Toll-like receptor 2 –196 to 174del polymorphism influences the susceptibility of Japanese people to gastric cancer

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Toll like receptors (TLR) play important roles in the signaling of many pathogen-related molecules and endogenous proteins associated with immune activation. The –196 to –174del polymorphism affects the *TLR2* gene and alters its promoter activity. We investigated the influence of the *TLR2* –196 to –174del polymorphism on the occurrence of non-cardiac gastric cancer (NCGC) in a Japanese population. The study was carried out with 289 patients with NCGC, 309 non-cancer patients with abdominal discomfort and 146 healthy controls. The –196 to –174del *TLR2* polymorphism was investigated using the allele-specific polymerase chain reaction method in all of the subjects. The –196 to –174del/del genotype of *TLR2* showed a significantly higher frequency in NCGC patients than in healthy controls (adjusted odds ratio [OR] = 6.06; 95% confidence interval [CI] = 1.86–19.72). Similarly, the frequency of the –196 to –174del/del genotype was significantly higher among NCGC patients than in non-cancer patients (adjusted OR = 2.02; 95% CI = 1.22–3.34). The same genotype was associated with an increased risk of both intestinal (OR = 2.00, 95% CI = 1.12–3.60) and diffuse-type (OR = 2.05; 95% CI = 1.11–3.79) histopathology. There were no significant associations between *TLR2* genotypes and tumor stage and anatomical location. Our data suggest that the –196 to –174del/del genotype of *TLR2* may increase the risk of gastric cancer in the Japanese population. (*Cancer Sci* 2007; 98: 1790–1794)

Helicobacter pylori infection is now accepted as a crucial event in the development of peptic ulcer disease and atrophic gastritis, and it is implicated in the development of gastric carcinoma, especially not located in cardia.[1–5] However, there is marked variation in the extent of gastric inflammation among *H. pylori*-infected patients, and only a small percentage of them actually develop peptic ulcer diseases or gastric cancer. This suggests that some genetic factors may also play an important role in the long-term outcome of *H. pylori* infection.[6–10]

Gastric epithelial cells respond to infection with *H. pylori* by activating numerous signal transduction cascades. To date, the responses that have been best characterized are those that lead to expression and activation of c-fos and c-jun via mitogen-activated protein kinase pathways,[11] and nuclear factor (NF)-κB.[12–14] One result of the activation of these pathways is the production of large amounts of interleukin (IL)-8 by the gastric epithelial cells.[12,14,15]

Cells of the innate immune system sense and respond to microbial products via the Toll-like receptors (TLR). These receptors recognize conserved molecular patterns that are expressed by infectious agents. In this way, TLR mediate the activation of transcription factors, mainly NF-κB and proinflammatory cytokines, resulting in inflammation.[16–20]

Recently, it was reported that TLR2 is expressed in *H. pylori*-infected gastric epithelial cells.[21] Furthermore, *H. pylori* induced NF-κB activation and chemokine expression by gastric epithelial cells through TLR2. Stable transfection of HEK293 cells with TLR2 resulted in extremely enhanced expression of IL-8, macrophage inflammatory protein (MIP)-3α and growth-regulated oncogene (GRO)-α.[22] TLR2 may play a significant role in gastric mucosal immunity to *H. pylori* infection.

Genetic studies on the *TLR2* gene have identified a number of polymorphisms, including one that causes a 22-bp nucleotide deletion, –196 to –174del. This substitution may significantly alter the function of the *TLR2* promoter, and thus may influence its activity. The *TLR2* –196 to –174del/del genotype has been reported to show decreased transactivation of responsive promoters.[23] Because of the important role that TLR2 plays with respect to the immune response against *H. pylori*, we hypothesized that polymorphisms in the *TLR2* gene may be important factors for determining the outcome of *H. pylori* infection.

In the present study, we investigated the influence of the *TLR2* –196 to –174del polymorphism on the risk of non-cardiac gastric cancer (NCGC) in a Japanese population. We also investigated its association with various subtypes and clinicopathological features of gastric cancer. Furthermore, we investigated the effect of this polymorphism on the risk of gastric ulcer, duodenal ulcer and *H. pylori*-induced gastritis.

