Genetic Variants in *Cyclooxygenase-2*: Expression and Risk of Gastric Cancer and Its Precursors in a Chinese Population

FEN LIU,* KAIFENG PAN,* XUEMEI ZHANG,† YANG ZHANG,* LIAN ZHANG,* JUNLING MA,* CAIXUAN DONG,* LIN SHEN,§ JIYOU LI,¶ DAJUN DENG,‖ DONGXIN LIN,† and WEICHENG YOU*

*Department of Cancer Epidemiology, Peking University School of Oncology, Beijing Institute for Cancer Research, Beijing Cancer Hospital, Beijing; †Department of Etiology and Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing; and §Department of Medical Oncology, ¶Department of Pathology, and ‖Department of Etiology, Peking University School of Oncology, Beijing Institute for Cancer Research, Beijing Cancer Hospital, Beijing, China

Background & Aims: **To screen the genetic variants in *cyclooxygenase-2* (*COX-2*), and evaluate their effects on COX-2 expression and risk of gastric cancer (GC) and its precursors, a population-based study was conducted in Linqu County, Shangdong Province, an area of China, a high-risk area of GC.** ***Methods:*** **Genotypes were determined by polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography analysis in 248 GC cases and 1523 subjects with precancerous gastric lesions. COX-2 expression was detected by immunohistochemical analysis of biopsy specimens of 593 subjects selected at random from 1523 subjects. *COX-2* transcriptional activity was examined by luciferase reporter gene assay.** ***Results:*** **We found an increased risk of GC in subjects with −1195 AA genotype (adjusted odds ratio [OR], 2.33; 95% confidence interval [CI]: 1.45–3.75). Stratified analysis indicated that an elevated risk of GC was observed in subjects carrying the AA genotype and *Helicobacter pylori* infection (OR, 3.88; 95% CI: 1.46–10.34) or smoking (OR, 7.02; 95% CI: 2.19–22.48), and a high expression of COX-2 was found in subjects with AA genotype (OR, 1.84; 95% CI: 1.09–3.10) compared with GG genotype. The prevalence of COX-2 expression positivity varied markedly by histologic status, and the OR (OR, 5.35; 95% CI: 2.64–10.8) was significantly increased for dysplasia compared with superficial gastritis/chronic atrophic gastritis. Furthermore, tissue homogenate with *H pylori* infection could significantly stimulate *COX-2* promoter activity driven by −1195A compared with the −1195G containing counterparts.** ***Conclusions:*** **These findings suggest that *COX-2* polymorphisms may play an important role, at least in part, in developing GC in this high-risk population.**

Evidence accumulated in the past decades revealed that gastric cancer (GC) is an end result of multistages of precancerous gastric lesions.[1,2] Chronic atrophic gastritis (CAG) can occur in young adults, mainly caused by *Helicobacter pylori* infection, and progress to intestinal metaplasia (IM), dysplasia (DYS), and GC.[3] Indeed, our previous studies showed that CAG was nearly universal, IM affected nearly half, DYS affected 20% of adult population, and risk of GC increased significantly with the severity of the lesions in Linqu County, Shangdong Province, an area of China with one of the highest GC mortality rates in the world ($70/10^5$ males and $25/10^5$ females per year).[4]

The cause of GC is still unclear in Linqu County, but a series of studies of GC and its precursors in this area identified several risk factors, including *H pylori* infection, high intake of sour pancakes, salted foods, cigarette smoking, and low consumption of fresh fruits and vegetables.[5] *H pylori* infection plays a crucial role in GC pathogenesis. A randomized intervention trial including antibiotic treatment of *H pylori* in Linqu County revealed a significant decrease in the prevalence of severe CAG, IM, and DYS, as well as favorable effects on GC.[6] Whether genetic variations may play a role in the process of GC carcinogenesis is another area to investigate. Therefore, the findings in Linqu County motivated us to select genetic polymorphisms of *cytochrome P450* (*CYP*), *glutathione-S-transferase* (*GST*), *Lewis*, and *interleukins* (*IL*) in our early studies[7–9] and *Cyclooxygenase* (*COX*)-*2* for this study.

COX, the enzyme that catalyzes the conversion of arachidonic acid to prostaglandins is involved in several biologic pathways.[10] COX-1 is constitutively expressed in tissues and produces prostaglandins, the active products in maintenance of the gastric mucosa, regulation of renal blood flow, and platelet aggregation.[11] Conversely, COX-2 is an inducible enzyme and produces prostaglan-

Abbreviations used in this paper: **CAG, chronic atrophic gastritis; COX, cyclooxygenase; DHPLC, denaturing high-performance liquid chromatography; DYS, dysplasia; GC, gastric cancer; IM, intestinal metaplasia; Ind DYS, indefinite dysplasia; OR, odds ratio; PGE_2, prostaglandin E_2; SG, superficial gastritis.**

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dins in response to various inflammatory stimulus or growth factors.[12] COX-2 overexpression has been associated with an inhibition of apoptosis and an increased metastatic potential and neoangiogenesis.[13–15] Furthermore, expression of COX-2 is elevated in a variety of human carcinomas including GC, and suppression of COX-2 reduces tumor development.[16–19] Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to protect against several tumors, such as colorectal cancer,[20] which leads to a hypothesis that it comes at least partly from inhibiting the activity of the COX enzymes.[20]

Several studies have suggested that genetic polymorphisms of *COX-2* were associated with the risk of colorectal, lung, and bladder cancer,[21–24] whereas a few of those polymorphisms may influence the function or alter the expression of COX-2. It has been shown that the −765G→C polymorphism has a functional effect and an association with risk of colorectal cancer and esophageal squamous-cell carcinoma.[25–27] Moreover, it has recently been proven that the −1195G→A polymorphism created a C-MYB binding site, which may result in higher transcription activity of the *COX-2* gene and shows an association with risk of esophageal squamous-cell carcinoma.[27] However, no study on *COX-2* polymorphism and risk of GC and its precursors has yet been reported.

