribution according to *H. pylori* seropositivity and the grade of gastric atrophy

Genotype	<i>H. pylori</i> seronegative			<i>H. pylori</i> seropositive		
	GA (–) (%)	GA (+) (%)	GA (++) (%)	GA (–) (%)	GA (+) (%)	GA (++) (%)
<i>ins/ins</i>	287 (43.9)	7 (41.2)	10 (35.7)	194 (43.8)	148 (45.4)	76 (45.2)
<i>ins/del</i>	292 (44.6)	7 (41.2)	17 (60.7)	202 (45.6)	140 (42.9)	72 (42.9)
<i>del/del</i>	75 (11.5)	3 (17.6)	1 (3.6)	47 (10.6)	38 (11.7)	20 (11.9)
Total	654 (100)	17 (100)	28 (100)	443 (100)	326 (100)	168 (100)

GA (–), subjects with no gastric atrophy; GA (+), subjects with gastric atrophy (PG I ≤ 70 ng/ml and PG I/PG II ≤ 3); GA (++) , subjects with severe gastric atrophy (PG I ≤ 30 ng/ml and PG I/PG II ≤ 2)

Table 4. Genotype frequencies for *TLR2* -196 to -174del polymorphism, age- and sex-adjusted odds ratios (ORs), and 95% confidence intervals (95% CIs) of gastric atrophy in *H. pylori* seropositive subjects

Genotype	<i>n</i>	All gastric atrophy (%)	OR ^a	95% CI	<i>P</i> value	Severe gastric atrophy (%)	OR ^a	95% CI	<i>P</i> value
<i>ins/ins</i>	418	224 (53.6)	1	Reference	—	76 (18.2)	1	Reference	—
<i>ins/del</i>	414	212 (51.2)	0.89	0.68–1.18	0.419	72 (17.4)	0.90	0.63–1.30	0.573
<i>del/del</i>	105	58 (55.2)	1.08	0.70–1.67	0.729	20 (19.0)	0.99	0.57–1.74	0.978
<i>ins/del</i> + <i>del/del</i>	519	270 (52.0)	0.93	0.71–1.21	0.574	92 (17.7)	0.92	0.65–1.30	0.631

^aAdjusted for age and sex**Table 5.** Genotype frequencies for *TLR2* -196 to -174del polymorphism, age- and sex-adjusted odds ratios (ORs), and 95% confidence intervals (95% CIs) of gastric cancer in subjects with gastric atrophy

Genotype	All gastric atrophy <i>n</i> = 539 (%)	Gastric cancer <i>n</i> = 583 (%)	OR ^a	95% CI	<i>P</i> value
<i>ins/ins</i>	241 (44.7)	243 (41.7)	1	Reference	—
<i>ins/del</i>	236 (43.8)	267 (45.8)	1.13	0.88–1.46	0.350
<i>del/del</i>	62 (11.5)	73 (12.5)	1.17	0.79–1.73	0.430
<i>ins/del</i> + <i>del/del</i>	298 (55.3)	340 (58.3)	1.14	0.89–1.45	0.294

^aAdjusted for age and sex

Discussion

In this study, the previously reported association of the *TLR2* -196 to -174del polymorphism with the risk of gastric cancer was not reproduced. Possible explanations for the discrepancies observed between the results of our study and the study reported in [17] can be speculated as follows. One explanation is that the significant increase in risk of gastric cancer reported for those with

the *TLR2*–196 to -174 *del/del* genotype in the study reported in [17] could have been a type-1 error, due to the relatively small number of study subjects with the *TLR2*–196 to -174 *del/del* genotype in that study. Another explanation is that the lack of association observed in the present study could be attributable to the heterogeneity of gastric cancer locations in the case subjects; the subsite distributions in the gastric cancer cases (cardia, fundus, body, pylorus, or antrum) were not

specified in this study. However, considering that more than 80% of gastric cancers in Japan are noncardia gastric cancers (NCGCs), as described previously [16], it would seem that the influence of this heterogeneity of gastric cancer subsites would not have been so large as to modulate the effect of this *TLR2* polymorphism on the risk of gastric cancer.

The unique point of the present study compared to other studies is that we investigated the association of the *TLR2* polymorphism with the risks of two steps leading to gastric cancer: *H. pylori* infection, gastric atrophy, and gastric cancer itself. Although this study failed to detect a statistically significant association, this type of analysis should be conducted in future studies so that we can clarify in which step the investigated factor has effects on *H. pylori*-related gastric carcinogenesis. In addition, we also conducted analyses in subjects older than 60 years of age to verify the reported association between the *TLR2* -196 to -174del polymorphism and the severity of intestinal metaplasia in those aged 60 years or more [17], and no significant associations were revealed (data not shown). The discrepancy observed between the present study and the previous report [17] could be mainly attributable to the lack of information on the presence of intestinal metaplasia in our study subjects. Further examinations of the association between this *TLR2* polymorphism and the risk of intestinal metaplasia are also required.

