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World J Gastroenterol 2005;11(21):3240-3244
World Journal of Gastroenterology ISSN 1007-9327

• *GASTRIC CANCER* •

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

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Received: 2004-03-23 Accepted: 2004-06-11

Abstract

AIM: To determine the correlation between methylation status of 5′ CpG island of cyclooxygenase-2 (COX-2) gene and protein expression in gastric cancer tissues for distinguishing the molecular characters of gastric cancers.

METHODS: Methylation status of 5′ CpG island of COX-2 gene was studied by PCR amplification after *Hpa*II and *Hha* I restrictive enzyme digestion; COX-2 expression was evaluated by immunohistochemical method.

RESULTS: *Hpa* II and *Hha*I site were all methylated in 12 normal gastric mucosa tissues, whereas they were demethylated in 77.27% (34/44) and 84.09% (37/44) gastric cancer tissues, respectively. Expression of COX-2 was detected in 68.18% (30/44) gastric cancer tissues, but no expression was found in normal gastric mucosa tissues. In gastric cancer tissues, COX-2 expression was correlated significantly with *Hpa*II site demethylation (29/30 *vs* 5/14, $P<0.001$ and *Hha*I site demethylation (28/30 *vs* 9/14, $P<0.05$).

CONCLUSION: The demethylation of 5′ CpG island of gene is necessary for COX-2 expression in human gastric cancer. The expression status of COX-2 may provide theoretical basis for COX-2 targeting gastric cancer treatments.

Key words: Gastric cancer; Methylation; Cyclooxygenase-2

Wang BC, Guo CQ, Sun C, Sun QL, Liu GY, Li DG. Mechanism and clinical significance of cyclooxygenase-2 expression in gastric cancer. *World J Gastroenterol* 2005; 11(21): 3240-3244
http://www.wjgnet.com/1007-9327/11/3240.asp

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 induced by cytokines, growth factors, mitogens, oncoproteins, *etc*.[1-4]. Overexpression of COX-2 has been reported in various types of tumors and some precancerous tissues[5-11]. Many epidemiological studies indicate that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) over one year reduces the risk of esophageal[12], gastric[13] and colorectal cancers[14]. Inhibiting COX-2 activity reduces the growth of polyps in APC$^{\triangle 716}$ knockout mice[15]. Sulindac and Celecoxib cause regression of colorectal adenomas in patients with familial adenomatous polyposis (FAP)[16,17]. 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[18], chromosome instability[19,20], mutation hotspots[21,22], and retrotransposon replacement[23]. 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[24,25], 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 ℃. Genomic DNA was isolated by proteinase-K digestion and phenol-chloroform extraction methods and stored at -20 ℃.

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 (*Hpa*II, *Hha*I) 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 *Hpa*II or *Hha*I (TaKaRa) in 20 μL volume for 8h at 37 ℃. 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 ℃ for 45 s, at 56 ℃ for 30 s, at 72 ℃ for 30 s, final extension at 72 ℃ 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 ℃ 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

χ^2 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 *Hpa*II and *Hha*I 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 specimens but positive in 30 (68.18%) of 44 gastric cancer tissue specimens. COX-2 protein was located in cytoplasm of cancer cells (Figure 2).

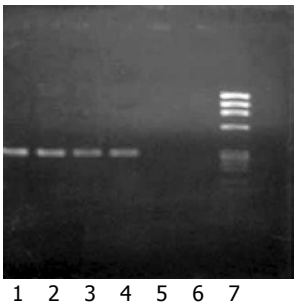

Figure 1 Methylation status of 5' CpG island of COX-2 gene in normal gastric mucosa and gastric cancer. Lanes 1-3: normal gastric mucosa; lanes 4-6: gastric cancer tissue; lanes 1 and 4: positive control; lanes 2 and 5: *Hpa*II digestion; lanes 3 and 6: *Hha*I digestion, lane 7: ΦX174-*Hae*III marker.

