

Association of cyclooxygenase-2 expression with *Hp-cagA* infection in gastric cancer

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Abstract

AIM: To observe the expression of cyclooxygenase-2 (COX-2) and to investigate the association between COX-2 expression and infection with cytotoxic-associated gene A (*cagA*) positive strain *Helicobacter pylori* (*Hp*) in human gastric cancer, and subsequently to provide fresh ideas for the early prevention of gastric cancer.

METHODS: 32 Specimens of gastric cancer and corresponding adjacent normal gastric mucosa were obtained from patients who had undergone surgical operations of gastric cancer. All the samples including 1 case of stomach malignant lymphoma and 31 cases of gastric adenocarcinoma were confirmed by pathology diagnosis. The expression of COX-2 in 32 specimens of gastric cancer and corresponding adjacent normal gastric mucosa was quantitatively determined and analyzed with Flow Cytometry, and the levels of COX-2 protein were compared between specimens with *cagA*⁺ *Hp* infection and those without *cagA*⁺ *Hp* infection. The *cagA* gene in 32 specimens of gastric cancer was detected by polymerase chain reaction (PCR) method.

RESULTS: Twenty-seven of 32 (84 %) specimens of gastric cancer showed over-expression of COX-2, compared with the adjacent normal gastric mucosa. *cagA*⁺ gene were detected from 19 specimens of gastric cancer, but not from the other 13 specimens. The levels of COX-2 protein in 19 specimens of gastric cancer with *cagA*⁺ *Hp* infection (the number of positive cells was 73.82±18.2) were significantly higher than those in the 13 specimens without *cagA*⁺ *Hp* infection (the number of positive cells was 35.92±22.1).

CONCLUSION: COX-2 is overexpressed in gastric cancer and *cagA*⁺ *Hp* infection could up-regulate the expression of COX-2 in gastric cancer in human. There may also exist another way or channel to regulate the expression of COX-2 in gastric cancer in addition to *cagA*⁺ *Hp* infection. Therefore, applying COX-2 selective inhibitors could be an effective and promising way to prevent gastric cancer.

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INTRODUCTION

The mortality rate of gastric cancer still takes the first place in eastern Asia, particularly in China. So it is important to study the mechanism of gastric carcinogenesis and to explore the effective and reasonable methods for early prevention of gastric cancer, subsequently reducing the mortality rate of gastric cancer.

Epidemiological studies show that gastric cancer is closely linked to *Helicobacter pylori* (*Hp*) infection^[1-5], because the incidence of gastric cancer increases 4-9 times after *Hp* infection, and more than 60 % patients with gastric cancer had been infected with *Hp*^[6]. Recent studies indicate that infection with *cagA*⁺ *Hp* possesses a potent toxin and high risk for causing gastric cancer^[7, 8]. There is a close association between *cagA*⁺ *Hp* and precancerous stage, even gastric cancer^[9-12]. But the mechanism that how *cagA*⁺ *Hp* actually leads to gastric cancer still remains unclear.

Cyclooxygenase-2 (COX-2) is one isoform of COX enzyme family, which is the rate-limiting enzyme for prostaglandin H synthesis. It is an inducible enzyme, and normally absent in cells, but its expression is rapidly and transiently induced in response to growth factors, tumor promoters or cytokines^[13, 14]. Recent studies indicate that COX-2 not only involves in inflammatory responses but also relates to carcinogenesis. Since COX-2 is overexpressed in many tumors, such as colon-rectum cancer^[15-17], esophageal cancer^[18, 19] etc., it may play an important role in the development and progression of cancers^[20-22].

It is well documented that *Hp* infection can cause inflammation, and COX-2 is often involved in inflammatory responses and also related to carcinogenesis. However, it is not clear whether *cagA*⁺ *Hp* infection induces COX-2 expression and whether there is an association between them during gastric cancer development and progression.

To investigate the possible association between the COX-2 expression level and *cagA*⁺ *Hp* infection, in this study, Flow Cytometry technique was employed to quantitatively determine the expression level of COX-2 in specimens of gastric cancer and adjacent normal gastric mucosa and PCR method was used to amplify the *cagA* gene in specimens of gastric cancer.

MATERIALS AND METHODS

Materials

Specimens of gastric cancer and corresponding adjacent normal gastric mucosa were obtained from 32 patients who had undergone surgical operations of gastric cancer at the Department of Tumor Surgery of the first affiliated hospital of China Medical University. All samples including 1 case of stomach malignant lymphoma and 31 cases of gastric adenocarcinoma were confirmed by pathology diagnosis. The 32 patients were made up of 19 males and 13 females ranging in age from 46 to 73 years old.