In the present study, we screened the *COX-2* gene promoter region and the entire coding region and investigated the genetic polymorphism of *COX-2* and risk of GC and precancerous lesions. We also evaluated the association between these polymorphisms and COX-2 expression and possible interactions between functional polymorphism and *H pylori* infection. Furthermore, we investigated whether *H pylori* could regulate COX-2 expression by functional polymorphism of the COX-2 promoter.

Materials and Methods

Study Population

In 2002, we launched a new intervention study of GC in Linqu County. A total of 2813 subjects participated in a baseline study on histopathology of gastric mucosa, representing 80% of eligible residents aged 35–64 years in 12 villages selected at random in this county. Briefly, 4 experienced gastroenterologists performed the endoscopic examinations. The gastric mucosa was examined, and 5 biopsy specimens were obtained from the standard location of the stomach: 2 in the body, 1 in the angulus, and 2 in the antrum. Three pathologists conducted the histopathologic diagnosis of this study, blinded to the laboratory tests. The detailed endoscope procedures with biopsy specimens taken from standard sites and criteria of gastric pathology along with photographs were described elsewhere.[4,28] Each subject was assigned a global diagnosis based on the most severe diagnosis among any of the 5 biopsy specimens. These criteria were as follows: superficial gastritis (SG), CAG, IM, indefinite dysplasia (Ind DYS), and DYS.

For the current study, a total of 1523 subjects were selected at random from each category of gastric pathology including SG/CAG (n = 427), IM (n = 414), Ind DYS (n = 541), and DYS (n = 141). A total of 248 GC cases were identified: 104 GC from our early 3400 screening population in 14 villages in Linqu County between 1994 and 2003, and 146 GC cases from the Cancer Hospital, Chinese Academy of Medical Sciences in Beijing, between 2001 and 2003.

Each case with precancerous gastric lesions was interviewed using a questionnaire to obtain information on cigarette smoking and alcohol consumption during the endoscope examination. For GC cases, interviews were conducted in the hospital before diagnosis. The study was approved by the Institutional Review Board of Peking University School of Oncology, and all subjects gave written informed consent.

Blood Sample Collection and DNA Preparation

A 5-mL blood sample was collected from each subject. The blood sample was allowed to clot for 30–40 minutes at room temperature and then centrifuged at 965*g* for 15 minutes. The resulting serum was separated into vials. The clot and serum were stored immediately at −20°C and then moved into a freezer at −70°C within 2 or 3 days after collection. High-molecular-weight genomic DNA was isolated by standard proteinase-K digestion and phenolchloroform extraction from the blood sample.

Genotyping

COX-2 polymorphisms were analyzed by PCR-based denaturing high-performance liquid chromatography (DHPLC). The promoter was amplified in 6 overlapping fragments, which cover the first 1300 bp upstream of the transcription initiation site. Each exon (including flanking intronic sequences) was amplified in a single reaction, except for exons 8 and 10 being amplified each in 2 overlapping fragments. PCR was accomplished with a 25-μL reaction mixture containing 100 ng of genomic DNA, 1.0 μmol/L of each primer, 0.1 mmol/L of dNTP, 2.0 mmol/L of $MgCl_2$, and 1.0 U Taq DNA polymerase in 1X reaction buffer (Promega, Madison, WI). The primer sequences, PCR annealing temperatures, and DHPLC oven temperatures are listed in Table 1. DHPLC analysis was performed on a Transgenomic WAVE System (Transgenomic Inc., Omaha, NE). The detailed genotyping process was described previously.[29] Briefly, PCR products for *COX-2* were denatured for 1 minute at 94°C and then gradually reannealed by decreasing the sample temperature from 94°C to 45°C over a period of 30 minutes to form homo- and/or hetero-duplexes. The PCR products were then applied to the DHPLC column at an optimal oven temperature and eluted with a linear acetonitrile gradient at a flow rate of 0.9

Table 1. Primer Sequences, PCR, and DHPLC Conditions for Detection of *COX-2* Gene Polymorphisms