Materials and Methods

Study population. We studied 598 patients attending the Endoscopy Center of Fujita Health University Hospital from January 2005 to October 2006. The 598 patients comprised 289 patients with NCGC (mean age 64.9 ± 11.7 years, male : female [M : F] ratio 0.71) and 309 non-cancer patients (mean age 62.4 ± 12.8 years, M : F ratio 0.59). Non-cancer patients underwent endoscopic examination for the complaint of abdominal discomfort and were diagnosed as having gastric ulcer, duodenal ulcer, gastritis or normal appearance. A diagnosis of gastritis was based on negative results for macroscopic lesions such as ulcer and cancer but positive results for *H. pylori* gastritis by culture, the rapid urease test, or antibodies to *H. pylori*. NCGC was diagnosed histologically and was classified according to Lauren's classification.[24] Detailed information about the stage and anatomical location was also investigated. Patients who had severe systemic disease and had received non-steroidal anti-inflammatory drugs were excluded from this study. The healthy control group comprised volunteers with no clinical history of gastroduodenal disease recruited from among Japanese medical students and staff of the Fujita Health University School of

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Table 1. Characteristics of the subjects

Characteristic	NCGC	Non-NCGC	Healthy control
No. subjects	289	309	146
Male/female (%/%)	204/85 (70.6/29.4)*	182/127 (58.9/41.1)	84/62 (57.5/42.5)
Mean age ± SD (year)	64.9 ± 11.7†	62.4 ± 12.8	36.0 ± 16.11
H. pylori infection positive ratio (%)	83.4‡	66.9	ND

†$P = 0.004$ (compared with non-cardiac gastric cancer [NCGC] patients), $P = 0.007$ (compared with healthy control subjects). ‡$P = 0.03$ (compared with non-NCGC patients), $P < 0.0001$ (compared with healthy control subjects). Mann–Whitney *U*-test. §$P < 0.0001$ (compared with non-NCGC patients). †,§χ^2-test. ND, not done.

Table 2. *TLR2* polymorphism and risk of gastric cancer (compared with healthy control subjects)

Variable	*TLR2* genotype (%)			OR (95% CI) ins/ins *vs* ins/del	*P*-value	OR (95% CI) ins/ins *vs* del carriers	*P*-value	OR (95% CI) del/del *vs* others	*P*-value
	ins/ins	ins/del	del/del						
Healthy control subjects ($n = 146$)	73 (50.0)	65 (44.5)	8 (5.5)	Reference		Reference		Reference	
NCGC ($n = 289$)	126 (43.6)	112 (38.8)	51 (17.6)	1.00 (0.66–1.52)	1	1.29 (0.87–1.93)	0.22	3.70 (1.70–8.02)†	0.0009†

†Age, sex-adjusted OR = 6.06 (95% CI = 1.86–19.72), $P = 0.003$. Statistical significance was assessed using the two-sided Fisher's exact test.
CI, confidence interval; del carriers, ins/del + del/del; NCGC, non-cardiac gastric cancer; OR, odds ratio.

Medicine. From April 2006 to October 2006, 146 volunteers (mean age 36.0 ± 16.1 years, M : F ratio 0.58) were enrolled. The Ethics Committee of Fujita Health University School of Medicine approved the protocol, and written informed consent was obtained from all of the subjects.

Detection of *H. pylori* infection. *Helicobacter pylori* infection was determined on the basis of the rapid urease test. Histological assessment used endoscopic biopsy specimens obtained from non-pathological mucosa of the greater curvature of the gastric antrum as well as upper corpus to avoid false-negative results as much as possible.[25] A biopsy could not be carried out for 27 of the patients because of their physical condition; in these patients, serum antibody was used instead for the detection of *H. pylori*.

Genotyping for the *TLR2* gene. Genomic DNA was extracted from non-neoplastic gastric biopsies or peripheral blood using the standard phenol–choloroform method. Polymorphisms at *TLR2* –196 to –174del were investigated using the allele-specific polymerase chain reaction (PCR) method. In brief, PCR was carried out in a reaction volume of 25 μL containing 200 ng genomic DNA, 10 pmol each primer, 200 ng each dNTP and 0.6 U *Taq* DNA polymerase (Toyobo, Osaka, Japan). The primers for *TLR2* were as follows: forward 5′-cacggaggcagcgagaaa and reverse 5′-ctgggccgtgcaaagaag. The DNA was denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 40 s. The final extension step was prolonged to 7 min. PCR products were visualized by electrophoresis on a 3.5% agarose gel and staining with ethidium bromide. A single band at 286 bp was judged as wild type, a single 264-bp band was judged as homozygous type, whereas the heterozygous type was two bands of 286 and 264 bp.