Methods

Flow cytometry determination (1) Obtaining single cell solution: 0.5 cm³ specimen of gastric cancer and tissue of adjacent normal gastric mucosa were put on separate 400 pore cleaning copper nets on top of small cups, and the samples

were cut into very small chips and washed with 0.9 % NaCl, then single cell solution was collected and confirmed with microscope that over 90 % cells were single cells. (2) Permeabilizing cells: after being washed 2 times with PBS, the single cells were resuspended with 2 mL 1×FACS permeabilizing solution (Becton Dickson), and incubated at R.T. for 10 min before being washed 1 time with PBS containing 0.5 % bovine serum albumin. The cells were then divided in 2 flow cytometer tubes, and made sure the number of cells in each tube is 10^6 . (3) Immunofluorescence staining: COX-2 was indirectly immunofluorescence stained and 2 tubes were set for each specimens of gastric cancer and tissues of adjacent normal gastric mucosa. 1 tube of each set was stained with FITC (polyclonal anti-rabbit IgG Becton Dickson-Pharmingen company), which was a second fluorescent antibody, as a second antibody self contro l. 2 μ L anti-COX-2 rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc.) were added to the other 1 tube of cells and mixed. The cells were incubated at R.T. for 30 min before being washed 1 time with PBS containing 0.5 % bovine serum albumin. 2 μ L secondary FITC antibody were then added and the cells were incubated in the dark at 2-8 °C for 30 min. After being washed 2 times with PBS, the cell pellet was resuspended in 500 μ L PBS for COX-2 detection by Flow Cytometer (FACSscan Becton Dickson). (4) FACS detection and analysis: Laser excitation was at 488nm, the data was obtained and analyzed with Cell Quest multiple function software, and the fragment interference was eliminated by drawing a gate.

PCR amplification The template from specimen of gastric cancer was amplified by polymerase chain reaction. The *cagA-Hp* primers used were: upstream: 5' -GTG CCT GCT AGT TTG TCA GCG; and the downstream: 5' -TTG GAA ACC ACC TTT TGT ATT AGC. (obtained from Dr Berg, Washington University). PCR reaction: the 20 μ L reaction mixture including 5 μ L template, 2 μ L PCR buffer, 2 μ L dNTP, 2 μ L MgCl₂, 0.5 μ L upstream and 0.5 μ L downstream primers, 0.25 μ L Taq DNA polymerase and dH₂O was subjected to denaturation at 94 °C for 5 min; 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C; with a final extension at 72 °C for 10 min. The amplification reaction was proceeded in a PerkinElmer Cetus Thermocycler (PE-9600).

Assay of PCR production 10 μ L of reaction mixture were loaded to 1.5 % agarose gel containing 0.5 μ g/ml ethidium bromide for electrophoresis, the gel was then placed under ultraviolet ray for detection. The amplified *cagA* gene product was about 390 bp.

Statistical analysis

The expression of COX-2 protein in gastric cancer was analyzed by paired T test with Excel 2000; the association between the levels of COX-2 expression and *cagA-Hp* infection in gastric cancer was analyzed with T test between the two groups.

RESULTS

Expression of COX-2 protein in gastric cancer

27 cases of gastric cancer (in 32 cases) expressed high levels of COX-2 with an average of 67.51 % \pm 21.11 % positive cells and the range was between 30.36-98.56 % (Figure 1: A₁, A₂, A₃), in contrast, the corresponding 27 cases of adjacent normal gastric mucosa only showed a weak expression of COX-2 with an average of 12.41 % \pm 8.16 % positive cells and the range between 3.55-29.16 % (Figure 1: B₁, B₂, B₃). The percentage of positive cells in gastric cancer was significantly higher than that in the normal gastric mucosa beside cancers ($P < 0.001$). Out of the 5 remaining cases, 3 did not show the expression of COX-2 in both specimen of gastric cancer and adjacent normal gastric mucosa, whereas in the other 2 cases, the percentage

of positive cells in specimen of gastric cancer was not higher than that in the adjacent normal gastric mucosa. In total, 27 cases of 32 gastric cancer overexpressed COX-2 protein and the positive rate was 84.4 %.

