Intratumoral *COX-2* Gene Expression Is a Predictive Factor for Colorectal Cancer Response to Fluoropyrimidine-Based Chemotherapy

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Abstract

Purpose: Cyclooxygenase-2 (*COX-2*) is generally elevated in tumors compared with normal tissue and apparently has an important role in tumor development. A number of studies have found high expression of *COX-2* to be an unfavorable prognostic factor for overall survival in several cancers. However, the influence of *COX-2* expression levels on tumor response to chemotherapy has been relatively little studied. The purpose of this study was to ascertain if *COX-2* gene expression is associated with tumor response in the clinical treatment of colorectal cancer with the fluoropyrimidine-based therapy S-1.

Experimental Design: Patients with advanced (stage IV) colorectal cancer were treated with S-1 twice daily based on the patient's body surface area (BSA; BSA < 1.25 m^2, 80 mg/d; 1.25 m^2 $\leq$ BSA $<$ 1.5 m^2, 100 mg/d; BSA $\geq$ 1.5 m^2, 120 mg/d) for 28 days followed by a 2-week period rest. mRNA was isolated from paraffin-embedded pretreatment primary tumor specimens and expression levels of *COX-2* relative to *β-actin* as the internal reference gene were measured using a quantitative reverse transcription-PCR (Taqman) system.

Results: The overall response rate in a group of 44 patients treated with S-1 was 40.9%. Sufficient tumor tissue was available from 40 of these patients for *COX-2* mRNA quantitation. *COX-2* gene expression was significantly lower in the responding tumors compared with the nonresponders ($P = 0.012$, Wilcoxon test). Patients with *COX-2* values above the cutoff value of 3.28×10^{-3} had a significantly shorter survival than those with *COX-2* gene expressions below the cutoff value (adjusted $P = 0.031$).

Conclusions: Intratumoral *COX-2* gene expression is associated with likelihood of response to chemotherapy with S-1 and is a prognostic factor for survival of patients after the start of S-1 chemotherapy.

Cyclooxygenases, which exist in two isoforms designated as COX-1 and COX-2, are rate-limiting enzymes in the formation of prostaglandins from arachidonic acid. Whereas *COX-1* is generally considered to be constitutively expressed, *COX-2* is highly inducible by various factors and is associated with inflammation (1). Cyclooxygenases are targets for the class of drugs known as the nonsteroidal anti-inflammatory drugs. Epidemiologic studies have shown that patients who regularly took nonsteroidal anti-inflammatory drugs had 40% to 50% decreased incidence and lower mortality from colorectal cancer and other cancers than those who did not take nonsteroidal anti-inflammatory drugs (2–4). Overexpression of *COX-2* (but not *COX-1*) has been reported in many human malignancies (5–9). Up-regulation and overexpression of *COX-2 in vitro* was shown to reduce apoptosis and to increase angiogenesis, invasiveness of malignant cells, and conversion of procarcinogens to carcinogens (10–16). Hida et al. (17) reported that a marked increase in COX-2 immunoreactivity in non–small-cell lung cancer, specifically adenocarcinomas, was associated with tumor-invasive lesions and lymph node metastasis, suggesting that increased *COX-2* expression may be associated with an invasive and more aggressive phenotype in this disease. Consistent with the implications of these findings, several studies have shown the unfavorable prognostic significance of elevated *COX-2* expression. Achiwa et al. (8) showed that high *COX-2* expression correlated with inferior survival in a subgroup of stage I lung adenocarcinomas. A previous study from this laboratory showed an association between high *COX-2* mRNA expression levels and worse survival in patients with curatively resected non–small-cell lung cancer (18).

Such observations implicating *COX-2* in many of the basic processes of tumor development have suggested that targeting *COX-2* with specific inhibitors may be an effective strategy for cancer treatment (19, 20). In particular, there has been much

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Received 8/16/04; revised 1/13/05; accepted 2/8/05.

