RESEARCH COMMUNICATION

Expression of Cyclooxygenase-1 and –2 and Clinicopathologic Features of Colorectal Cancer in Northern Thailand

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Abstract

Two isoforms of cyclooxygenase, COX-1 and COX-2, have been identified and shown to be involved in tumorigenesis. Although, overexpression of COX-2 in human cancers has been repeatedly reported, no data have hitherto been available for Thai patients. To cast light on the role(s) of COX enzymes in the development and progression of colorectal cancers and to determine the incidence of COX-2 overexpression, the expression levels of COX-1 and COX-2 proteins using Western blot analysis in tumor tissues and adjacent normal tissues obtained from 44 Thai patients with colorectal cancer.

Compared with paired normal tissues, COX-2 was overexpressed in 13 of 44 colorectal tumor tissues (29.5%). Overall, COX-2 levels in colorectal tumor specimens were significantly correlated with histological differentiation, in particular in the tumors with poor differentiation ($p<0.05$). In addition, overexpression of COX-2 was found more frequently in colorectal tumors with lymphatic invasion, regional lymph node metastasis and larger size, althoughwithout statistical significance. In contrast to the relatively consistent alteration in COX-2 expression, the level of COX-1 expression was quite varied in tumor tissues. Forty-eight percent of colorectal tumors exhibited a decreased level of COX-1 in comparison to normal tissues and overexpressed in 23%. Thus both isoforms may both play roles in promoting tumorigenesis. However, there was no significant relationship between the alteration of COX-1 protein levels and any pathological features of tumors.

Key Words: COX-1 - COX-2 - colorectal cancer - clinicopathologic features - Thailand

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Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Three enzyme isoforms have been so far identified and referred as COX-1, COX-2 and the last one COX-3, about which which very little is known . While COX-1 is constitutively expressed, COX-2 gene expression has been demonstrated to increase following treatment with a variety of stimuli, such as pro-inflammatory cytokines, growth factors and ultraviolet B light (reviewed in Gasparini et al., 2003).

Several studies have shown that the levels of mRNA and protein of COX-2, but not COX-1, are elevated in colorectal cancers compared with the adjacent normal mucosa (Eberhart et al., 1994; Ristimaki et al., 1997; Cianchi et al., 2001). One of the most important studies showing the role of COX-2 in colorectal carcinogenesis was the determination of the effects of COX-2 gene knockout on intestinal polyposis development using adenomatous polyposis coli (Apc^{-716}) gene knockout mice, a mouse model of human FAP (Familial Adenomus Polyposis). Breeding of Apc^{-716} knockout mice with $Cox\text{-}2^{-/-}$ led to the progeny in which both the number of intestinal polyps and the induction of angiogenic stimuli were significantly reduced (Oshima et al., 1996). COX-2 also plays an important role in the tumour progression. One previous study thus showed the levels of COX-2 mRNA to be significantly higher in tumours with larger size and those with deeper invasion in comparison to normal tissues (Fujita et al., 1998), suggesting that the

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COX-2 expression level increases significantly upon progression of adenomas to carcinomas. In addition, COX-2 affects many processes that are important in carcinogenesis; for instance, COX-2–generated prostaglandins have been demonstrated to be immunosuppressive that help the tumour cells to escape from immunologic surveillance and stimulate cell proliferation (Williams et al., 1999). The overexpression of COX-2 led to alterations in the phenotype of intestinal epithelial cells involving an increase in cell matrix adhesion and inhibition of apoptosis that could enhance their tumorigenic potential (Tsujii et al., 1997). Moreover, COX-2 may contribute to tumour angiogenesis due to several reasons including: (1) COX-2 increased expression of vascular endothelial growth factor (VEGF), (2) the ecosanoid products from COX-2, i.e., TxA2, PGE2, and PGI2 can directly stimulate endothelial cell migration and growth factor-induced angiogenesis and (3) COX-2 inhibited endothelial cell apoptosis by stimulation of Bcl-2 or Akt activation (reviewed in Gately, 2000).

However, a role of COX-1 in intestinal polyposis was also demonstrated in a similar model as in Apc^{-716} knockout mice (Chulada et al., 2000) and a role for COX-1 in angiogenesis was suggested using inhibitors and antisense oligonucleotides in in vitro models (Tsujii et al., 1998). Hence, whereas the role of COX-2 in cancer and angiogenesis has been clearly demonstrated by molecular, pharmacological and genetic methods, additional studies are required to establish the importance of COX-1.