Statistical analysis. Hardy–Weinberg equilibrium of the *TLR2* gene allele in the healthy controls and non-cancer patients were assessed by χ^2 statistics. Differences of *TLR2* genotype frequencies among gastric cancer, gastric ulcer, duodenal ulcer or gastritis patients and healthy controls were determined by the two-sided Fisher's exact test. The χ^2 statistics was used for comparison of *TLR2* genotype frequencies between the NCGC and non-cancer patients. The odds ratios (OR) and 95% confidence intervals (CI) were also calculated by logistic regression with adjustment for age, sex and *H. pylori* infection status. A probability value of less than 0.05 was considered statistically significant in all analyses.

Results

Study population. A total of 289 NCGC patients, 309 non-cancer patients and 146 healthy controls subjects participated in this study. The characteristics of the subjects are summarized in Table 1. Age, male sex and *H. pylori* infection-positive ratios were significantly higher in the NCGC group than in the non-cancer patients. Age and male sex ratios were also higher in the NCGC group than in the healthy control subjects. Non-cancer patients had 80 gastric ulcers (25.9%), 38 duodenal ulcers (12.3%) and five gastric + duodenal ulcers (1.6%), 105 had gastritis (34.0%) and 80 had *H. pylori*-negative normal healthy stomachs (26.2%).

***TLR2* genotype.** The –196 to –174del polymorphism of *TLR2* was investigated in all 744 subjects. The frequency of *TLR2* polymorphism in the healthy controls and non-cancer patients did not deviate significantly from those expected under the Hardy–Weinberg equilibrium ($P = 0.18$ and 0.71, respectively). In another Japanese study, the distribution of *TLR2* genotypes was ins/ins 49%, ins/del 40% and del/del 11%. The frequency of the del/del genotype seemed to be comparatively higher in our healthy control subjects, but the distribution of the other genotypes in healthy control subjects and non-NCGC patients was not significantly different.

In the comparison of genotype frequency between the NCGC and healthy control groups, the frequency of the *TLR2* –196 to –174del/del genotype was significantly higher in NCGC patients (OR = 3.70; 95%CI = 1.70–8.02) by Fisher's exact test, and this significant association remained after logistic regression analysis with adjustment for age and sex (OR = 6.06; 95%CI = 1.86–19.72; Table 2). Similarly, the frequency of the –196 to –174del/del genotype was also significantly higher among NCGC patients than in non-cancer patients by logistic regression analysis with adjustment for age, sex and *H. pylori* infection status (OR = 2.02; 95% CI = 1.22–3.34; Table 3). Meanwhile, no significant differences in –196 to –174 ins/del genotype (NCGC vs healthy controls, OR = 1.0, 95% CI = 0.66–1.52; NCGC vs non-cancer patients, OR = 0.90, 95% CI = 0.63–1.30) or

Table 3. *TLR2* polymorphism and risk of gastric cancer (compared with non-NCGC patients)

Variable (%)	*TLR2* genotype (%)			OR (95% CI) ins/ins *vs* ins/del	*P*-value	OR (95% CI) ins/ins *vs* del carriers	*P*-value	OR (95% CI) del/del *vs* others	*P*-value
	ins/ins	ins/del	del/del						
Non-NCGC patients (*n* = 309)	142 (46.0)	137 (44.3)	30 (9.7)	Reference		Reference		Reference	
NCGC (*n* = 289)	126 (43.6)	112 (38.8)	51 (17.6)	0.90 (0.63–1.29)	0.57	1.08 (0.78–1.51)	0.64	2.02 (1.22–3.34)	0.006

All data were adjusted for age, sex and *Helicobacter pylori*-positive ratio. CI, confidence interval; del carriers, ins/del + del/del; NCGC, non-cardiac gastric cancer; OR, odds ratio.

Table 4. Association between *TLR2* polymorphism and clinicopathological features of gastric cancer (compared with non-non-cardiac gastric cancer patients)

Variable (%)	Genotype			OR (95% CI) ins/ins *vs* others	*P*-value
	ins/ins	ins/del	del/del		
Patients without gastric cancer (*n* = 309)	142	137	30	Reference	
Lauren's histological subtypes					
Intestinal type (*n* = 171)	69	72	30	2.00 (1.12–3.60)	0.02
Diffuse type (*n* = 118)	57	40	21	2.05 (1.11–3.79)	0.02
Tumor location					
Upper third (*n* = 17)	10	4	3	1.70 (0.46–6.25)	0.43
Middle third (*n* = 148)	60	62	26	2.12 (1.17–3.83)	0.01
Lower third (*n* = 124)	53	50	21	1.92 (1.03–3.60)	0.04
Tumor stage					
Early stage (*n* = 170)	66	76	28	1.99 (1.11–3.55)	0.02
Advanced stage (*n* = 119)	59	36	23	2.22 (1.20–4.08)	0.01

All data were adjusted for *Helicobacter pylori*-positive ratio. CI, confidence interval; OR, odds ratio.