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interest recently in using COX-2 inhibitors along with conventional anticancer therapy (21) based on the idea that many of the COX-2–regulated genes that contribute to tumor progression may also be determinants of tumor chemo- or radiosensitivity. For example, the putative role of COX-2 in promoting antiapoptotic signaling (16, 22) suggests that tumors that highly express COX-2 should be less sensitive to agents that act by inducing apoptosis. This group includes many of the commonly used anticancer agents. The finding that COX-2 up-regulates the multidrug resistance gene *MDR1/pgp70* (23) suggests another mechanism by which COX-2 might promote chemoresistance. A further reason for thinking that COX-2 inhibitors will improve conventional cancer treatment derives from the role of COX-2 in promoting angiogenesis, suggesting the prospect that COX-2 inhibitors will inhibit neoangiogenesis (21, 24).

Clinical trials combining selective COX-2 inhibitors such as celecoxib and rofecoxib have been initiated based on the credible *ab initio* rationale discussed above and on *in vitro* and animal model data showing that the COX-2 inhibitors do increase the cytotoxic activity of some anticancer agents (25, 26). However, current information on the effect of *COX-2* expression on the intrinsic chemosensitivity of tumors is still very limited. In one of the few clinical studies to address directly the relationship between *COX-2* expression and tumor chemosensitivity, Ferrandina et al. (27) showed that increased *COX-2* expression was associated with resistance to cisplatin-based chemotherapy in both ovarian and cervical cancer.

The aim of the present study was to determine whether intratumoral *COX-2* expression is a predictive factor for tumor response to fluoropyrimidine-based chemotherapy. The hypothesis based on the above discussion would be that high COX-2 levels should render the tumors less responsive to the drugs. The subject population was a group of colorectal cancer patients treated with S-1, an oral drug regimen widely used in Japan to treat various solid tumors such as colorectal cancer, gastric, and breast cancer, which consists of the 5-fluorouracil prodrug tegafur and two modulators, 5-chloro-2,4-dihydroxypyridine and potassium oxonate (28).

Materials and Methods

Patient population. Eligible patients had (*a*) a diagnosis of recurrent colorectal cancer after surgical operation or disseminated colorectal cancer; (*b*) Eastern Cooperative Oncology Group performance status of 0 to 2 with adequate hematologic, hepatic, and renal function; (*c*) no treatment during the preceding 4 weeks; and (*d*) a lesion that was measurable by radiological examination.

Treatment. Patients were treated at the Tokyo Women's Medical University with S-1 twice daily for 28 days, followed by a 2-week period rest. The S-1 was given orally after breakfast and dinner. As in previous phase II studies of S-1 in advanced gastric cancer (29, 30), BSA was used to determine the dose of S-1 administered as follows: BSA < 1.25 m^2, 80 mg/d; 1.25 $m^2 \leq$ BSA < 1.50 m^2, 100 mg/d; BSA $\geq$ 1.5 m^2, 120 mg/d. These treatments were repeated until disease progression as determined by the treating physician or at the physician's discretion.

The protocol was reviewed and approved by an institutional review board and an ethics committee before study activation, and informed consent was obtained from every patient according to the institutional regulations.

Clinical evaluation and response criteria. After two cycles of treatment, measurable disease was reassessed. Response criteria were based on the standard definitions for bimeasurable disease (31). Response was assessed by computer-assisted tomography in liver, lymph node, and lung metastases, as well as in primary lesions. To be classified as a responder, a tumor had to have a 50% reduction in the sum of the products of the perpendicular diameters of the of the indicator lesion without growth of other disease or the appearance of new lesions (31). Overall survival was calculated as the period from the initiation of chemotherapy with S-1 to death. Patients who were still alive at the last follow-up were censored at that time.

Laboratory methods; microdissection. A representative formalin-fixed, paraffin-embedded pre-S1 treatment tumor specimen was selected by a pathologist after examination of the H&E-stained slides. Ten-micron-thick sections were stained with nuclear fast red to enable visualization of histology for laser capture microdissection (PALM Microlaser Technologies AG, Bernried, Germany), which was done to ensure that only tumor cells were studied.

