

Increased expression of cyclooxygenase-2 in first-degree relatives of gastric cancer patients

Jin-Ting Zhang, Ming-Wei Wang, Zhen-Long Zhu, Xiao-Hui Huo, Jian-Kun Chu, Dong-Sheng Cui, Liang Qiao, Jun Yu

Jin-Ting Zhang, Ming-Wei Wang, Zhen-Long Zhu, Xiao-Hui Huo, Jian-Kun Chu, Dong-Sheng Cui, Jun Yu, Department of Medicine, the First Affiliated Hospital of Hebei Medical University, Shijiazhuang 050017, Hebei Province, China
Liang Qiao, Jun Yu, Storr Liver Unit, Westmead Millennium Institute, University of Sydney at Westmead Hospital, NSW 2145, Sydney, Australia

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Co-first-authors: Jin-Ting Zhang and Ming-Wei Wang
Correspondence to: Dr. Jun Yu, Storr Liver Unit, Westmead Millennium Institute, University of Sydney at Westmead Hospital, NSW 2145, Sydney, Australia. jun_yu@wmi.usyd.edu.au
Telephone: +61-2-98459142 Fax: +61-2-98459103
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INTRODUCTION

Gastric cancer is one of the most common malignancies in China and the most frequent cause of cancer-related death^[1]. Although its incidence is increasing, it is still the second most commonly diagnosed fatal cancer worldwide^[2]. Unfortunately, gastric tumors are usually diagnosed at their advanced stage and the current 5-year survival rate is only 17%. Development of gastric cancer, like many other malignancies, is a multi-step process involving the accumulation of genetic alterations. However, much remains to be learned about the molecular pathogenesis of gastric cancer progression and new molecular targets are needed for the prevention and treatment of gastric cancer, especially in its early stage.

Previous studies showed that about 10% of gastric cancer patients exhibit familial clustering^[3,4], and a high prevalence of intestinal metaplasia has been found to be in the first-degree relatives of patients with gastric cancer in contrast to age-matched controls. Thus, the first-degree relatives are considered to be in pre-malignant state^[5]. Therefore, identification of genes predisposing to familial cancer is an essential step towards understanding the molecular events underlying tumorigenesis and is critical for the clinical management of affected families.

Prostaglandins play an important role in the protection of the upper gastrointestinal tract mucosa against injurious agents. Prostaglandin production in the gastro-duodenal mucosa is due to a single isoform of cyclooxygenase (COX)^[6]. Two COX isoforms, COX-1 and -2, have been identified^[7]. COX-1 is a housekeeping gene that is constantly expressed in a number of cell types, whereas COX-2 is an early responsive gene that is rapidly induced by growth factors, tumor promoters, oncogenes, and carcinogens^[8]. COX-2 products have also been shown to be mutagenic^[9] and tumorigenic^[10]. Multiple lines of evidence suggest that COX-2 plays an important role in the malignant transformation of cells^[11] including various cancer cells^[12]. In contrast, levels of COX-1 are relatively constant. Recently, the importance of COX-2 in gastro-intestinal carcinogenesis has been recognized. In humans, COX-2 is frequently detected in the majority of colon tumor samples and is involved in early events of colon carcinogenesis^[13,14]. Indeed, increased COX-2 expression has been observed in gastric carcinoma

Abstract

AIM: To study the expression of cyclooxygenase-2 (COX-2) in human gastric cancer tissues and their paired adjacent mucosa, as well as mucosa from gastric antrum and corpus of the first-degree relatives of the recruited cancer patients.

METHODS: The expression of COX-2 mRNA in 38 patients with gastric cancer and their 29 first-degree relatives and 18 healthy controls was assessed by the real time RT-PCR. The expression of COX-2 protein was determined by Western blot.

RESULTS: A marked increase in COX-2 mRNA expression was found in 20 of 37 (54%) cancerous tissues compared to their respective paired normal mucosa ($P < 0.001$). Interestingly, increased COX-2 mRNA expression was also found in mucosa of the corpus (6/29) and antrum (13/29) of their first-degree relatives. Increased COX-2 mRNA expression was more frequently observed in the antrum biopsies from cancer patients than in the antrum biopsies from healthy controls ($P < 0.05$). In addition, 3 of 23 (13%) patients with atrophic mucosa and 6 of 35 (17%) patients with intestinal metaplasia showed increased COX-2 mRNA expression. Furthermore, COX-2 expression increased in *H pylori*-positive tissues, especially in antrum mucosa.

CONCLUSION: Increased COX-2 expression is involved in gastric carcinogenesis, and may be necessary for maintenance of the malignant phenotype and contribute to *Helicobacter pylori*-associated malignant transformation.