Table 5. *TLR2* polymorphism and risk of gastric ulcer, duodenal ulcer and gastritis

Variable (%)	*TLR2* genotype			OR (95% CI) ins/ins *vs* ins/del	*P*-value	OR (95% CI) ins/ins *vs* del/carriers	*P*-value	OR (95% CI) ins/ins *vs* others	*P*-value
	ins/ins	ins/del	del/del						
Healthy control subjects (*n* = 146)	73	65	8	Reference		Reference		Reference	
Patients without gastric cancer (*n* = 309)	142	137	30	1.08 (0.66–1.52)	0.75	1.18 (0.79–1.74)	0.42	1.85 (0.83–4.15)	0.15
Gastric ulcer (*n* = 80)	41	31	8	0.85 (0.48–1.51)	0.66	0.95 (0.55–1.64)	0.89	1.92 (0.69–5.32)	0.28
Duodenal ulcer (*n* = 38)	19	18	1	1.06 (0.51–2.20)	1	1.00 (0.50–2.04)	1	0.47 (0.06–3.85)	0.69
Gastric and duodenal ulcer (*n* = 5)	2	3	0	1.68 (0.27–10.40)	0.67	1.50 (0.24–9.24)	1	ND	ND
Gastritis (*n* = 105)	41	52	12	1.42 (0.84–2.42)	0.23	1.56 (0.94–2.60)	0.1	2.23 (0.88–5.66)	0.1
H. pylori negatives (*n* = 81)	39	33	9	0.95 (0.45–2.01)	0.89	1.08 (0.63–1.85)	0.9	2.16 (0.80–5.83)	0.19

Statistical significance was assessed using the two-sided Fisher's exact test. CI, confidence interval; del carriers, ins/del + del/del; ND, not done; OR, odds ratio.

ins/del carriers (NCGC vs healthy controls, OR = 1.29, 95% CI = 0.87–1.93; NCGC vs non-cancer patients, OR = 1.08, 95% CI = 0.78–1.51) were found. To investigate whether the *TLR2* polymorphism influenced the clinicopathological features of gastric cancer, the tumor location, stage and Lauren's histological classification were included in a stratified analysis.

Compared with non-cancer patients, logistic regression analysis revealed that there were similar OR and 95% CI between the –196 to –174del/del genotype and the middle-third location (OR = 2.12, 95% CI = 1.17–3.83) and lower anatomical location (OR = 1.92, 95% CI = 1.03–3.60) (Table 4). No significant association was found between the same genotype and upper-third locations, possibly because the number of patients with upper-third cancer was small.

With regard to tumor stage and Lauren's histological classification, the same genotype increased both early and advanced intestinal and diffuse type gastric cancer (early stage, OR = 1.99, 95% CI = 1.11–3.55; advanced stage, OR = 2.22, 95% CI = 1.20–4.08; intestinal type, OR = 2.00, 95% CI = 1.12–3.60; diffuse type, OR = 2.05, 95% CI = 1.11–3.79). There were no significant genotype differences among the healthy controls and non-cancer patients and similar tendencies were also observed among the patients with gastric ulcer, duodenal ulcer and gastritis when compared with healthy controls (Table 5).

Discussion

In the present study, we found that polymorphism of *TLR2* is associated with an increased risk of NCGC in a Japanese population. The –196 to –174del/del genotype frequency was significantly higher in patients with NCGC than in healthy

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controls. Furthermore, statistical significance was also observed when compared with non-cancer patients with abdominal discomfort by logistic regression analysis.

In 2004, Noguchi *et al*. reported that the –196 to –174del/del genotype of *TLR2* was associated with reduced transcriptional activity by the luciferase reporter assay.[23] Although we did not investigate the effect of *TLR2* polymorphism on TLR2 activity in human gastric epithelial cells, it is possible that the polymorphism might have altered the activity of TLR2. Because TLR2 plays important roles with respect to the immune response against *H. pylori*, this altered function may be relevant in carcinogenesis of the stomach.

Regarding the histological differences in gastritis between gastric cancer, gastric ulcer and duodenal ulcer, it has been suggested that patients with gastric ulcer have an increased risk of gastric cancer compared with those with duodenal ulcer.[26–28] Gastritis characterized by remarkable infiltration of neutrophils with severe damage to the surface epithelium, which may lead to multifocal atrophic gastritis, is often shown in patients with gastric ulcer and is considered to indicate a high risk of developing gastric cancer.