RNA isolation and cDNA synthesis. RNA isolation from formalin-fixed paraffin embedded specimens was done according to a proprietary procedure of Response Genetics, Inc. (Los Angeles, CA) (US patent no. 6,248,535). Following RNA isolation, cDNA was prepared from each sample as described previously (32).

Reverse transcription-PCR. Relative cDNA quantitation for *COX-2* and an internal reference gene (*β-actin*) was done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (TaqMan), Applied Biosystems, Foster City, CA], as described previously (32–34).

The primers and probe sequences used are given in Table 1. The PCR reaction mixture consisted of 600 nmol/L of each primer, 200 nmol/L probe, 2.5 units AmpliTaq Gold Polymerase, 200 μmol/L each dATP, dCTP, dGTP, 400 μmol/L dUTP, 5.5 mmol/L $MgCl_2$, and 1× Taqman Buffer A containing a reference dye to a final volume of 25 μL (Applied Biosystems). Cycling conditions were 50°C for 10 seconds, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. Colon, liver, and lung RNAs (Stratagene, La Jolla, CA) were used as control calibrators on each plate.

Statistical analysis. The *COX-2* gene expression value was expressed as the ratio between two absolute measurements (gene of interest divided by an internal reference gene, *β-actin*). *COX-2* gene expression values in the responding and nonresponding groups of patients were analyzed using the Wilcoxon test. To compare *COX-2* gene expression values relative to demographic and clinicopathologic characteristics, the Wilcoxon test was used for two groups and the Kruskal-Wallis test was used for three or more groups. Medians and ranges were used to summarize *COX-2* gene expression values

Table 1. Sequences of PCR primers and probes

Cyclooxygenase-2 (COX-2)	
Forward primer (COX2-633F)	5′-GCTCAAACATGATGTTTGCATTC-3′
Reverse primer (COX2-711R)	5′-GCTGGCCTCGCTTATGA-3′
Probe (COX2-658T)	5′-(FAM)TGCCCAGCACTTCACGCATCAGTT(TAMRA)-3′
β-Actin	
Forward primer (b-actin-592F)	5′-TGAGCGGGCTACAGCTT-3′
Reverse primer (b-actin-651R)	5′-TCCTTAATGTCACGCACGATTT-3′
Probe (b-actin-611T)	5′-(FAM)ACCACCACGGCCGAGCGG(TAMRA)-3′

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within groups of patients. The associations between demographic and clinicopathologic variables and response were examined using Fisher's exact tests.

To evaluate the association of *COX-2* gene expression with overall survival, COX-2 value was categorized into a low and a high value. The optimal cut point was determined by adapting the maximally selected χ^2 method of Miller and Siegmund (35) and Halpern (36). To determine a *P* value that would be interpreted as a measure of the strength of the association, 2,000 bootstrap-like simulations were done to estimate the distribution of the maximal χ^2 statistics. The association of each of the demographic and clinicopathologic variables with survival was tested using the log-rank test. A Cox proportional hazards model was used to investigate the effect of *COX-2* gene expression on overall survival considering potentially important prognostic factors in colorectal cancer. All reported *P* values are two sided.

Results

A total of 44 primary pre-S1 treatment colorectal cancer specimens from 44 patients, all of whom had stage IV disease, were studied. Eighteen (40.9%) of the patients were classified as responders and 26 patients (59.1%) were nonresponders. *COX-2* mRNA level values were successfully obtained for 40 (90.9%) of the 44 patients. The remaining four tumor specimens had levels of *β-actin* (the internal reference gene) below our accepted cutoff for the assay and were therefore excluded. Nineteen of the 40 patients (47.5%) included in the study were male and the median age for all patients was 64 (range 39-74). Fifteen and 25 patients received S-1 chemotherapy administered at 100 and 120 mg/d, respectively, based on their BSA. The median cycle of S-1 chemotherapy patients received was 6 (range 2-14).

Eighteen patients (45%) for whom *COX-2* expressions were measured responded to S-1 chemotherapy and 22 (55%) were nonresponders. The median overall survival for the 40 patients was 15.3 months (95% confidence interval, 12.1-19.8) with a median follow-up period of 8.7 months (15 patients were still alive at the time of the analysis of the data).