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with no alteration in the levels of COX-1^[15]. Whether COX-2 is associated with early gastric carcinogenesis in humans, especially in gastric mucosa of the first-degree relatives of gastric cancer patients, remains unclear. In this study, we aimed to detect the expression level of COX-2 in gastric cancer and non-cancerous tissues to look for a possible relationship between the development of gastric cancer and COX-2 expression.

MATERIALS AND METHODS

Subjects

Fifty patients with gastric cancer (mean age 66 years, range 50-87 years), 29 of their first-degree relatives (mean age 50 years, range 27-74 years) and 18 healthy subjects without gastric cancer family history (mean age 58 years, range 24-86 years) were recruited in this study. Tissues were collected from tumor area and their adjacent non-tumor corpus and antrum of cancer patients, their first-degree relatives and healthy subjects. Two or three biopsies were snap-frozen in liquid nitrogen for mRNA and protein analysis, and two additional specimens were fixed in 10% buffered formalin for routine histology. Written informed consent was obtained from all participants before commencement of the study.

Histology

Formalin fixed tissues were processed and stained with hematoxylin and eosin, for routine histological evaluation. *Helicobacter pylori* (*H. pylori*) was detected by Warthin-Starry staining^[16]. Histological classification of gastric cancer type was based upon Lauren system which divides gastric cancer into intestinal or diffuse type. The severity of gastritis and *H. pylori* colonization status of the non-tumorous gastric mucosa were determined by updated Sydney System^[17]. All histological sections were reviewed by an experienced gastrointestinal pathologist.

RNA and protein isolation

Gastric tissue specimens were homogenized with an ultrasonographic homogenizer. Total RNA and proteins were sequentially extracted using TRIzol reagents (CINNA/MRC, Cincinnati, OH, USA), according to the manufacturer's instructions.

RT-PCR

One microgram of total RNA was reverse transcribed using dNTPs (1 mol/L), 5× reverse transcription (RT) buffer (500 mol/L Tris-HCl, pH 8.3, 250 mol/L KCl, 50 mol/L MgCl₂, and 50 mol/L DTT), 16 units RNasin, and 2.5 units of AMV reverse transcriptase (Gibco-BRL, Life Technologies). mRNA expression of COX-2 was first determined by conventional RT-PCR. One microliter of reverse-transcription product (cDNA) was amplified by PCR using 1 U of Ampli-Taq DNA polymerase (Gibco-BRL), and 6 pmoL each of COX-2 forward and reverse primers, with 6 pmoL each of forward and reverse β-actin primers included in the same multiplex PCR reaction, as an internal control for efficiency of RT and amount of RNA. Each PCR cycle consisted of a denaturation at 94 °C for 28 s, an annealing at 60 °C for 48 s and an elongation at 72 °C for 1 min. A

total of 30 cycles were performed with an additional extension at 72 °C for 5 min. The primer sequences and PCR product sizes were as follows: *cox-2*, sense 5'-AGATCATCTCTGCCTGAGTATCTT-3', anti-sense 5'-TTCAAATGAGATTGTGGGAAAAT-3', with a 305-bp amplification; β-actin, sense, 5'-TGACGGGGTCACCC-ACACTGTGCCCATCTA-3', anti-sense, 5'-CTAGAAGC-ATTTGCGGTGGACGATGGAGGG-3' with a 654-bp amplification. PCR products were separated on 1.5% agarose gels with 0.5 μg/mL of ethidium bromide, and stained bands were visualized under UV light, photographed, and digitized with a scanner.

Real-time quantitative PCR was then performed on ABI PRISM 7000 sequence detection system using Sybrgreen, PCR mastermix (Perkin Elmer, Branchburgh, NJ, USA) and primers. Primer sequences were designed from the GenBank as follows: COX-2, (forward) 5'-GCCCTTCC-TCCCTGTGCC-3', (reverse) 5'-AATCAGGAAGCTGC-TTTTAC-3'; and β-actin, (forward), 5'-CTAATGGGCA-CCCAGCACAATG-3', (reverse) 5'-GCCGATCCACA-CGGAGTACT-3'. A 24-μL reaction mix was aliquot with 1 μL/replicate of cDNA. A DNA-free template control (containing water) was included and each sample was added in duplicate. Reaction tubes were sealed with optical caps, and the PCR reaction was run at 50 °C for 2 min, at 95 °C for 10 min, followed by 40 cycles at 96 °C for 45 s, at 60 °C for 45 s and at 72 °C for 1 min. Specificity of PCR products was characterized by melting curve analysis and followed by gel electrophoresis. Quantification was determined by the threshold cycle. Actin was used as a housekeeping gene to normalize mRNA levels and compared to mRNA expression levels in normal control stomach.