The relation levels of COX-2 and COX-1 mRNA and/or protein expression in colorectal cancer have been evaluated by a number of different groups which have reported increased levels of COX-2 expression in this carcinomas (Ristmaki et al., 1997; Uefuji et al., 1998; Murata et al., 1999, Eberhart et al., 1994). Eberhart et al. determined COX mRNA level by Northern blot and found that COX-2 mRNA, but not COX-1, overexpressed in 86% of carcinomas and in 43% of polyps compared with accompanying normal mucosa (Eberhart et al., 1994). Using immunohistochemistry, Sano et al. demonstrated that the levels of COX-2 protein expression were much greater in tumor tissues, whereas the COX-1 expression was weak in both normal and cancerous tissues (Sano et al., 1995). Western blot analysis is also a widely method used for identification and quantitation of COX protein expression. A study carried out in colorectal adenocarcinomas showed that there was higher protein level of COX-2 in neoplastic tissues, whereas the expression of COX-1 was decreased in 12 out of the 15 cancerous specimens compared with paired normal mucosa (Cianchi et al., 2001). Thus, while COX-2 protein was overexpressed, COX-1 protein have been found to be equalled or reduced in cancerous in comparison to normal tissues. Therefore, there are still no clear consensuses of the role of COX-1 in tumorigenesis. In additional, although overexpression of COX-2 was repeatedly reported in human cancer, none of them was investigated in Thai cancer patients. In addition, although this cancer is often found in Thai population, they have not been extensively studied. Moreover, knowing the incidence of COX-2 overexpression in Thai patients may provide primary information whether COX-2 inhibition will be useful for Thai population. Thus, this study aimed to investigate the expression levels of COX protein in Thai patients with colorectal cancer by Western blot analysis. Furthermore, the relationship between expression of COX-2 and COX-1, and the pathological features were investigated in order to determine the roles of COX enzyme in tumour progression.

Materials and Methods

Selection of Patients and Samples

All tissues in this study were obtained from Thai patient who had undergone curative surgical for primary colorectal cancer at Maharaj Nakorn Chiang Mai Hospital during April 2002 to June 2003. In each case, adjacent normal mucosa was collected for comparison. These specimens were immediately placed in vials, frozen in embedded medium for the preservation of cell integrity, and stored at –80 ^{o}C until analyzed. They were diagnosed by a pathologist according to pathological features of the tumors, which included tumor size in maximal diameter, depth of invasion, venous invasion, lymphatic invasion, perineural invasion, histological grading, lymph node metastasis, distant metastasis, and tumor staging (the AJCC TNM classification). Such patients' data were searched from the outpatient department (OPD) card at the administration and clerical section, Maharaj Nakorn Chiang Mai Hospital. The study processes were thoroughly accepted by the ethical committee of the faculty of medicine, Chiang Mai University according to document number 56/2545.

Western Blotting

Western blotting was performed to evaluate the expression of COX proteins in each tissue. Frozen tissues were thawed, cut into small pieces and homogenized in SDS lysis buffer (0.5M Tris-HCl pH 6.8, 2% SDS (w/v) and 10% glycerol (v/v)) containing a protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2.2 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64; Sigma, U.S.A). The tissue homogenate was then centrifuged at 10000g for 15 minutes at 4^{o}C, after which the supernatant was removed and the protein concentration of the supernatant was estimated by using the BCA protein assay kit (PIERCE, U.S.A). Twenty-five micrograms of protein from the tumor tissue and normal tissue from each patient was resolved on a 10% SDS polyacrylamide gels under reducing conditions and electrotransferred onto a nitrocellulose membrane (Biorad, U.S.A). The menbrane was blocked with 5% non-fat milk in TBS containing 0.05%Tween-20 (TBS-Tween) for 1 hour before incubated with monoclonal antibodies specific for COX-1 and COX-2 (Cayman Chemical. USA) at 4^{o}C overnight, and with horseradish peroxidase-conjugated goat anti-mouse IgG (Dako, U.S.A) for 2 hour at RT, respectively. After extensive washing with TBS-

Tween, immunoreactive protein was visualized with a chemiluminescence-based procedure using the ECL detection kit according to the manufacturer's protocol (Amersham, U.S.A). In order to examine the equality of protein loaded in each lane, a protein encoded from the house keeping gene 'actin' was used as a loading control. The detected membrane was submerged in the stripping buffer (62.5 mM Tris-HCl (pH6.7) containing 2% SDS and 100 mM 2-mercaptoethanol) and incubated at 50°C for 1 hour in order to eliminate the bonding of the previous antibodies, after which the membrane was re-probed with anti-actin mAb (Sigma, U.S.A). The intensity of each lane indicating COX-1, COX-2 and actin was semi-quantified using the densitometer (Helena Lab Science, U.S.A) and expressed as a ratio of the target to actin protein level.