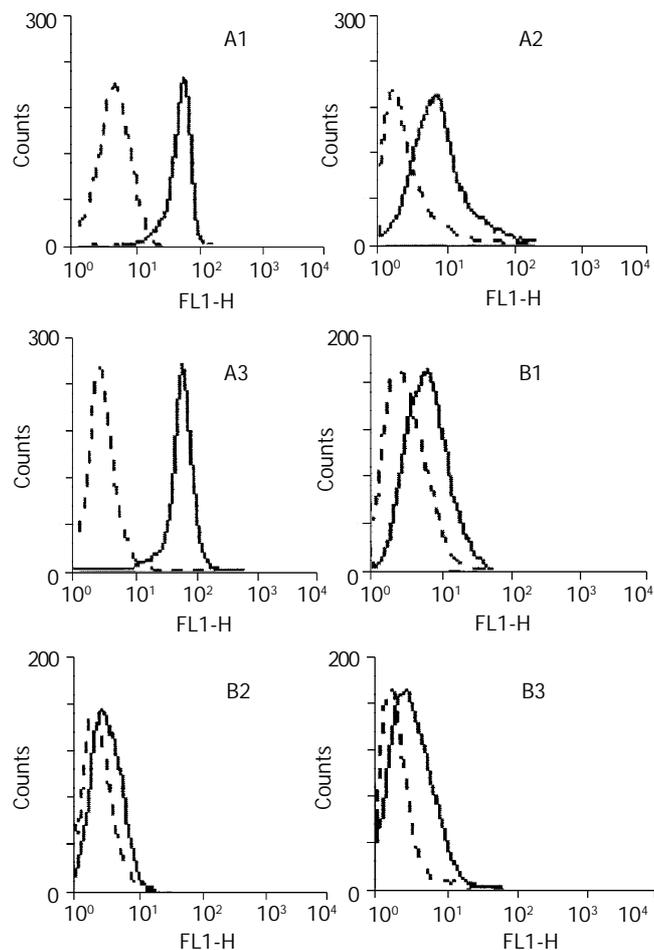


Figure 1 Analysis of COX-2 expressions in gastric cancer tissues and relevant adjacent normal gastric tissues by FACS. A₁, A₂, A₃: Gastric cancer tissues; B₁, B₂, B₃: Relevant adjacent normal gastric tissues. Dotted lines represent results generated with anti-rabbit-FITC; Solid lines represent results generated with rabbit-anti COX-2 and anti-rabbit-FITC.

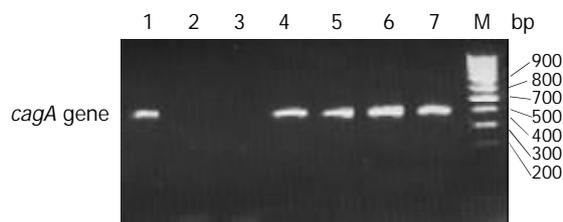


Figure 2 Agarose gel electrophoresis of PCR products of *cagA* gene in gastric cancer tissues. M: DNA marker; 1, 4, 5, 6 and 7: positive PCR products; 2 and 3: negative PCR products.

The association between the levels of COX-2 expression and *cagA-Hp* infection in gastric cancer

The *cagA⁺Hp* was detected in 19 out of 32 (59.3 %) gastric cancers (Figure 2). According to the *cagA* detection results, 32 cases of gastric cancer were then divided into two groups, the *cagA* positive group and *cagA* negative group, so that we could analyze the difference in the levels of COX-2 expression between the two groups. As shown in Table 1, the levels of COX-2 expression in *cagA* positive group were much higher than those in *cagA* negative group, and *cagA* gene was not

detected in the 5 cases with no obvious COX-2 expression. The results suggest that the expression of COX-2 is associated with the *cagA*⁺ *Hp* infection in gastric cancer and *cagA*⁺ *Hp* infection can up-regulate the expression of COX-2.

Table 1 The expression of COX-2 protein in gastric cancer

Grope	n	Cells expressed COX-2($\bar{x}\pm s$)%
Gastric cancer	32	67.51±21.11 ^a
Normal gastric mucosa	32	12.41±8.16
Gastric cancer with <i>cagA</i> ⁺ <i>Hp</i>	19	73.82±18.2 ^b
Gastric cancer without <i>cagA</i> ⁺ <i>Hp</i>	13	35.92±22.1

^a*P*<0.001 vs normal gastric mucosa; ^b*P*<0.001 vs without *cagA*⁺ *Hp* group.