Clinicopathologic characteristics and COX-2 mRNA expression levels. *COX-2* mRNA levels by demographic and clinicopathologic characteristics were analyzed (Table 2). No significant differences in *COX-2* mRNA expression levels for these variables were found (Table 2).

Response to S-1 chemotherapy and COX-2 mRNA expression levels. There was a significant difference in median *COX-2* mRNA levels in responders versus nonresponders ($P = 0.012$, Wilcoxon test; Fig. 1). *COX-2* gene expression levels were significantly lower in tumor tissue of responders (median: 1.22×10^{-3}; range: 0.04×10^{-3} to 6.07×10^{-3}) than those in the nonresponders (median: 3.32×10^{-3}; range: 0.39×10^{-3} to 7.73×10^{-3}). As can be seen in Fig. 1, 3 of the 15 patients with *COX-2* mRNA expression level above 3×10^{-3} experienced a response to S-1 chemotherapy compared with 15 of the 25 patients with *COX-2* mRNA expression $\leq 3 \times 10^{-3}$. None of demographic and clinicopathologic variables (Table 2) were statistically significantly associated with tumor response to S-1

Table 2. Association between *COX-2* gene expression and demographic clinical and pathologic characteristics

		COX-2 gene expression		
Variables	***n***	**Median**	**Range (min-max)**	***P****
Sex				
Male	19	1.44	(0.39-7.20)	0.10
Female	21	3.28	(0.04-7.73)	
Age, y				
<65	21	2.43	(0.41-7.73)	0.30
≥65	19	1.80	(0.04-5.86)	
Relapse category				
Liver metastasis	19	2.03	(0.52-5.65)	0.54
Peritoneal and lymph node metastasis	11	1.80	(0.39-7.20)	
Lung metastasis	6	3.38	(0.39-5.86)	
Local recurrence	4	5.18	(0.04-7.73)	
Histology				
Well-differentiated adenocarcinoma	30	2.29	(0.39-7.73)	0.89
Moderately differentiated adenocarcinoma	7	1.80	(0.04-7.20)	
Mucinous adenocarcinoma	3	1.44	(0.61-4.21)	
Performance status				
0	3	5.40	(0.04-5.65)	0.82
1	22	2.37	(0.39-7.73)	
2	15	1.80	(0.41-7.20)	
Dose level				
100 mg/d	15	3.28	(0.04-7.20)	0.14
120 mg/d	25	1.80	(0.39-7.73)	

*Based on the Wilcoxon or Kruskal-Wallis test.

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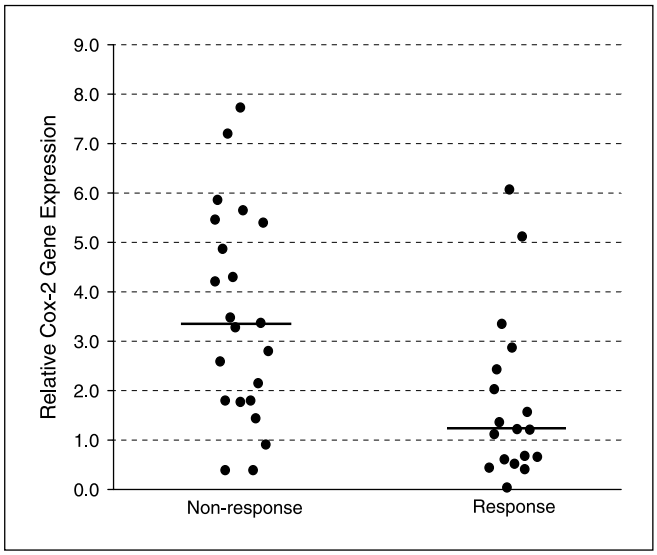

Fig. 1. *COX-2* expression in colorectal tumors according to response (>50% tumor shrinkage) or nonresponse (<50% tumor shrinkage). *COX-2* expression values in responding tumors are significantly lower than those in nonresponding tumors (Wilcoxon test, $P = 0.012$). Median values are indicated by horizontal bar.

chemotherapy at the significance level of 0.10 based on Fisher's exact tests (data not shown).

