Mechanism and clinical significance of cyclooxygenase-2 expression in gastric cancer

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INTRODUCTION
Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins. COX-1 is constitutively expressed in a variety of tissues; COX-2 is inducible by cytokines, growth factors, mitogens, oncoproteins, etc. Overexpression of COX-2 has been reported in various types of tumors and some precancerous tissues. Many epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) over one year reduces the risk of esophageal, gastric, colorectal cancers. Inhibiting COX-2 activity reduces the growth of polyps in APC knockout mice. Sulindac and Celecoxib cause regression of colorectal adenomas in patients with familial adenomatous polyposis (FAP). The effects of NSAIDs will bring about a new approach to the prevention and treatment of cancers, especially digestive cancers. Unfortunately, the mechanisms of COX-2 expression have not been defined.

Aberrant DNA methylation exists in carcinoma universally and is manifested as wide DNA hypomethylation and local CpG island hypermethylation (mainly in the promoter region). CpG island demethylation facilitates gene transcription, resulting in oncogene activation, chromosome instability, mutation hotspots, and retrotransposon replacement. CpG island hypermethylation in the promoter is one of the predominant mechanisms of inactivating various tumor suppressor genes in tumorigenesis. Thus aberrant DNA methylation is regarded as the third tumorigenesis pathway. In recent years, it was reported that some cancer cell lines without COX-2 expression exhibit hypermethylation of CpG island in the promoter or exon 1 region, and methylation-inhibiting agents restore expression of COX-2, suggesting that COX-2 expression may be related to the methylation status of 5′-CpG island of COX-2 gene. Accordingly, we attempted to compare the methylation status of 5′-CpG island around the transcriptional starting site of COX-2 gene in the normal gastric mucosa and gastric cancer in order to clarify the mechanisms for COX-2 expression, distinguish the molecular characteristics of gastric cancers, and provide the theoretical basis for COX-2 in the prevention and treatment of gastric cancer.

MATERIALS AND METHODS

Materials
Forty-four primary gastric cancer tissue specimens were obtained from patients undergoing gastrectomy in the First Affiliated Hospital of Zhengzhou University and Luoyang Oriental Hospital. The age of patients ranged from 35 to
71 years (a mean of 55.7 years). Twelve normal gastric mucosal specimens adjacent to cancer were used as controls. None of the patients received chemotherapy or radiation therapy. All tissues were immediately frozen in liquid nitrogen and stored at -80 °C. Genomic DNA was isolated by proteinase-K digestion and phenol-chloroform extraction methods and stored at -20 °C.

**Methods**

**COX-2 gene methylation status analysis** DNA methylation status of CpG island at the 5’ end of COX-2 gene was determined by restriction enzyme PCR as described previously[26]. This method for distinguishing methylated from unmethylated alleles in a gene is based on cutting by methylation-sensitive restrictive enzymes (HpaII, HhaI) and subsequently amplifying the gene fragment by PCR using primers specific to sequences flanking the restrictive enzyme cut sites. Design of COX-2 primers was based on the following published sequences (D28235, AF044206): 5’-CAGCTTCCTGGGTTTCCGATT-3’ (sense) and 5’-TTTGCTGTCTGAGGGCGTCT -3' (antisense), 292 bp product. One microgram genomic DNA was cut by 12 U HpaII or HhaI (TaKaRa) in 20 μL volume for 8h at 37 °C. PCR was performed using primer pairs described above, under the following conditions: 25 μL volume, PCR mix containing 1× GC buffer, deoxynucleotide triphosphates (0.3 mmol/L each), primers (1 μmol/L each), enzyme-cut DNA 200 ng, and 1.5 U LA Taq DNA polymerase (TaKaRa). Amplification was carried out for 30 cycles at 94 °C for 45 s, at 56 °C for 30 s, at 72 °C for 30 s, final extension at 72 °C for 5 min. Positive control was performed using genomic DNA lacking enzyme digestion. Four microliters of PCR products were loaded onto 20 mg/L agarose gel, stained with ethidium bromide, and visualized under UV illumination.