Western blotting

Total protein concentration was determined by the method of Bradford (DC protein assay, Bio-Rad, Hercules, CA, USA). Fifteen micrograms of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto equilibrated polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. Membranes were blocked using 5% skim milk, and then incubated with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against COX-2 (1:1 000) or β-actin (1:1 000) overnight at 4 °C. After incubation with secondary antibody, proteins were detected by enhanced chemiluminescence (ECL, Amersham Corporation), and bands were quantified by scanning densitometry using the SCAN Control (Scanco~1.lnk) imaging system.

Statistical analysis

Statistical association between COX-2 expression and various clinicopathological factors was determined using the χ^2 test. An exact comparison densitometric analysis of COX-2 PCR products was performed by Student's *t*-test. *P*<0.05 was considered statistically significant.

RESULTS

COX-2 expression in gastric cancer

Increased COX-2 mRNA expression was detected in 37 of

50 (74%) gastric cancer tissues, COX-2 mRNA expression was found to be at very low levels in adjacent gastric mucosa, with 6 of 34 (18%) corpus tissues and 2 of 35 (6%) antrum tissues showing positive COX-2 expression (Table 1 and Figure 1). Quantitative analysis showed that tumor tissues expressed significantly higher levels of COX-2 mRNA compared to the adjacent antrum or corpus samples (Figure 2). COX-2 mRNA was expressed in both intestinal and diffuse types of gastric cancer and did not show any significant difference. By Western blot, COX-2 protein expression was found in 15 of 24 (62.5%) cancers and 4 of 24 (16.7%) adjacent non-tumor specimens (Figure 3). There was a significant correlation between COX-2 mRNA and COX-2 protein expression in gastric tumor samples ($r = 0.522$; $P = 0.001$).

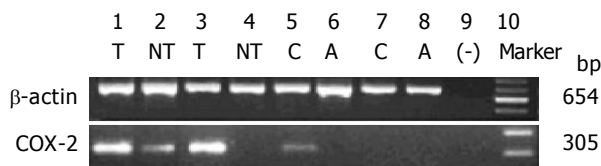


Figure 1 RT-PCR analysis of COX-2 mRNA in gastric tissues. Lanes 1-4: gastric cancer; lanes 5 and 6: first-degree relatives; lanes 7 and 8: healthy control; lane 9: negative control; lane 10: 100-bp DNA ladder. T: Tumor; NT: adjacent non-tumor; C: corpus, and A: antrum.

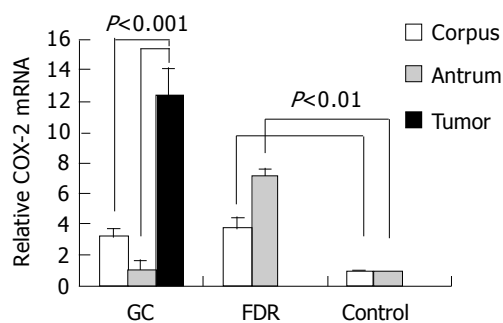


Figure 2 Quantitative RT-PCR analysis of COX-2 mRNA in gastric tissues. GC: gastric cancer; FDR: first degree relatives.

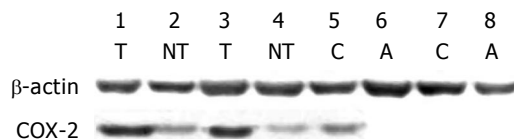


Figure 3 COX-2 protein expression in gastric tissues. T: Tumor; NT: adjacent non-tumor; C: corpus, and A: antrum.

Expression of COX-2 in gastric mucosa of first degree relatives

We also studied the COX-2 expression in 29 first-degree relatives of gastric cancer patients, and in 18 normal subjects without family history of gastric cancer. Increased COX-2 mRNA expression was observed in 6 of 29 (20.1%) corpus tissues and in 13 of 29 (44.8%) antrum tissues of

first-degree relatives (Figure 1). In contrast, only 1 of 18 normal controls exhibited very low COX-2 mRNA expression in both corpus and antrum tissues. Quantitative analysis showed that expression of COX-2 mRNA increased in corpus and antrum tissues of first-degree relatives compared to healthy controls ($P < 0.01$, Figure 2). Weakly positive COX-2 protein was detected in the mucosa of first degree relatives (Figure 3), whereas it was undetectable in normal controls.

We then proceeded to investigate the COX-2 expression in two pre-cancerous conditions: atrophic mucosa and mucosa with intestinal metaplasia, to determine whether upregulation of this gene occurred at an earlier stage in malignant transformation. COX-2 mRNA was upregulated in 3 of 23 (13%) cases of mucosal atrophy and 6 of 35 (17%) cases of intestinal metaplasia.