Survival in relation to COX-2 expression. Using 3.28×10^{-3} as a cutoff value, 26 patients (65%) had low COX-2 expression and 14 (35%) had high COX-2 expression. The overall survival by COX-2 expression (low versus high) is shown in Fig. 2. Survival for colorectal cancer patients with *COX-2* values below the cutoff value was significantly longer than for those patients with *COX-2* above this value (simulated $P = 0.031$; Fig. 2). The univariate analysis indicated that none of demographic or clinicopathologic factors (Table 2) were statistically significantly associated with survival at the significance level of 0.10 on the basis of the log-rank test (data not shown). Nonetheless, for completeness of the analysis, the association between COX-2, using the 3.28 cut point, and survival was reexamined stratifying for each of the clinicopathologic factors. In these reanalyses, the degree of the association (i.e., observed hazard ratio) was basically unchanged when adjusting each of demographic or clinicopathologic factors (data not shown).

Discussion

In this study, we found a significant association between high *COX-2* expression in colorectal tumors and less favorable clinical outcome for patients treated with S-1 chemotherapy, both in terms of tumor response and patients' survival. Most previous clinical correlative studies have concentrated on the prognostic value of *COX-2* overexpression for overall survival and only a few have directly addressed the effect of *COX-2* expression on tumor response to chemotherapy. The most definitive of these, which involved treatment of ovarian and cervical tumors with platin-based therapy (27), showed that nonresponding tumors had a significantly higher percentage of *COX-2*–positive tumors than did the responders, whereas almost all progressing tumors were *COX-2* positive. The present study shows that *COX-2* expression is also associated with tumor response for fluoropyrimidine therapy in colorectal cancer, suggesting that the influence of COX-2 on tumor chemosensitivity may be a general effect acting on a variety of agents with different mechanisms of action in various tumor types.

The mechanism responsible for COX-2–induced desensitization of tumors to chemotherapy (i.e., whether it is due to apoptosis modulation by *Bcl-2* induction or to effects on some other process) will require further study. It is also possible that COX-2 may directly influence the expression levels of target enzymes for some drugs, although for the present case, we did not see any correlation between *COX-2* and any of the putative response markers for fluoropyrimidines such as *TS* or *DPD* gene expressions. Regardless of the mechanism, however, the finding that intratumoral *COX-2* overexpression seems to lower the intrinsic chemosensitivity of tumors provides a more substantial experimental underpinning for the use of COX-2 inhibitors with conventional anticancer agents to increase the efficacy of the chemotherapy. Assessment of *COX-2* expression status before chemotherapy could be used to identify patients that have a lower probability of response to chemo- or radiotherapy, who may therefore require a different treatment than those with low *COX-2* for optimal benefit.

The critical question for the strategy of combining COX-2 inhibitors with chemotherapy is whether administration of a COX-2 inhibitor can reverse the effects of *COX-2* overexpression and cause the tumor to acquire the more responsive low-*COX-2* phenotype. The indications from both *in vivo* and *in vitro* studies thus far are encouraging. For example, whereas artificially increasing the levels of prostaglandin E2 in human colon cancer HCA-7 cells caused up-regulation of the expression of *Bcl-2* (12), a COX-2 inhibitor, NS398, down-regulated *Bcl-2* expression and concomitantly increased the amount of apoptosis in LNCaP human prostate cancer cells (37). Similarly, inhibition of COX-2 was shown to up-regulate the proapoptotic molecules par-4 (21) and caspase-3 (38) and to enhance drug-induced apoptosis *in vitro* (39). Such an effect on *Bcl-2* expression should in theory increase the sensitivity of the cells to a variety of agents and, indeed, Hida et al. (40) found that a *COX-2* inhibitor (nimesulide) increased the potency