**Immunohistochemistry**

Paraffin-embedded gastric tumor tissues were cut into 4 μm sections, then deparaffinized in xylene and rehydrated through a series of alcohol and water. The slides were placed in 10 mmol/L citrate buffer (pH 6.0) and microwaved for 15 min to enhance antigen exposure. The sections were incubated in 30 mL/L hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Slides were then washed in PBS (pH 7.6) and incubated with PBS containing normal rabbit serum for 30 min, followed by incubation with primary goat antibody to COX-2 (SantaCruz) at 4 °C overnight. Sections were then incubated with a second biotinylated antibody for 30 min before they were reacted with DAB solution. In control slides, PBS was used instead of the primary antibody. On the basis of the intensity and the number of cells stained, expression of COX-2 was defined as moderate to strong staining affecting more than 30% of the tumor area. The COX-2 staining was reviewed by two immunohistochemistry experts independently.

**Statistical analysis**

χ² or Fisher’s exact test was used, P<0.05 was considered statistically significant. All analyses were performed using SPSS 10.0 software.

**RESULTS**

Demethylation of HpaII and HhaI site was found in 34 (77.27%) and 37 (84.09%) of 44 gastric cancer tissue specimens, respectively. Both sites were methylated in 12 normal gastric mucosa specimens (Figure 1). Expression of COX-2 was negative in normal gastric mucosa but positive in 30 (68.18%) of 44 gastric cancer tissue specimens. COX-2 protein was located in cytoplasm of cancer cells (Figure 2).

![Figure 1](image1.png)  
**Figure 1** Methylation status of 5’ CpG island of COX-2 gene in normal gastric mucosa and gastric cancer. Lanes 1-3: normal gastric mucosa; lanes 4-6: gastric cancer tissue; lanes 1 and 4: positive control; lanes 2 and 5: HpaII digestion; lanes 3 and 6: HhaI digestion, lane 7: ΦX174-HaeIII marker.

![Figure 2](image2.png)  
**Figure 2** Immunohistochemical analysis of COX-2 in gastric cancer. A: Negative staining for histological normal gastric mucosa (×100); B: COX-2 expression in high differentiated cancer (×200); C: poorly differentiated cancer (×400).
The study showed that demethylation of HpaII and HhaI site was not significantly correlated with the tumor cell differentiation degree, TNM staging, and lymph node (LN) metastasis (P>0.05). COX-2 expression was significantly higher in III/IV stage group than in I/II stage group (24/31 vs 6/13, P<0.05). In gastric cancer with LN metastasis, COX-2 expression was statistically higher than that without LN metastasis (22/27 vs 8/17, P<0.05). There was no significant difference in COX-2 expression between high/moderate and poor differentiation groups (16/21 vs 14/23, P<0.05, Table 1).

Among the 30 cases of 44 gastric cancers with positive COX-2 expression, 28 had demethylation of both HpaII and HhaI site, one had methylation of HhaI site, and one had methylation of both HpaII and HhaI site. In 14 COX-2 negative gastric cancer tissue specimens, four had methylation of HpaII and HhaI site, five had demethylation of HhaI site and HpaII site, one had demethylation of HpaII site and methylation of HhaI site, and four had demethylation of both HpaII and HhaI site. Demethylation of DNA at HpaII and HhaI site was correlated significantly with COX-2 expression in gastric cancer tissue (P<0.001, Table 2).

### Table 1 Relationship between HpaII and HhaI demethylation, COX-2 expression and clinical parameters in gastric cancers

| Differentiation | HpaII | HhaI | COX-2 expression |
|-----------------|-------|------|------------------|
|                 | D     | M    |                  |
| High/moderate   | 21    | 18   | 3 19 2 16 5 5    |
| Poor            | 23    | 16   | 7 18 5 14 9 9    |
| TNM staging     |       |      |                  |
| I/II            | 13    | 8    | 5 9 4 6 7 7      |
| Lymph node metastasis | 31 | 26 | 5 28 3 24 7a |
| No              | 17    | 11   | 6 12 5 8 9 9    |
| Yes             | 27    | 23   | 4 25 2 22 5 5    |

*a P<0.05 vs I/II stage group, b P<0.05 vs no LN metastasis group, D: demethylation, M: methylation.

### Table 2 Correlation of HpaII and HhaI site demethylation and COX-2 expression

| COX-2 expression | HpaII | HhaI |
|------------------|-------|------|
|                  | D     | M    |
| +                | 29    | 1    |
| -                | 5     | 9a   |

*a P<0.05 vs HhaI, b P<0.001 vs HpaII, D: demethylation, M: methylation.