DISCUSSION

Cyclooxygenases (COXs) are the key enzyme in arachidonate metabolism and catalyze the biosynthesis of prostaglandin H₂, which is the precursor for prostanoids. COX family consists of the classical COX-1 enzyme, which is constitutively expressed in many tissues, and involved in the homeostasis of various physiologic functions, and its isozyme COX-2, which was discovered in 1991^[13] and is involved in many inflammatory reactions with its expression rapidly induced by growth factors, tumor promoters or cytokines. Under normal conditions, COX-2 is absent in tissue cells. Since *Hp* infection causes inflammatory reaction, it may also induce the expression of COX-2. Romano and associates incubated MKN28 gastric mucosal cells with broth culture filtrates or bacterial suspensions from wild-type *Hp*, after 24 hours COX-2 mRNA levels increased by 5-fold and the synthesis of PGE₂, the main product of COXs, increased by 3-fold, whereas COX-1 mRNA levels remained unchanged^[23]. This effect was specifically related to *Hp* because it was not observed with *Escherichia coli*. This study indicates that *Hp* could induce the expression of COX-2 in gastric mucosal cells *in vitro*. However, whether *Hp* induces the expression of COX-2 *in vivo*, particularly in gastric cancer, and whether there is an association between *cagA*⁺ *Hp* infection and the expression of COX-2 still remain unclear. Our results showed that 27 out of 32 cases (84 %) of human gastric cancer expressed COX-2, which was similar to the status in colon-rectum cancer^[24], and indicated that the expression of COX-2 was closely associated with gastric cancer. The average percentage of cells that positively express COX-2 was 73.82 % in 19 cases of gastric cancer with *cagA*⁺ *Hp* infection; whereas it was 35.92 % in 13 cases of gastric cancer without *cagA*⁺ *Hp* infection. The levels of COX-2 expression in gastric cancer with *cagA*⁺ *Hp* infection were much higher than those without *cagA*⁺ *Hp* infection (*P*<0.001). 5 cases of gastric cancer did not show expression of COX-2 also were not detected for *cagA* gene PCR product. The result indicates that *cagA*⁺ *Hp* infection can up-regulate the expression of COX-2 in gastric cancer, and *cagA*⁺ *Hp* infection may play an important role during gastric carcinogenesis by mediating the expression of COX-2. McCarthy *et al*^[25] analyzed the COX-2 expression in gastric antral mucosa before and after eradication of *Hp* infection by immunohistochemistry and the results indicate that acute and chronic antral inflammation is associated with *Hp* as well as the expression of COX-2 protein in epithelial cells. The expression of COX-2 was reduced, but not eliminated, in the epithelium after successful eradication of *Hp*. Despite the reduction in COX-2 expression after *Hp* eradication, expression of COX-2 in epithelial cells remained and strongly correlated with the extent of the chronic inflammatory cell infiltration. This conclusion supports our

results that COX-2 was also expressed in some cases of gastric cancer without *cagA*⁺ *Hp* infection and that the expression of COX-2 in gastric cancer without *cagA*⁺ *Hp* infection is weaker than that with *cagA*⁺ *Hp* infection. Recent studies indicate that *Hp* can induce COX-2 expression in gastritis^[26-30], and COX-2 expression in gastric cancer with *Hp* infection has also been reported recently^[31,32], but most of the studies were conducted with immunohistochemistry, Western blot and RT-PCR methods^[26-32], and to quantitatively determinate COX-2 expression and to divide *Hp* into subtypes for analysis has seldom been reported so far.

Recently, evidence has been presented that COX-2 is induced in human colorectal cancers and in the polyps of mouse FAP models. When the COX-2 gene is inactivated in FAP model mice, both the number and size of polyps are reduced dramatically. In addition, selective inhibitors of COX-2 cause results similar to those caused by COX-2 gene knockout mutations. These genetic and pharmacological data open up the possibility of effectively treating human FAP and various human cancers with COX-2 selective inhibitors^[33-35].

The fact that COX-2 is overexpressed in gastric cancer and that the expression of COX-2 in gastric cancer infected by *cagA*⁺ *Hp* is much higher than that without *cagA*⁺ *Hp* infection indicates that *cagA*⁺ *Hp* infection up-regulate the expression of COX-2 in human gastric cancer, and the expression of COX-2 is closely associated with gastric cancer. But the fact that the specimens of gastric cancer without *cagA*⁺ *Hp* infection also express COX-2 at different levels implies that there may exist another way or channel to up-regulate the expression of COX-2 in gastric cancer besides *cagA* positive strain infection. Therefore, with the successful eradication of *Hp* infection, applying COX-2 selective inhibitors could be an effective and promising way to prevent gastric cancer.

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