Primer sequence 5′→3′	PCR product size (bp)	PCR annealing temperature (°C)	DHPLC temperature (°C)
Promoter			
A F: GTG TAG TTT TAT TTC AGG TTT TA	387	60.4	56.5
R: CTG TCC ACT TTT CCA AGA			
B F: TTC CTC ATC CAA CTA TGT TC	383	55.5	55.0
R: CTC ATA ATA CTG GTC CTA A			
C F: CCT ATG AAG GGC TAG TAA C	359	55.6	57.6
R: AAA CTC TTA TTT TGT GGA ATG AA			
D F: AAA GCA ACT TAG CTA CAA AG	233	60.0	55.7
R: TGG TCT GTA CGT CTT TAG AG			
E F: CAG CCT ATT AAG CGT CGT CAC TA	362	62.0	62.5
R: CCC ACT CTC CTG TCT GAT CCC			
F F: CCA GAA CTG GCT CTC GGA A	377	62.0	59.2
R: CTG ACT CTC ACT GCA AGT CGT AT			
Exons			
1 F: AGG CGG AAA GAA ACA GTC AT	335	64.0	68.0
R: CCA GGA GGT CAG AGC GGA AAC			
2 F: TTG TAA AGT TGA TTC ATA GT	324	53.6	57.0
R: CAA GAA GAC GAA GAA AGG			
3 F: AAG TTT GTC CTT TGG TTG C	339	56.0	55.1
R: TAG ATT AGG CTT ACA GTA TTA T			
4 F: CCA CTT TCC ACA TTT TAC A	281	53.6	57.1
R: GCA GCC CGT CTT ATA GTT A			
5 F: ATT CTT GGT AGA TTG ACA G	316	56.0	56.0
R: CTT GAC TAT GAT TTG GTA T			
6 F: AAA ACT TCA ACA GCA ACA A	179	56.4	54.5
R: TAT GGG TAT AAG CGG TAA T			
7 F: AAT GTG TTC CTT AAC TTT TTA ACT G	349	62.1	58.9
R: TAA TTT TCC CTG GGG AAG AGG			
8 AF: CAA GGA AGA AAA CAG AAA T	289	55.5	56.0
AR: TGA AAG GTG TCA GGC AGA A			
BF: ATT TGA CCC AGA ACT ACT T	256	56.0	56.5
BR: TAG TCT TTT GTT TTG GTT TT			
9 F: CCT AAG GCA AGC TGA ATA CAA A	301	60.0	56.1
R: TCC ATC TCG AAA AGA AAA CCA AA			
10 AF: AGT TGG CAG CAA ATT GAG CA	392	60.0	57.2
AR: GGA ACA CTG AAT GAA GTA AAG G			
BF: GCC TAC TGG AAG CCA AGC AC	372	62.0	57.2
BR: TGA CTC CTT TCT CCG CAA C			

mL/min. The genotypes revealed by DHPLC analysis were further confirmed by DNA sequencing with ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

H pylori Antibody Assays

Details of serologic assay were described previously.[30] Briefly, *H pylori* stains cultured from gastric biopsies of 5 patients in the study subjects were used to provide a local antigen preparation for serology. Serum levels of anti–*H pylori* immunoglobulin (Ig)G and IgA were measured separately in duplicate with enzyme-linked immunosorbent assay (ELISA) procedures. Quality control samples were assayed at Vanderbilt University, Nashville, Tennessee. An individual was determined to be positive for *H pylori* infection if the mean optical density for either the IgG or the IgA was >1.0, a cut-off value from the examination of a group of *H pylori*–negative persons and reference sera.

Immunohistochemical Analysis

To evaluate the association between *COX-2* polymorphisms and expression, a total of 593 subjects with precancerous gastric lesions was randomly selected from 1523 subjects (parts of paraffin-embedded tissue specimens were not available on 248 GC cases). Tissue specimens were fixed in 10% neutral-buffered formalin and were paraffin embedded according to standard procedures. Four-micrometer-thick sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity. All sections were subjected to heat-induced epitope retrieval in a microwave oven. Sections were incubated with anti-COX-2 mouse monoclonal antibody (1:100; Cayman Chemical Co., Ann Arbor, MI) at 4°C overnight. The sections were treated with polyperoxidase-anti-mouse/rabbit immunoglobulin (GBI) for 30 minutes at 37°C, and antibody-binding sites were visualized by

DAB kit (Zhongshan Golden Bridge Co, Beijing, China). Negative controls were done by omitting the primary antibody. The immunohistochemical expression of COX-2 was examined independently by 2 pathologists. The percentage of positive cells was graded semiquantitatively, and each sample was assigned to one of the following categories: grade 1, <5% cells showed immunoreactivity; grade 2, 5%–30% positive cells; and grade 3, >30% positive cells. COX-2-positive expression was defined as cells with grade 2 or above (≥5%) expression levels.

H pylori Strain and Growth Conditions

To examine whether *H pylori* could regulate COX-2 expression by functional polymorphism of the COX-2 promoter, *H pylori* TN2 strain (Takeda Chemical Industries, Ltd., Osaka, Japan) was used in our study. TN2, a cagA-positive *H pylori* strain isolated from a patient with gastric ulcer, could induce invasive gastric adenocarcinoma of Mongolian gerbils at 62 weeks after inoculation.[31] The TN2 strain was cultured on Columbia agar containing 8% sheep blood and *H pylori*–selective supplement (Oxoid Ltd, Hampshire, United Kingdom) for 3–5 days at 37°C under microaerobic conditions as described.[32] *H pylori* colonies on agar plates were washed and suspended in brain-heart infusion (Oxoid, UK) containing 10% glycerol. The number of organisms was quantified by measuring the optical density of the suspension at 450 nm (optical density reached 1.2 absorbance units, corresponding to a bacterial concentration of 5×10^8 colony-forming units/mL). Organisms were then suspended in Dulbecco's modified Eagle medium (DMEM) with serum prior to each experiment.