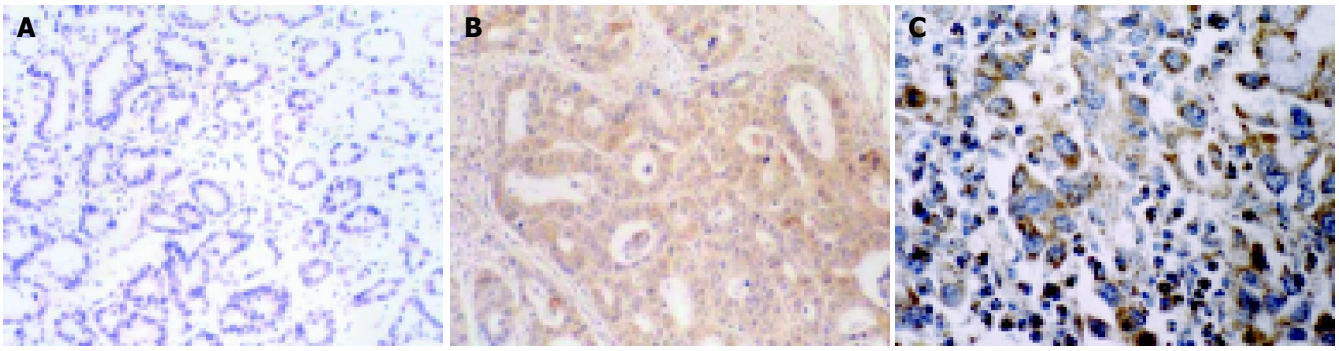

Figure 2 Immunohistochemical analysis of COX-2 in gastric cancer. **A**: Negative staining for histological normal gastric mucosa (×100); **B**: COX-2 expression in high differentiated cancer (×200); **C**: poorly differentiated cancer (×400).

The study showed that demethylation of *Hpa*II and *Hha*I 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 *Hpa*II and *Hha*I site, one had methylation of *Hha*I site, and one had methylation of both *Hpa*II and *Hha*I site. In 14 COX-2 negative gastric cancer tissue specimens, four had methylation of *Hpa*II and *Hha*I site, five had demethylation of *Hha*I site and *Hpa*II site, one had demethylation of *Hpa*II site and methylation of *Hha*I site, and four had demethylation of both *Hha*I and *Hpa*II site. Demethylation of DNA at *Hpa*II and *Hha*I site was correlated significantly with COX-2 expression in gastric cancer tissue ($P<0.001$, $P<0.05$, Table 2).

Table 1 Relationship between *Hpa*II and *Hha*I demethylation, COX-2 expression and clinical parameters in gastric cancers

	n	*Hpa*II		*Hha*I		COX-2 expression	
		D	M	D	M	+	–
Differentiation							
High/moderate	21	18	3	19	2	16	5
Poor	23	16	7	18	5	14	9
TNM staging							
I/II	13	8	5	9	4	6	7
	31	26	5	28	3	24	7[a]
Lymph node metastasis							
No	17	11	6	12	5	8	9
Yes	27	23	4	25	2	22	5[c]

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

Table 2 Correlation of *Hpa*II and *Hha*I site demethylation and COX-2 expression

COX-2 expression	*Hpa*II		*Hha*I	
	D	M	D	M
+	29	1	28	2
-	5	9[b]	9	5[a]

[a]$P<0.05$ *vs* *Hha*I, [b]$P<0.001$ *vs* *Hpa*II, D: demethylation, M: methylation.

DISCUSSION

The present study indicates that the demethylation of 5' CpG 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 $1q^{25.2\text{-}25.3}$, 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-κB, Sp-1, TATA box, *etc.*[27]. 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*[25], reported that CpG island is completely methylated in human gastric carcinoma cell line SUN-601, and treatment of the demethylating agent 5-aza-deoxycitidine reactivates the expression of COX-2 and restores IL-1β sensitivity. Akhtar *et al*[30], 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 suggest that 5' CpG island demethylation may be a prerequisite factor for expression of COX-2 gene. It has been suggested that many stimulators can regulate the expression of COX-2 gene through complex signal transduction pathways acting on transcriptional-regulatory elements, or improving COX-2 mRNA stability through binding to AU-rich element in 3' untranslated region[31,32]. 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[24]. Interestingly, K-ras mutations are frequently found in $CIMP^+$ colorectal cancer, compared with $CIMP^-$ cases having higher P^{53} mutations[35], and P^{53} could suppress the expression of COX-2[36]. Furthermore, overexpression of COX-2 is less frequent in gastric cancer with microsatellite instability (MSI) than in that without MSI[37] which is mainly resulted from methylation of $hMLH_1$[38]. 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 cancers are COX-2 positive, COX-2 inhibitors may get favorable curative effects[16,17]. The other cases are COX-2 negative, which mainly resulted from transcriptional silencing caused by 5' CpG island methylation of the gene. Demethylating agents may exert beneficial therapeutic effects[39], but further study is needed to address these deductions.

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