### DISCUSSION

The present study indicates that the demethylation of 5′ CpgG islands of COX-2 gene may be a major cause for COX-2 expression in human gastric cancer. Human COX-2 gene is located in 1q25.2-25.3, consisting of 10 exons and 9 introns. In the 5′-flanking region, there is a CpG island containing many transcription factor binding sites including cAMP response element (CRE), NF-kB, Sp-1, TATA box, etc. We chose a 292 bp region in the up- and downstream of the transcriptional starting codon from -194 to +98 (containing 14 CpG sites with G+C content of 51% and an observed/expected presence of CpG of 0.75), which meets the established criteria for a CpG island [28,29]. According to our results, unlike that being fully methylated in normal gastric mucosa specimens, the 5′ CpG island of COX-2 was demethylated in most gastric cancer tissue specimens. Moreover, the demethylation was correlated significantly with COX-2 expression. Song et al [30], reported that CpG island is completely methylated in human gastric carcinoma cell line SUN-601, and treatment of the demethylating agent 5-aza-deoxycytidine reacts the expression of COX-2 and restores IL-1β sensitivity. Akhtar et al [31], found that gastric carcinoma cell lines ASG and KATO III, possessing methylated promoters, do not express COX-2, and have no response to H pylori stimulation, but treatment with 5-aza-cytidine and H pylori subsequently causes significant COX-2 expression. These results lead us to assume that in the early stage of gastric cancer, COX-2 gene is firstly demethylated by unknown mechanisms and then begins to transcript under the co-effects of many transcriptional factors. In our study, 5′ CpG island of COX-2 gene was partially or completely demethylated in 10 cancer tissue specimens without COX-2 expression, but was methylated in two cancer tissue specimens with COX-2 expression, suggesting that the interaction between suppressive effects of CpG island methylation and activation effects of transcriptional factors may influence the transcription of COX-2, namely, a COX-2 gene with demethylated CpG island, if there is no activation of transcriptional factors, may also be in transcriptional silencing.

We found that in 22.73% (10/44) gastric cancer tissue specimens, COX-2 gene exhibited a methylated CpG island. Toyota et al [33,34], reported that a subset of gastric and colorectal cancers present a CpG island methylator phenotype (CIMP), which is characterized by simultaneous methylation of multiple CpG islands of many genes, including p16, THBS1, and hMLH1. It was suggested that CpG island methylation of COX-2 is strongly correlated with CIMP in gastric cancer [35]. Interestingly, K-ras mutations are frequently found in CIMP colorectal cancer, compared with CIMP cases having higher P16 mutations [36] and P53 could suppress the expression of COX-2 [37]. Furthermore, overexpression of COX-2 is less frequent in gastric cancer with microsatellite instability (MSI) than in that without MSI [38], which is mainly resulted from methylation of hMLH1 [39]. These findings suggest that the COX-2 expression status represents different pathways of...
gastric carcinogenesis. COX-2 unexpressed cases have abnormally high methylating potential of CpG island of many genes, including COX-2 gene.

Based on this research, we propose to divide gastric cancer into two groups according to the expression status of COX-2. The clinical treatment targeting COX-2 correspondingly need different strategies. Most gastric carcinogenesis COX-2 unexpressed cases have negative, which mainly resulted from transcriptional silencing caused by 5’ CpG island methylation of the gene. Demethylating agents may exert beneficial therapeutic effects[69], but further study is needed to address these deductions.