Tissue Homogenate Preparation

To determine whether *H pylori* stimulates transcriptional activity by functional polymorphism of *COX-2* promoter dependently upon the release of proinflammatory cytokines and growth factors, 4 tissues were selected from 300 biopsy specimens frozen in liquid nitrogen and stored at −80°C for the purpose of the prostaglandin E_2 (PGE_2) assay. Four tissue specimens were matched in the following categories: Ind DYS or DYS, *H pylori* infection, COX-2-positive expression, and high levels of PGE_2 (300−400 pg/mL/mg tissue, by Enzyme Immunoassay Kit, Cayman Chemical Co. Inc). Prior to assay, tissues were thawed, weighed, and placed in 700 μL DMEM with serum. Tissues were then homogenized with a homogenizer (Tissue Tearor, Biospec Products, Inc).

Luciferase Reporter Gene Assay

The human gastric adenocarcinoma cell line AGS (ATCC, CRL-1739) was used to examine the transcriptional activity of *COX-2*. The detailed assay processes for construction of reporter plasmids and transient transfection were described previously.[27] Briefly, AGS cells were seeded in 10-mm, 24-multiwell plates at a density of 6×10^4 cells/well. After overnight confluence, cells were cotransfected with 150 ng of reporter plasmid and 1.0 ng of pRL-SV40 (Luciferase Assay System; Promega). Prior to assay of luciferase activity, cells were incubated in DMEM without serum (14 hours) and treated with gastrin (100 nmol/L, G17, Sigma), *H pylori* suspensions (optical density, 1.2), and tissue homogenate (8.6 mg/mL) for 8 hours. AGS cells cotransfected with reporter plasmid and pRL-SV40 without treatment served as a control. Luciferase activity was measured using a manual luminometer as described previously.[27] Fold increase was calculated by defining the activity of empty pGL3 Basic vector as 1. For each plasmid construct, pA-G-G and pA-A-G (G→A at −1195, as described previously[27]), 2 independent transfection experiments were performed, and each was done in triplicate.

Statistical Methods

The Hardy–Weinberg equilibrium equation was used to determine whether the proportion of each genotype obtained was in agreement with expected values as calculated from allele frequencies. The differences in age among GC, DYS, Ind DYS, IM, and CAG/SG groups were evaluated with the Mann–Whitney test. The Pearson's χ^2 test was used to examine the differences among GC, DYS, Ind DYS, IM, and CAG/SG groups in sex, *H pylori* infection, smoking, and drinking. Odds ratios and 95% confidence intervals for the polymorphisms under consideration and gastric lesions were computed by unconditional logistic regression, adjusting for age, sex, *H pylori* infection, smoking pack-years, and drinking frequency-years. We tested the null hypotheses of additivity and multiplicativity and evaluated the departures from additive and multiplicative interaction models. These analyses were carried out with Statistical Analysis System Software (version 6.12; SAS Institute, Cary, NC). Haplotype frequencies and linkage disequilibrium coefficients were estimated using EH (EH-plus) software.

Results

The frequency distribution of gender, age, *H pylori* infection, cigarette smoking, and alcohol drinking in subjects with GC and precancerous lesions is presented in Table 2. The mean age was significantly higher in GC cases than those in SG/CAG. The percentages of other variables in IM, Ind DYS, DYS, and GC cases were significantly different from those in SG/CAG. Among the GC cases, 104 (42%) were intestinal type, 100 (40%) were diffuse type, and 44 (18%) were mixed type.

To screen the profile of *COX-2* polymorphisms, a total of 150 cases were randomly selected from the 1771 study subjects. Two allelic variants were detected within the *COX-2* coding region, in which one (Val102Val C→G) was a silent polymorphism in exon 3, and another (Gly587Arg G→A) was a missense polymorphism located in exon 10, causing a glycine-to-arginine change at codon 587. The frequencies of variant alleles were 0.04 and 0.12, respectively. PCR-DHPLC and sequencing

Table 2. Selected Characteristics and Risk Factors in Subjects With Gastric Cancer and Precancerous Lesions

Variable	SG/CAG[a] n = 427	IM n = 414	Ind DYS n = 541	DYS n = 141	GC n = 248
Mean age in years (SD)	51.1 ± 6.6	50.6 ± 6.4	50.3 ± 6.7	51.8 ± 6.4	59.0 ± 12.3
P value		.25	.05	.35	<.0001
Sex (%)					
Male	268 (62.8)	185 (44.7)	271 (50.1)	85 (60.3)	181 (73.0)
Female	159 (37.2)	229 (55.3)	270 (49.9)	56 (39.7)	67 (27.0)
P value		<.0001	<.0001	.60	.007
Helicobacter pylori infection (%)	n = 427	n = 413	n = 540	n = 141	n = 248
Yes	299 (70.0)	281 (68.0)	485 (89.8)	102 (72.3)	175 (70.5)
No	128 (30.0)	132 (32.0)	55 (10.2)	39 (27.7)	73 (29.5)
P value		.53	<.0001	.60	.88
Smoking status (%)	n = 426	n = 412	n = 539	n = 141	n = 248
Yes	188 (44.1)	138 (33.5)	221 (41.0)	80 (56.7)	141 (56.9)
No	238 (55.9)	274 (66.5)	318 (59.0)	61 (43.3)	107 (43.1)
P value		.002	.33	.009	.001
Drinking (%)	n = 416	n = 403	n = 525	n = 137	n = 248
Yes	183 (44.0)	128 (31.8)	193 (36.8)	67 (48.9)	129 (52.0)
No	233 (56.0)	275 (68.2)	332 (63.2)	70 (51.1)	119 (48.0)
P value		.0003	.03	.32	.05

[a]SG/CAG as a reference group.

revealed 5 single-nucleotide polymorphisms and 1 insertion polymorphism in the promoter region. Among 6 variants, the −741G→A, −671A→G, −655 to −654 TAG, and −287G→A were rare (≤1.0%), and the −1195G→A (49.1%) and −765G→C (8.5%) variants were common in our study. Because the frequencies of −741G→A, −671A→G, −655 to −654 TAG, and −287G→A were less or equal to 1%, we selected −1195G→A, −765G→C, and Gly587Arg variants to compare the frequencies of genotypes in subjects with GC and precancerous gastric lesions.