However, in the present study no association was found between *TLR2* polymorphism and gastric ulcer. The frequencies of the *TLR2* genotypes were not significantly different in patients with gastric ulcer and duodenal ulcer when compared with healthy controls. Although a weak correlation was found between –196 to –174del/del genotype and gastritis, it was not statistically significant. We also investigated the association between *TLR2* polymorphism and *H. pylori* infection in non-NCGC patients as well as in the NCGC group. In the non-NCGC patients, the *TLR2* genotype distribution was ins/ins 43.5%, ins/del 47.3% and del/del 9.2% in *H. pylori* positives, and ins/ins 51.0%, ins/del 38.2% and del/del 10.8% in *H. pylori* negatives. Meanwhile, in the NCGC group, the *TLR2* genotype distribution was ins/ins 41.9%, ins/del 41.1% and del/del 17.0% in *H. pylori* positives, and ins/ins 52.1%, ins/del 27.1% and del/del 20.1% in *H. pylori* negatives. No association was observed between *TLR2* genotype and *H. pylori* infection ($P = 0.32$ and 0.19, respectively, by 3×2 tables using the χ^2-test). In this case-control study, to investigate the genetic factor that affects the risk of carcinogenesis but not the risk of gastric ulcer and atrophic gastritis, we compared *TLR2* polymorphisms among NCGC, healthy controls and non-cancer patients with peptic ulcer diseases and gastritis with *H. pylori* infection. Only a small group of patients with gastric ulcer and atrophic gastritis actually develop gastric cancer, although they are accepted as a high-risk group for the development of gastric cancer. Our data suggest that *TLR2* polymorphism may be an important factor that modifies the outcome of *H. pylori* infection rather than the risk of *H. pylori* infection itself. Furthermore, *TLR2* polymorphism may be associated with the risk of developing gastric cancer even in patients with gastric ulcer and atrophic gastritis.

Gastric cancer is one of the most common malignancies worldwide and remains a leading cause of death in Asia and some European countries.[29] Many epidemiological and experimental data suggest the impact of *H. pylori* infection as a risk factor for gastric cancer,[2,30,31] and some investigators have studied the efficacy of *H. pylori* eradication in order to reduce its risk and mortality.[32,33] Other investigators have also studied the efficacy of endoscopic examination for early detection of gastric cancer.[34] However, whether *H. pylori* eradication can reduce the risk or mortality of gastric cancer is highly controversial, and considering the potential risk and cost of *H. pylori* eradication and endoscopic examination, implementation reflecting an individual's risk of developing gastric cancer would be ideal. In this context, our new finding that the *TLR2* polymorphism increases the risk of NCGC suggests the potential for *H. pylori* eradication to suppress gastric carcinogenesis in subjects with the *TLR2* –196 to –174del/del genotype.

We also investigated the effect of the *TLR2* –196 to –174del/del genotype on the characteristics of NCGC by stratified analysis, and found that the same genotype was associated with a higher risk of both intestinal- and diffuse-type NCGC. Correa *et al*. reported that gastric atrophy and metaplasia following severe inflammation is an especially strong risk factor for developing the intestinal type of gastric cancer.[26,35] Uemura *et al*. also reported that severe gastric atrophy, corpus-predominant gastritis and intestinal metaplasia are strong risk factors for the development of intestinal-type gastric cancer.[2] *TLR2* polymorphism may be associated with the risk of carcinogenesis but not the risk of developing mucosal atrophy. Therefore, the –196 to –174del/del genotype may increase the risk of both intestinal- and diffuse-type histopathology.

As a recent report demonstrated that *H. pylori*, acting thorough *TLR2*, stimulates HEK293 cells to release IL-8,[22,36] which induces angiogenesis in carcinoma cells, we also investigated whether the –196 to –174del/del genotype might affect the progression of NCGC. However, we could not found any association between the same genotype and stage of NCGC. It is possible that TLR2 plays a critical role in carcinogenesis but not in tumor progression.

In conclusion, the present study demonstrates that the *TLR2* –196 to –174del/del polymorphism is associated with an increased risk of NCGC, both intestinal- and diffuse-type, middle-third and lower-third cancer. However, we only investigated the *TLR2* polymorphism in a limited region of Japan. Because the *TLR2* gene polymorphism may show variations in different ethnic groups, further studies will be 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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