Statistical Analysis

The data were analyzed using SPSS for Window version 7.5 (SPSS, Inc., Chicago, IL, USA)

Results

Quality Controls of the Study

In this study an adjacent normal tissue was also collected together with the tumor tissue and used as a normal control for each patients. In addition, control tissue homogenate was prepared from pooled tissue homogenate of colorectal and subjected to examination along with the unknown sample for the quality control of the protein assay and Western blot analysis in each assay. In order to examine the equality of protein loaded in each lane, several studies used ß-actin protein which is classified as one of the house keeping gene as an internal loading control (Molina et al, 1999; Konturek et al., 2001). In the present study, the detected membrane for COX-2 and COX-1 protein expression was finally reprobed with a monoclonal antibody specific for ß-actin and the detected protein level was expressed as a ratio of the target protein to actin level. Finally, the specificity and cross-reactivity of antibodies specific for COX-1 or COX-2 was tested with the alternate recombinant COX protein purchased from Cayman chemical and found no cross-reactivity with the alternate COX isoform of the two antibodies (data not shown).

Patient and Tumor Characteristics

Representative sections from each paraffin-embedded block of colorectal cancer tissues were routinely processed and stained with hematoxylin and eosin for morphological examination by a pathologist who was unaware of the results of Western blotting. Patient characteristics and pathologic features of each of the lesions are listed in Table 1. Of 44 patients with colorectal cancers, 22 had metastases to lymph nodes. Of five patients with distant metastases, three had liver tumors, one had lung and liver tumors, and another had ovarian tumors. Tumor sizes of colorectal specimen varied from 2.5 to 9 cm in maximum diameter, with a mean value of 4.9 ± 1.7 cm.

Table 1. Characteristics of the Colorectal Cancer Cases

Parameters	Colorectal cancer
Sex (cases)	
Male	15
Female	29
Age (years)	
Mean	60.5 + 15.7[a]
Range	22-89
Tumor size (maximum diameter; cm)	4.9 + 1.7
(Small:Large)	24:20
Histological differentiation (cases)	
(WD:MD:PD)	21:16:7
Depth of invasion (cases)	
(M:SM:MP:SS:SE)	0:1:2:28:13
Lymph node metastasis (cases)	
(Present:Absent)	22:22
Distant metastasis (cases)	
(Present:Absent)	5:39
Lymphatic invasion (cases)	
(Present:Absent)	39:5
Venous invasion (cases)	
(Present:Absent)	14:30
Perineural invasion (cases)	
(Present:Absent)	3:41
Stage grouping of tumor	
(I:II:III:IV)	2:17:20:5

[a]Mean + SD. (WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; M, mucosa; SM, submucosa; MP, muscular propria, SS, subserosa, SE, serosa)

Expression of COX Proteins in Colorectal Tumors

Representative immunoblots of colorectal samples using a specific anti-COX-2 antibody were shown in Figure 1a. The same membrane was then stripped and reprobed in order to assess the expression of COX-1 (Fig. 1b) and ß-actin (Fig. 1c), respectively. COX-2 protein was detected in 13 out of 44 tumor tissues (29.5%), whereas no COX-2 protein was detected in any of the normal colorectal tissue samples. While the level of COX-2 expression in normal tissues was

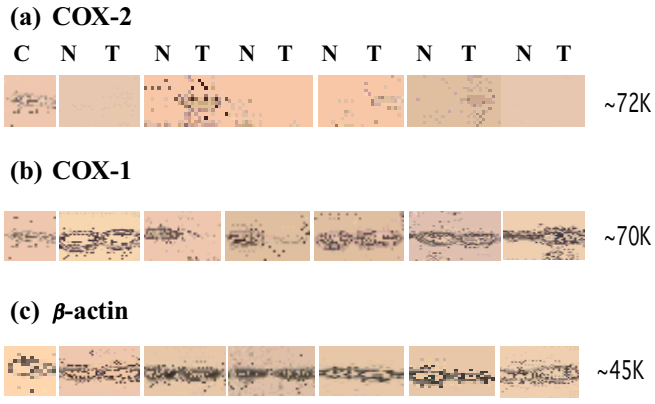

Figure 1. Protein Expression of COX-2, COX-1, and ß-actin Assessed by Western Blotting in Colorectal Cancer Tissues and the Corresponding Adjacent Normal Tissues. (T, tumor; N, normal tissue).