The frequencies of each of the 3 genotypes fit the Hardy–Weinberg equilibrium law. Comparing SG/CAG with IM, Ind DYS, or DYS and GC, we found that the frequencies of 3, −1195 genotypes in GC cases (GG, 17.7%; GA, 46.8%; AA, 35.5%) were significantly different from those in SG/CAG (GG, 24.6%; GA, 51.8%; AA, 23.7%) ($\chi^2 = 12.0$, $P = .003$) (Table 3). Multivariate analysis showed that subjects carrying the AA genotype were at a 2-fold elevated risk for GC (OR, 2.33; 95% CI: 1.45–3.75) compared with subjects carrying the GG genotype. An increased OR for DYS was observed in subjects carrying GA or AA genotype, but confidence intervals included the null value.

The risks of GC associated with the −1195G→A polymorphism by *H pylori* infection, smoking, and drinking status are shown in Table 4. Risk of GC was elevated in subjects who carried the AA genotype and *H pylori* infection (OR, 2.64; 95% CI: 1.47–4.74), smoked (OR, 2.81; 95% CI: 1.43–5.53), or drank (OR, 3.89; 95% CI: 1.92–7.87). Similar results were observed among subjects carrying at least 1 A allele and *H pylori*

Table 3. Genotype Frequencies of −1195G→A, −765G→C, and Gly587Arg Polymorphisms Among Gastric Cancer and Precancerous Lesions

		IM		Ind DYS		DYS		GC	
Genotype	SG/CAG n (%)	n (%)	OR (95% CI)[a]	n (%)	OR (95% CI)[a]	n (%)	OR (95% CI)[a]	n (%)	OR (95% CI)[a]
Gly587Arg									
GG	384 (94.3)	379 (96.4)	1.0	492 (93.4)	1.0	128 (92.8)	1.0	240 (96.8)	1.0
GA	22 (5.4)	12 (3.1)	0.52 (0.25–1.08)	32 (6.1)	1.19 (0.66–2.14)	9 (6.5)	1.19 (0.52–2.75)	8 (3.2)	0.65 (0.27–1.53)
AA	1 (0.3)	2 (0.5)	2.50 (0.22–28.5)	3 (0.5)	1.72 (0.18–16.7)	1 (0.7)	2.06 (0.13–33.7)	0 (0)	—
−765G→C									
GG	384 (89.9)	373 (90.1)	1.0	472 (87.2)	1.0	126 (89.4)	1.0	220 (88.7)	1.0
GC	43 (10.1)	38 (9.2)	0.86 (0.54–1.38)	68 (12.6)	1.30 (0.85–2.00)	15 (10.6)	1.04 (0.55–1.97)	27 (10.9)	1.04 (0.61–1.76)
CC	0 (0)	3 (0.7)	—	1 (0.2)	—		—		—
−1195G→A									
GG	105 (24.6)	106 (25.6)	1.0	137 (25.3)	1.0	29 (20.6)	1.0	44 (17.7)	1.0
GA	221 (51.8)	208 (50.2)	0.86 (0.61–1.21)	264 (48.8)	1.04 (0.75–1.44)	78 (55.3)	1.54 (0.92–2.58)	116 (46.8)	1.28 (0.83–1.98)
AA	101 (23.7)	100 (24.2)	0.87 (0.58–1.30)	140 (25.9)	1.07 (0.73–1.58)	34 (24.1)	1.43 (0.78–2.61)	88 (35.5)	2.33 (1.45–3.75)[b]

[a]ORs and 95% CIs were calculated by logistic regression and adjusted for age, sex, *Helicobacter pylori* infection, smoking pack-years, and drinking frequency-years.
[b]$P < .05$.

Table 4. Risk of Gastric Cancer Related to −1195 Genotypes by *Helicobacter pylori* Infection, Tobacco Smoking, and Alcohol Drinking

			OR (95% CI)[a]			
Subjects	SG/CAG	GC	GG	GA	AA	GA + AA
All subjects, n	427	248	1.00	1.28 (0.83–1.98)	2.33 (1.45–3.75)[b]	1.60 (1.06–2.42)[b]
H pylori infection, n						
Negative	128	73	1.00	0.51 (0.21–1.25)	1.41 (0.54–3.71)	0.73 (0.31–1.70)
Positive	299	175	1.00	1.50 (0.88–2.58)	2.64 (1.47–4.74)[b]	1.87 (1.13–3.09)[b]
Smoking status, n						
Nonsmoker	238	107	1.00	0.99 (0.48–2.05)	1.98 (0.95–4.14)	1.35 (0.69–2.63)
Smoker	188	141	1.00	1.34 (0.74–2.42)	2.81 (1.43–5.53)[b]	1.72 (0.99–3.00)
Drinking status, n						
Nondrinker	233	119	1.00	0.90 (0.48–1.71)	1.56 (0.79–3.07)	1.12 (0.62–2.03)
Drinker	183	129	1.00	1.79 (0.96–3.35)	3.89 (1.92–7.87)[b]	2.36 (1.31–4.26)[b]

[a]ORs and 95% CIs were calculated by logistic regression, with GG genotype as the reference group and adjusted for age and sex.
[b]$P < .05$.

infection (OR, 1.87; 95% CI: 1.13–3.69) or who drank (OR, 2.36; 95% CI: 1.31–4.26).