TLRs have been shown to have the ability to recognize pathogens or pathogen-derived products and to initiate signals leading to the activation of innate immunity responses [18, 19]. In *H. pylori* infection, it is generally accepted that TLRs are involved in the initial recognition of this bacterium. However, it is still controversial whether *H. pylori*'s LPS is recognized by TLR4 or TLR2. Some studies have provided strong evidence that TLR4 is expressed by gastric epithelial cells and the LPS of *H. pylori* is involved in the signaling pathways leading to the production of proinflammatory cytokines [20–22]; however, some other studies have shown contradictory results; e.g., the study by Smith et al. [11] revealed that *H. pylori* LPS is a TLR2 ligand and not a TLR4 ligand. Later, it was also shown that TLR4 signaling initiated by *H. pylori* LPS induced TLR2 expression in gastric epithelial cells [23]. For the association between polymorphisms in the *TLR* genes and the risk of gastric cancer, associations between polymorphisms in the *TLR4* gene and the risk of gastric cancer are well documented, although there exists only one report regarding the association between polymorphisms in the *TLR2* gene and the risk of gastric cancer [12]. Considering the important roles of TLRs in *H. pylori* infection, further investigations into the associations of genetic polymorphisms in *TLR* genes with the risk of *H. pylori* infection, gastric atrophy, or gastric cancer,

along with further in vivo studies of the roles of TLRs in *H. pylori*-induced gastric carcinogenesis are expected to elucidate the precise molecular mechanisms involved in human *H. pylori*-induced gastric carcinogenesis, and it is hoped that such studies will lead to effective methods of individualizing the prevention of *H. pylori*-related gastric cancer.

Four polymorphisms in the *TLR2* gene have been reported to date: -196 to -174del, -191G/A, 597T/C, and 1350T/C [24]. Although the functional roles of the polymorphisms other than the *TLR2* -196 to -174del polymorphism have not yet been clarified, it would also be intriguing to examine the associations between these polymorphisms and the risk of gastric cancer.

The present study has several limitations. Although the *H. pylori* status of the control subjects was examined with a serology test, we did not check the CagA status. As reported in Japan, nearly 100% of *H. pylori* strains possess a functional *cag* pathogenicity island (*cagPAI*), which encodes and produces the CagA protein [25]. A previous study also confirmed that almost all strains isolated from the subjects in our Japanese dataset were East Asian *cagA*-positive strains [26, 27], indicating that the *H. pylori* strains in our present study subjects also possessed CagA. Another limitation is related to the diagnosis of atrophic gastritis. This was done entirely on the basis of serum pepsinogen (PG) levels and not through histological assessment. However, the PG method is well established as a surrogate marker of atrophic gastritis [28–30]. The validated criterion is PG I 70 ng/ml or less and a PG I/PG II ratio of 3.0 or less, and that for severe atrophic gastritis is PG I 30 ng/ml or less and a PG I/PG II ratio of 2.0 or less, both of which are assumed to be reliable because they are widely used in practice in Japan [31, 32]. *H. pylori* seropositivity and PG levels were not examined in the gastric cancer cases in this study, based on the assumption that most of the gastric cancer cases would be *H. pylori*-positive with atrophic gastritis [33, 34]. Considering that the intestinal type of gastric cancer, the predominant type of gastric cancer in Japan, arises from gastric atrophy caused by *H. pylori* infection, and diffuse-type gastric cancer occurs regardless of gastric atrophy [35, 36], and non-cardia gastric cancer is associated with *H. pylori* infection and gastric atrophy while cardia gastric cancer is not [37], it would be intriguing to perform subgroup analysis according to these two histological types or tumor locations. These analyses might help to unveil the association of this *TLR2* polymorphism with the steps of gastric carcinogenesis more clearly. However, we could not perform these analyses because of the unavailability of histological data and data about tumor location.

The *del* allele frequency of the *TLR2* -196 to -174del polymorphism in our control subjects was 33.6% (1098

del alleles/3272 alleles), which was similar to the *del* allele frequency in the controls in a previous report in Japan; 31.3% (117 *del* alleles/374 alleles) [24], and slightly higher than that in the report by Tahara et al. [12]; 27.7% (81 *del* alleles/292 alleles). This discrepancy in the *del* allele frequency among the controls might partly explain the contradictory results of our study and the study reported by Tahara et al. [12]. The *del* allele frequency in people of other ethnicities has not yet been reported even in the Database of Single Nucleotide Polymorphisms (dbSNP) and the International HapMap Project (HapMap) databases [38, 39]. In the present study we found a new long insertion allele, which, as far as we know, is reported here for the first time. The function of this new long insertion allele is not yet known, and this warrants further biological and epidemiological investigations. The genotype distribution among our controls was not significantly different from Hardy-Weinberg equilibrium ($P = 0.9796$), suggesting that no selective mechanisms existed among the control subjects.

In this study, 699 *H. pylori* seropositive control subjects and 937 *H. pylori* seronegative control subjects were compared for an estimation of the risk of *H. pylori* seropositivity; 443 subjects without gastric atrophy and 494 subjects with gastric atrophy among *H. pylori* seropositive control subjects were compared for the risk estimation of gastric atrophy, and 539 control subjects with gastric atrophy and 583 subjects with gastric cancer were compared for the risk estimation of gastric cancer. The statistical power for a sample with 400 cases and 400 controls was more than 65% for an OR of 1.5 or 0.67 with a two-sided α -error of 0.05, when a genotype frequency among the controls was between 30% and 70%, and the statistical power was more than 50% when a genotype frequency among the controls was between 20% and 80%, making the statistical power for the present sample enough to detect a definite association.

In summary, this study has revealed that the *TLR2* -196 to -174del polymorphism is not significantly associated with the risk of *H. pylori* infection, atrophic gastritis, or gastric cancer in Japanese, suggesting that the influence of this *TLR2* polymorphism on *H. pylori*-induced gastric carcinogenesis is limited in Japanese people. However, considering the important roles of *TLR2* in *H. pylori* infection, it would be intriguing to investigate the roles of *TLR2* polymorphisms in *H. pylori*-induced gastric carcinogenesis. Further examinations with larger numbers of study subjects are expected to verify our findings.

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