below the detection limit of Western blot analysis used in this study, COX-1 expression was detectable in both tumor and normal tissues. Interestingly, it was found that there was an alteration of COX-1 expression level in tumor tissues compared with normal tissue. Therefore, COX-1 expression levels were expressed as an expression ratio between the band density ratio of COX-1/ ß-actin of tumor tissue and normal tissue. To ensure that the alterations of COX-1 protein detected by Western blotting was due to the actual changes of COX-1 expression in tumor cells, not due to an experimental variation, only more than two-fold alteration was considered significant, as conducted previously (Dimberg et al., 1999). Therefore when the ratio was within the range of 0.5-2, it was assigned to a group which COX-1 expression was not changed. If the ratio was lesser than 0.5 or higher than 2, it would be assigned to a group of decreasing or increasing of COX-1 expression, respectively (see Table 2 for data).

COX Protein Expression in Relation to Classification of Pathological Features

Pathological parameters including tumor sizes in maximum diameter, depth of invasion lymph node metastasis, distant metastasis, and stage grouping of the cancers in relation to COX expression are shown in Table 2. Overexpression of COX-2 was significantly correlated with histological differentiation of colorectal tumors ($P<0.05$) and found more frequently in colorectal tumors with regional lymph node metastasis and larger size, although it was not statistically significant. Unlike COX-2, the level of COX-1 expression was found to be quite varied in tumor tissues. Forty-eight percent of colorectal tumors exhibited a decreased level of COX-1 in comparison to normal tissues. Interestingly, COX-1 was also found to be overexpressed in 23% of colorectal tumors indicating the possibility that COX-2 and COX-1 may both play important roles in promoting tumorigenesis. However, there was no significant relationship between the alterations of COX-1 protein levels and the pathological features of tumors was observed in both cancers.

Discussion

Colorectal cancers are the most common causes of cancer death in the world. Originally, several epidemiological studies have suggested that NSAIDs reduce the incidence of and mortality from colorectal cancers (Thun et al., 1993; Husain et al., 2002). Although, the exact mechanisms of NSAIDs on cancer prevention have not been clarified, one of the possible roles is via the inhibition of COX enzymatic activity.

There have been many studies on the levels of COX-2 protein expression in gastrointestinal tract cancers using Western blot analysis and have shown an enhanced level of COX-2 expression in colorectal tissues as compared with normal tissues such as overexpressed COX-2 levels in in 19 of 25 (75%) (Kargman et al., 1995) and 12 of 15 (80%) (Cianchi et al., 2001) of colorectal carcinomas. These results suggest that COX-2 plays an important role in tumorigenesis of the large bowel. However, the present study demonstrated that overexpression of COX-2 protein was presented in only 13 out of 44 (29.5%) colorectal tumor tissues from Thai patients and none of the adjacent normal tissues was found to possess a detectable level of COX-2. The low incidence

Table 2. Summary of Relationship between COX-1 and COX-2 Expression and Pathologic Features of Colorectal Tumors

Pathological features	COX-2 overexpression	COX-1 alteration Decrease:Not change:Increase
No. of patients	13 (29.5%)	21:13:10
Tumor size		
<5 cm (n=24)	6 (25%)	13:5:6
>5 cm (n=20)	7 (35%)	8:8:4
Histological differentiation*		
Well or Moderate (n=35)	8 (21.6%)	16:12:9
Poor (n=7)	5 (71.4%)	5:1:1
Depth of invasion		
Early cancer (M or SM) (n=1)	-	1:0:0
Advanced cancer (MP or SS or SE) (n=43)	13 (30.2%)	20:13:10
Lymph node metastasis		
Absent (n=22)	5 (22.7%)	12:4:6
Present (n=22)	8 (36.3%)	9:9:4
Distant metastasis		
Absent (n=39)	11 (28.2%)	18:12:9
Present (n=5)	2 (40%)	3:1:1
Tumor stage grouping		
Early stage (I or II) (n=19)	4 (21%)	10:4:5
Late stage (III or IV) (n=25)	9 (36.1%)	11:9:5

*P<0.05, according to Chi-square test.

of COX-2 overexpressed in colorectal cancer in Thai population, lead to the question whether COX-2 inhibitor will be useful as a anti-cancer drug in Thai population, since it has been demonstrated that tumor growth and angiogenesis could be suppressed by selective COX-2 inhibitor only if the tumor cells expressed COX-2 (Sawaoka et al., 1999).