The risks of GC related to −1195 genotypes were further examined with stratification by *H pylori* infection, smoking, and drinking status (Table 5). The OR of GC for subjects carrying the AA genotype or *H pylori* infection alone was 1.49 (95% CI: 0.54–4.11) or 1.16 (95% CI: 0.45–3.01), respectively. However, the OR was elevated in subjects carrying the AA genotype and *H pylori* infection (OR, 3.88; 95% CI: 1.46–10.34). There was an interaction between the AA genotype and *H pylori* infection, with a relative risk because of the interaction of 2.23 and a synergy index of 4.43. A similar result was observed between the AA genotype and smoking status. The OR was significantly increased in subjects carrying the AA genotype and smoking (OR, 7.02; 95% CI: 2.19–22.48), and an interaction between the AA genotype and smoking was observed (a relative risk because of the interaction of 2.91 and a synergy index of 1.94).

To elucidate further the relevance of *COX-2* polymorphism, haplotypes for the 3 polymorphisms were reconstructed using EH software. In total, 8 distinct haplotypes were found. The results showed that the haplotype effects essentially coincide with the effect of the −1195G→A polymorphism alone. The only A_{-1195}-G_{-765}-G_{G587R} haplotype that carries the variant allele of the −1195G→A polymorphism was associated with an increased GC risk (OR, 1.48; 95% CI: 1.16–1.88).

The associations between COX-2 expression and the −1195 genotypes are shown in Table 6. Comparing the GG genotype, the COX-2 expression was significantly increased in subjects who carried the AA genotype (OR, 1.84; 95% CI: 1.09–3.10) or at least 1 A allele (OR, 1.53; 95% CI: 1.02–2.31). The associations between COX-2 expression and the −1195 genotypes were further examined by *H pylori* infection and histologic status. We found an increased expression of COX-2 in subjects who carried at least 1 A allele and *H pylori* infection (OR,

Table 5. Risk of Gastric Cancer Associated With −1195 Genotypes by *Helicobacter pylori* Infection, Tobacco Smoking, and Alcohol Drinking

	−1195 Genotype			
	GG[a]	OR (95% CI)[b]	AA[a]	OR (95% CI)[b]
H pylori				
Negative	11/20	1.00	25/24	1.49 (0.54–4.11)
Positive	33/85	1.16 (0.45–3.01)	63/77	3.88 (1.46–10.34)[c]
Smoking status				
Nonsmoker	15/52	1.00	41/63	1.83 (0.87–3.87)
Smoker	29/52	3.28 (0.95–11.34)	47/38	7.02 (2.19–22.48)[c]
Drinking status				
Nondrinker	21/47	1.00	40/61	1.54 (0.77–3.09)
Drinker	23/55	0.59 (0.21–1.65)	48/35	2.25 (0.93–5.44)

[a]GC/CAG.
[b]ORs and 95% CIs were calculated by logistic regression, with GG genotype as the reference group and adjusted for age and sex.
[c]$P < .01$.

Table 6. COX-2 Expression Associated With −1195 Genotypes by *Helicobacter pylori* Infection and Histologic Status

Subjects	COX-2 expression (−)	COX-2 expression (+)	OR (95%CI)[a] GG	GA	AA	GA + AA
All subjects, n	217	376	1.00	1.41 (0.92–2.18)	1.84 (1.09–3.10)[b]	1.53 (1.02–2.31)[b]
H pylori infection, n						
Negative	60	72	1.00	0.92 (0.33–2.57)	2.97 (0.82–10.8)	1.25 (0.47–3.33)
Positive	157	304	1.00	1.62 (0.98–2.65)	1.66 (0.92–2.98)	1.63 (1.02–2.59)[b]
Histologic status, n						
SG/CAG	98	64	1.00	0.99 (0.44–2.21)	1.61 (0.60–4.31)	1.13 (0.53–2.44)
IM	46	107	1.00	1.34 (0.57–3.16)	1.82 (0.63–5.24)	1.47 (0.65–3.32)
Ind DYS	58	155	1.00	2.94 (1.37–6.32)[b]	4.39 (1.75–11.0)[b]	3.38 (1.65–6.91)[b]
DYS	15	50	1.00	0.11 (0.11–1.53)	0.04 (0.002–0.72)	0.09 (0.01–1.14)

[a]ORs and 95% CIs were calculated by logistic regression and adjusted for age, sex, smoking pack-years, and drinking frequency-years.
[b]$P < .05$.

1.63; 95% CI: 1.02–2.59). Furthermore, we found a high expression of COX-2 in subjects who carried the GA genotype (OR, 2.94; 95% CI: 1.37–6.32) and the AA genotype (OR, 4.39; 95% CI: 1.75–11.0) or at least 1 A allele (OR, 3.38; 95% CI: 1.65–6.91) and Ind DYS. However, no significant different expression was found in different genotypes and DYS.