Table 1 COX-2 mRNA expression in gastric tissues

Tissues	COX-2 mRNA expression		%
	+	-	
Gastric cancer	20	17	54
Paired adjacent corpus	6	28	18
Paired adjacent antrum	2	33	6
Corpus of first-degree relatives	6	23	21
Antrum of first-degree relatives	13	16	45
Corpus of healthy control	1	17	6
Antrum of healthy control	1	17	6

Correlation of COX-2 expression with *H pylori* infection

We compared the COX-2 mRNA expression in *H pylori* infected and *H pylori*-negative tissues (Table 2). RT-PCR analysis of the gastric tissues showed that the expression of COX-2 was significantly upregulated in *H pylori* infected tissues compared to *H pylori*-negative tissues ($P < 0.05$). Because *H pylori* is most commonly colonized in gastric antrum^[18], we also compared the levels of COX-2 expression in *H pylori* infected antrum and corpus tissues, and found that the expression of COX-2 mRNA was significantly higher in the antrum ($P < 0.05$).

Table 2 Correlation between COX-2 mRNA expression and *H pylori* infection

<i>H pylori</i>	COX-2 mRNA expression					
	Antrum	%	Corpus	%	Total	%
Positive	12/34	35	8/36	22	20/70	29
Negative	4/44	9	4/39	10	8/83	10
<i>P</i>		<0.005		>0.05		<0.005

DISCUSSION

Gastric cancer is one of the most commonly encountered malignancies worldwide. Several of its pre-cancerous conditions, such as atrophic gastritis and intestinal metaplasia, have been well recognized. The concern with these gastric cancer precursor conditions or lesions is related to the attempt to prevent carcinoma or to detect it at an early

stage. Atrophic gastritis is defined as the loss of gastric glands, which can occur either in antrum or in corpus. Increased prevalence of this type of gastritis has been found in subjects who are at high risk for gastric cancer^[19]. Intestinal metaplasia is defined as the replacement of gastric mucosa by glands that have the characteristics of the small intestine^[20], and this condition has been strongly associated with development of gastric cancer. Atrophic gastritis and intestinal metaplasia have been accepted as pre-cancerous conditions for some years. Generalized genetic instability has been shown to occur early in this process^[21,22]. In contrast, the relative contributions of inherited susceptibility and environmental effects to familial gastric cancer are poorly understood, because little is known about the genetic events that predispose to gastric cancer. The first-degree relatives of gastric cancer patients have a three-fold risk for developing gastric carcinoma^[3,9]. Therefore, the identification of genes predisposing to familial cancer is an essential step towards understanding the molecular events underlying tumorigenesis and critical for the clinical management of affected families.

In our study, COX-2 gene expression was undetectable in normal control gastric mucosa. In the majority of gastric carcinomas studied, COX-2 expression was clearly upregulated compared to the levels of expression in accompanying antrum and corpus mucosa devoid of cancer cells. A similar pattern of COX-2 mRNA expression has previously been found in human gastric carcinoma^[23]. More importantly in this study, upregulation of COX-2 mRNA and protein was found in first-degree relatives. Furthermore, we detected COX-2 expression in atrophic mucosa and in intestinal metaplasia. These results suggest that COX-2 overexpression constitutes an early event in the gastric neoplastic transformation process which occurs at the pre-cancerous stage. Similarly, expression of COX-2 is found in some non-malignant hyperplastic gastric glands that may represent pre-malignant lesions stained for the COX-2 protein^[23]. Expression of COX-2 is also found in rat epidermis during hyperplastic transformation^[24]. In addition, colonic epithelium expresses only low levels of COX-2 mRNA and elevated levels are found in more than 40% of pre-malignant colonic adenomas. COX-2 expression is detected in hyperplastic bronchial epithelium and atypical alveolar epithelium in lung specimens^[25]. COX-2 is involved in Barrett's-associated metaplastic and dysplastic specimens^[17]. All these findings strongly suggest that COX-2 may play a role in early carcinogenesis.

H. pylori has been epidemiologically linked to gastric cancer and classified as a class I carcinogen by the World Health Organization^[26]. In the present study, expression of COX-2 mRNA was upregulated in gastric mucosa from *H. pylori*-positive tissues compared to *H. pylori*-negative tissues, suggesting that induction of COX-2 may be a specific response to *H. pylori* infection. COX-2 mRNA expression was significantly correlated with *H. pylori* infected antrum tissues, but not with *H. pylori* infected corpus tissues. A more important factor may be the consistent finding that *H. pylori* infection density is greater in gastric antrum than in corpus^[27,28]. Therefore, the overexpression of COX-2 may be a direct effect of *H. pylori*.

In conclusion, gastric cancer and pre-cancerous lesions

express COX-2. COX-2 mRNA is detectable in gastric mucosa of first-degree relatives of cancer patients. COX-2 may contribute to *H. pylori* associated neoplastic transformation. Further investigation is necessary to determine the putative role of this gene in gastric carcinogenesis.

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