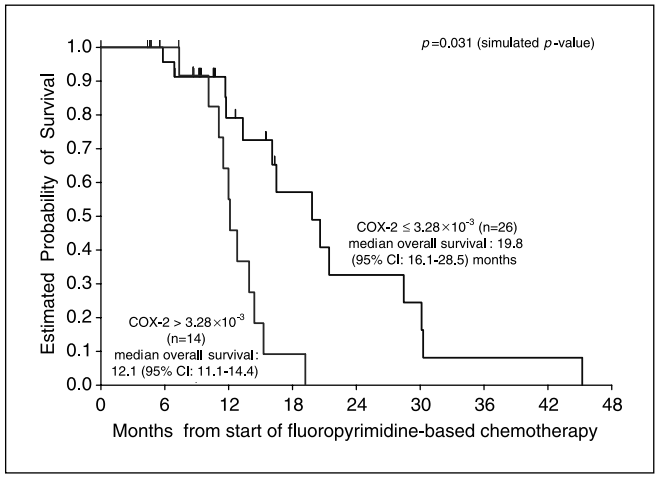

Fig. 2. Survival (Kaplan-Meier) plots indicating probability of survival for patients with *COX-2* expressions above or below the cutoff *COX-2* expression value for all patients (*COX-2*: *β-actin*, 3.28×10^{-3}).

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(decreased the IC50) of a number of anticancer drugs of various different mechanistic classes against non–small-cell lung cancer cells *in vitro*. In animal models and cell culture (41–43), *COX-2* inhibitors were found to enhance the effect of radiotherapy. The mechanism of this effect still remains unclear, however, because at least in one case studied (44), the COX-2 inhibitor, although enhancing the radiation effect, did not seem to change the apoptotic fraction of cells. Interestingly, some studies have found that selective COX-2 inhibitors seem to produce anticarcinogenic effects by COX-2–independent pathways as well (45). A recent clinical study found that celecoxib inhibited an induction of prostaglandin E production in lung cancer patients treated with paclitaxel/carboplatin (46). Comparison with historical response rates suggested that the addition of celecoxib may have increased the response rate as well (46). Because of the role of COX-2 in promoting angiogenesis, COX-2 inhibitors might also enhance the effectiveness of angiogenesis-directed therapy in a manner similar to that of epidermal growth factor receptor–targeted agents (47).

The strategy of targeting COX-2 during conventional therapy is attractive from several points of view. First, because *COX-2* overexpression is generally observed in tumor tissue but not normal tissue, any enhancement of drug efficacy due to COX-2 inhibition should occur preferentially in tumors, thereby resulting in a gain in therapeutic index. In addition, as discussed previously, *COX-2* overexpression seems to be a common phenomenon in cancer, and thus the use of COX-2 inhibitors may provide a means to enhance the effectiveness of treatment of a variety of different tumor types with a variety of different therapies. So far, if the present study is included, we know that high COX-2 levels are associated with lower tumor sensitivity to both cisplatin- and fluoropyrimidine-based therapies. It has also been observed that the responsiveness of cancer cells to selective COX-2 inhibitors is proportional to the degree of *COX-2* expression (48), suggesting that patients with low intratumoral *COX-2* expression may not benefit from addition of a COX-2 inhibitor to their therapy. Quantitative measurement of *COX-2* expression status such as that described here during clinical trials should eventually establish levels of *COX-2* expression in tumors at which addition of COX-2 inhibitors can be expected to be of benefit, and thus identify before treatment those patients who would be the best candidates for treatment with COX-2 inhibitors along with their conventional therapy.

In summary, this study shows that *COX-2* expression may be a determinant of drug efficacy for fluoropyrimidine therapy of colorectal cancer, thus bolstering the concept that coadministration of COX-2 inhibitors may improve the clinical outcome of conventional chemo/radiotherapy, but with the caveat that the beneficial effects of using COX-2 inhibitors probably vary according to the initial intratumoral *COX-2* expression level. Independent studies are necessary to confirm the role of COX-2 expression in predicting tumor response to fluoropyrimidine-based chemotherapy in patients with colorectal cancer. Among further directions for research in this area would be to quantitatively determine the effects of COX-2 inhibitors on the antitumor efficacy of different classes of drugs and to elucidate the molecular mechanism of this enhancement.

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