Table 7 shows that the prevalence of COX-2 expression positivity varied markedly by histologic status. Positivity was lowest (39.5%) among those with SG/CAG, rose to 69.9% among those with IM, and peaked at 76.9% with more advanced lesion (DYS). The ORs for each of the advanced lesions significantly increased after adjusting for age, sex, *H pylori* infection, smoking, and drinking status.

Because the −1195G→A polymorphism creates a C-MYB binding site, it may result in higher transcription activity of the *COX-2* gene. Furthermore, our studies have indicated that the −1195G→A polymorphism was associated with increased risk of GC, and this association was even higher in subjects carrying the AA genotype and *H pylori* infection. To test whether *H pylori* can regulate transcriptional activity of *COX-2* by −1195G→A, we examined *COX-2* transcriptional activity by luciferase asssay. As shown in Figure 1, reporter gene expression driven by all the −1195A-containing *COX-2* promoters were greater than those driven by the −1195G-containing counterparts. No significant difference of *COX-2*-promoter activity driven by −1195A or −1195G treated with *H pylori* strain and without treatment control was observed. A similar result was observed between gastrin treatment and control (data not shown). However, interestingly, treatment with tissue homogenate significantly increased transcriptional activity of *COX-2* ($P < .05$, Figure 1).

Table 7. Prevalence of COX-2 Expression According to Precancerous Lesions Status

Histologic status	n	COX-2 expression (%)	OR (95% CI)[a]
SG/CAG	162	39.5	1.0
IM	153	69.9	4.20 (2.53–6.98)
Ind DYS	213	72.8	3.64 (2.29–5.76)
DYS	65	76.9	5.35 (2.64–10.8)

[a]ORs and 95% CIs were calculated by logistic regression, with SG/CAG as the reference group and adjusted for age, sex, smoking pack-years, and drinking frequency-years.

Discussion

In an area of high risk of GC, we investigated a profile of *COX-2* polymorphisms, its expression, and their associations with GC and its precursors, which is, to

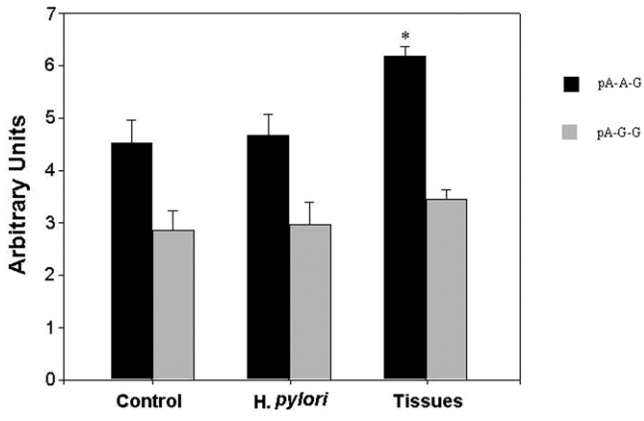

Figure 1. Luciferase reporter gene expression assays. Luciferase expression of the 2 constructs in AGS cells cotransfected with pRL-SV40 to standardize transfection efficiency. Luciferase levels of pGL-3 Basic and pRL-SV40 were determined in triplicate and standardized for transfection efficiency. Fold increase was measured by defining the activity of the empty pGL-3 Basic vector as 1. Data shown are the means fold increase ±SD from 2 independent transfection experiments, each performed in triplicate. *$P < .05$ compared with tissue homogenate treatment and control.

our best knowledge, the first study on the role of COX-2 in the natural history of GC so far. We found that subjects who carried the −1195AA genotype had an increased risk of GC, and COX-2 expression was also significantly associated with the AA genotype. These results suggested that the −1195G→A polymorphism has a functional relevance.

The *COX-2* promoter region contains several key cis-acting regulatory elements, including cAMP-responsive element, nuclear factor-κB, nuclear factor-IL-6, stimulatory protein-1 (SP1), and a transforming growth factor-β response element, suggesting that a complex array of factors is involved in its regulation.[33] The −1195G→A variant is not located within any of these transcription factor binding sites; however, it has just been proven that the −1195G→A change creates a c-MYB binding site, which results in higher transcription activity of the *COX-2* gene. A 1.72-fold excess risk of developing esophageal squamous-cell carcinoma for the −1195AA carriers was also observed in that study.[27] In our study, we found that subjects who carried the −1195AA genotype had an increased risk of GC. Furthermore, we found a significant association between COX-2 expression and the −1195AA genotype. Our findings strongly support the experimental results and provide direct population-based evidence that the −1195G→A polymorphism in the promoter region could regulate the expression of COX-2, thus modifying the risks of GC and precancerous gastric lesions.