It has been reported the relationship between COX-2 levels and pathological features of colorectal tumor,. i.e., larger sizes and deeper invasion, but was not correlated with whether the patients had distant metastasis or not (Fujita et al., 1998). In this study, COX-2 expression was found more frequently in tumor with larger size, with deeper invasion and with lymph node metastasis, although it was not statistically significant. This may due to the fact that there was a small number of tumors in Thai population exhibit COX-2 overexpression (only 29.5%). Although larger group of cancer patients needed to be studied, this observation implies that the rate of overexpression of COX-2 may be partly dependent the race and genetic backgrounds of the patients.

The majority of previous studies have reported that COX-1 protein levels were quite similar between in tumor tissues and normal tissues (Molina et al., 1999; Murata et al., 1999). However some studies have noted that COX-1 level can be either reduced or increased in tumor tissues (Murata et al., 1999; Cianchi et al., 2001). Therefore, there is still no clear consensus about the role of COX-1 in tumorigenesis. The present study demonstrated that the levels of COX-1 protein in tumor tissues were varied either equlled reduced or increased expression in comparison to normal tissues. However, the majority of colorectal tumors (47.8%) possess a decreased level of COX-1 protein compared to normal tissue. Interestingly, 10 of 44 tumor tissues (22.7%) showed an increased level of COX-1 protein, indicating that COX-1 may also play an important role in promoting and maintaining the neoplastic state as well as COX-2.

COX-1 expression was considered to be constitutive and generated prostaglandin for normal physiological function. However, a number of studies have recently shown that COX-1 expression can be induced in vitro by tobacco carcinogen (Rioux et al., 2000) and VEGF (Bryant et al., 1998). In addition, an elevated level of COX-1 expression has been reported in mouse lung tumors (Bauer et al., 2000), human breast cancer (Hwang et al., 1998) and human ovarian cancer (Gupta et al., 2003). This leads the question whether it is worthy to try to develop a selective COX-2 inhibitor for the purpose of using them as an anti-cancer drug.

A study performed by Sales and his group has recently demonstrated that COX-1 may regulate COX-2 expression through its enzyme product (Sales et al, 2002). From their results, the authors proposed that COX-1 may act in autocrine/paracrine fashion to regulate COX-2 expression. Taken together with our results, it is possible that expression of the two isoforms of COX is regulated by each other. During the early stage of tumorigenesis, a small increase of COX-2 expression may be compensated by the reduction of COX-1 expression as cells try to maintain the total enzyme activity with in the limited range. However, once the tumor has progressed, this balance may be broken. Therefore, at later stage of cancer, tumor cells possess an increase level of COX-1 or COX-2 as either of them can promote and maintain tumor growth. More than 50% of tumor tissues investigated in this study exhibited a decreased level of COX-1, with a small proportion of colorectal tumors (23%) appeared to overexpress COX-1. If our hypothesis is true, overexpression of either COX-1 or COX-2 should be enough to maintain tumor growth; therefore, cancers that overexpressed COX-1 should not overexpressed COX-2 and vice versa. Of 10 colorectal tumors that exhibited an increased level of COX-1, only 3 tumors had overexpressed level of COX-2. On the other hand, of 13 tumors overexpressing COX-2, only 3 tumors were found to overexpress COX-1. However, the only drawback of this hypothesis is that no significant relationship between overexpression of COX-1 and any of the pathological features was found in this study.

In conclusion, this study has demonstrated that COX-2 was overexpressed in the colorectal tumor tissues from Thai patients and their presence was significantly correlated with poor differentiation of the cancer cell. In addition, overexpression of COX-2 was found more frequently the tumors with larger size, lymph node metastasis, and lymphatic invasion of colorectal cancers suggesting that COX-2 may be involved in the development and/or progression of these cancers. Although alteration of COX-1 in tumor tissues was not significantly correlated with any of the pathological features, the results obtained from this study raise the possibility that it may play important roles in tumorigenesis. If that was the case, it is necessary to inhibit both isoforms of COX in order to antagonize tumor growth.

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