REFERENCES
1. Sheng H, Shao J, Dixon DA, Williams CS, Prescott SM, DuBois RN, Beauchamp RD. Transforming growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. J Biol Chem 2000; 275: 6628-6635
2. Ramsay RG, Friend A, Vazantios Y, Freeman R, Sicurella C, Hamnett F, Armes J, Venter D. Cyclooxygenase-2, a colorectal cancer nonsteroidal anti-inflammatory drug target, is regulated by m-CpB. Cancer Res 2000; 60: 1805-1809
3. De Lorenzo MS, Yamaguchi K, Subbaramia K, Dannenberg AJ. Bryostatin-1 stimulates the transcription of cyclooxygenase-2: evidence for an activator protein-1-dependent mechanism. Clin Cancer Res 2003; 9: 5036-5043
4. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NF-kappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J 2003; 17: 2115-2117
5. Yip-Schneider MT, Barnard DS, Billings SD, Cheng L, Heilman DK, Lin A, Marshall SJ, Crowell PL, Marshall MS, Sweeney CJ. Cyclooxygenase-2 expression in human pancreatic adenocarcinomas. Carcinogenesis 2000; 21: 139-146
6. Tanji N, Kikugawa T, Yokoyama M. Immunohistochemical study of cyclooxygenases in prostate adenocarcinoma; relationship to apoptosis and Bcl-2 protein expression. Cancer Res 2000; 60: 2313-2319
7. Chan G, Boyle JO, Yang EK, Zhang F, Sacks PG, Shah JP, Edelstein D, Soslowsky RJ, Koki AT, Woerner BM, Masferrer JL, Dannenberg AJ. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res 1999; 59: 991-994
8. Kulkarni S, Rader JS, Zhang F, Liapis H, Koki AT, Masferrer JL, Subbaramia K, Dannenberg AJ. Cyclooxygenase-2 is overexpressed in human cervical cancer. Clin Cancer Res 2002; 7: 429-434
9. van der Woude CJ, Jansen PH, Tiebosch AT, Beuving A, van der Woude CJ, Jansen PH, Tiebosch AT, Beuving A, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 1999; 402: 187-191
10. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Daum BN, Ma J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. Science 2003; 300: 489-492
11. Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 1999; 402: 187-191
12. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Daum BN, Ma J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. Science 2003; 300: 489-492
13. Feng Z, Hu W, Rom WN, Beland FA, Tang MS. N-hydroxy-4-aminobiphenyl-DNA binding in human p53 gene: sequence preference and the effect of C5 cytosome methylation. Biochemistry 2003; 42: 6144-6142
14. Florl AR, Lower R, Schmitz-Dragzer BJ, Schulz WA. DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urethral and renal cell carcinomas. Br J Cancer 2001; 83: 1312-1321
15. Toyota M, Shen L, Ohe-Toyoda M, Hamilton SR, Sinicrope FA, Issa JP. Aberrant methylation of the Cyclooxygenase 2 CpG island in colorectal tumors. Cancer Res 2000; 60: 4044-4048
16. Song SH, Jung HS, Choi IH, Inoue H, Tanabe T, Kim NK, Bang YJ. Transcriptional silencing of Cyclooxygenase-2 by hyper-methylation of the 5’ CpG island in human gastric carcinoma cells. Cancer Res 2001; 61: 4628-4635
17. Hattori M, Sakamoto H, Satoh K, Yamamoto T. DNA demethylation is expressed in ovarian cancers and the expression correlates with methylation of CpG sites in the promoter region of c-erbB-2 and survivin genes. Cancer Lett 2001; 169: 155-164
18. Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the human cyclooxygenase-2 gene. Biochem J 1994; 302(Pt 3): 723-727
19. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. J Mol Biol 1987; 196: 261-282
20. Brown WR, Bird AP. Long-range restriction site mapping of mammalian genomic DNA. Nature 1986; 322: 477-481
21. Akhtar M, Cheng Y, Magno RM, Ashktorab H, Smaoo DT, Meltzer SJ, Wilson KT. Promoter methylation regulates Helicobacter pylori-stimulated cyclooxygenase-2 expression in gastric epithelial cells. Cancer Res 2001; 61: 2399-2403
31 Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA, Prescott SM. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3'-untranslated region. *J Biol Chem* 2000; 275: 11750-11757

32 Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA, Prescott SM. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J Clin Invest* 2001; 108: 1657-1665

33 Toyota M, Ahuja N, Suzuki H, Itoh F, Ohe-Toyota M, Imai K, Baylin SB, Issa JP. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999; 59: 5438-5442

34 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; 96: 8681-8686

35 Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci USA* 2000; 97: 710-715

36 Subbaramaiah K, Altorki N, Chung WJ, Mestre JR, Sampat A, Dannenberg AJ. Inhibition of cyclooxygenase-2 gene expression by p53. *J Biol Chem* 1999; 274: 10911-10915

37 Yamamoto H, Itoh F, Fukushima H, Hinoda Y, Imai K. Overexpression of cyclooxygenase-2 protein is less frequent in gastric cancers with microsatellite instability. *Int J Cancer* 1999; 84: 400-403

38 Momparler RL, Ayoub J. Potential of 5-aza-2'-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. *Lung Cancer* 2001; 34 Suppl 4: S111-S115

39 Karpf AR, Moore BC, Ririe TO, Jones DA. Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2'-deoxycytidine. *Mol Pharmacol* 2001; 59: 751-757