Our study observed an elevated risk for the −1195AA genotype and GC in subjects with *H pylori* infection and an interaction between the −1195AA genotype and *H pylori* infection. Moreover, subjects carrying the AA genotype also expressed higher COX-2 than the GG genotype. Previous studies demonstrated that *H pylori* infection could up-regulate gene expression of growth factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) and, therefore, resulted in the up-regulation of COX-2 expression.[34,35] In vitro experiments also showed that bacterial virulence factors located outside the *H pylori* cag pathogenicity island (cag PAI) could activate the MEK/ERK1/-2 signaling to mediate bacterial effects on the *COX-2* promoter.[36] In addition, gastrin is a key molecule that plays an important role in regulation of COX-2 expression and that stimulates the expression of HGF and COX-2.[34,37] To examine whether there was a difference between the −1195G and A allele in *H pylori*-stimulated transcriptional activity of *COX-2*, *H pylori*, gastrin, and homogenate of tissue with *H pylori* infection were tested in a luciferase assay system. Strikingly, we found that, by treatment with the tissue homogenate, the reporter gene expression driven by the −1195A allele was significantly higher than that driven by the −1195G allele. These results are consistent with our population data, showing an interaction between *H pylori* infection and the −1195A allele in identifying GC risk. Although some in vitro experiments have shown that *H pylori* could stimulate host COX-2 expression through multiple signaling pathways, our results showed that not *H pylori* itself but tissues with *H pylori* infection increased the *COX-2* promoter activity because the homogenate of tissues with *H pylori* infection contains various growth factors and proinflammatory cytokines, which may mediate *H pylori* effects on the *COX-2* promoter by −1195G→A through a complex mechanism.

Our study observed an interaction between the −1195G→A polymorphism and smoking. We also found an increased expression of COX-2 in smokers, but confidence interval included the null value (data not shown). Prior study has shown that smoking was associated with COX-2 expression. A study indicated that tobacco smoke stimulated epidermal growth factor receptor tyrosine kinase activity, leading, in turn, to enhanced transcription of *COX-2* in the oral mucosa of smokers.[38] Because COX-2 expression can be induced by smoking, and COX-2 may play important roles in the development of GC, it would be expected that subjects who carried the AA genotype and smoked were more susceptible to developing GC.

A few case-control studies have suggested that genetic variants in *COX-2* were associated with an increased risk of certain cancers.[21–24,27] The −765G→C polymorphism is located within a putative SP1 binding site and may have lower promoter activity and result in the down-regulation of COX-2 expression.[26] Previous studies demonstrated an association between the −765C allele and increased risk to esophageal squamous-cell carcinoma.[27] Another study also showed an increased risk of colon cancer in Singapore Chinese with high consumers of n-6 polyunsaturated fatty acids.[39] However, a study in Japan showed no association between the −765G→C polymorphism and risk of colorectal cancer.[40] In our study, this polymorphism also failed to show the associations with GC and precancerous lesions risk. It may be that the variant genotypes (CC) of the −765G→C are rare in the population of our study (<1%). Moreover, a rare variant Val511Ala was reported to be weakly associated with colorectal cancer.[22] However, this polymorphism is absent in this population. There were also some other studies that reported an association between polymorphisms in the 3′-UTR of *COX-2* and risk of colorectal and lung cancer,[21,23] whereas we did not examine the variants in the 3′-UTR in our study.

We found that COX-2 expression was increased from SG/CAG to IM, and DYS, supporting that overexpression of COX-2 may play an important role in GC development,[41,42] because the COX-2 expression may occur earlier than that of histologic changes in the gastric mucosa, suggesting that COX-2 expression can be used as a surrogate end point in an intervention trial to inhibit the progression of gastric lesions. However, the number of DYS with different genotypes (Table 6) was small in our study and thus failed to detect an association between the expression of COX-2 in subjects with −1195AA genotype and DYS.

Little has been published on associations between genetic polymorphisms and precancerous gastric lesions. However, we did not find statistically significant associations between any genotype from these polymorphisms and advanced gastric lesions. Because there were very few subjects with normal gastric mucosa in this population, we combined SG with CAG as a reference group. If these variants affected transitions primarily from SG to CAG, these effects would not be detected by this design. In addition, there may be many factors to contribute to a multiple pathway involved in the early stage of gastric carcinogenesis, which could reduce the power to detect the effects of any one variant.

The design of our study has been strengthened in certain aspects. The screening of *COX-2* polymorphism allowed us to obtain the profile of the *COX-2* gene and might reflect the other population in northern China. Second, the relatively large samples selected at random from a well-defined, high-incidence population allowed us to evaluate the association between genetic polymorphisms and severity of gastric lesions (IM, DYS, and GC).

Our study has some limitations. Potential drawbacks such as selection bias may have occurred because some GC cases were from hospitals, whereas SG/CAG cases were from the Linqu County general population. However, the genotype frequencies among the Linqu County population fit the Hardy–Weinberg law. Moreover, a separated analysis with the GC cases from Linqu County yielded a similar association between GC and the −1195 AA genotype (OR, 2.31; 95% CI: 1.14–4.68) (data not shown).

In summary, this study provided evidence that the −1195G→A polymorphism increases the genetic susceptibility of GC in a Chinese population. We also found an interaction between *H pylori* infection and the −1195AA genotype. This study further indicated that tissue homogenate with *H pylori* infection could significantly stimulate *COX-2* promoter activity driven by the −1195A compared with the −1195G. Moreover, a significant association between COX-2 expression and the −1195AA genotype was observed, supporting that the −1195G→A polymorphism has a functional relevance. To our knowledge, this is the first study to indicate an association between *COX-2* polymorphism and risk of GC.

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Address requests for reprints to: Weicheng You, MD, Department of Cancer Epidemiology, Peking University School of Oncology, Beijing Institute for Cancer Research, Beijing Cancer Hospital, No. 52 Fucheng Road, Haidian District, Beijing 100036, China. e-mail: weichengyou@yahoo.com; fax: (86) 10-88122437.

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