Increased Expression of COX-2 in Nontumor Liver Tissue Is Associated with Shorter Disease-free Survival in Patients with Hepatocellular Carcinoma

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ABSTRACT

Recent studies have shown increased levels of cyclooxygenase-2 (COX-2) in a variety of human malignancies including hepatocellular carcinoma (HCC), but little is known about the prognostic value of COX-2 in HCC or its associated nontumor liver tissue. We examined the expression of COX-2 protein by immunohistochemistry in 53 patients with HCCs whose corresponding nontumor tissues were hepatitis C virus-related chronic hepatitis (n = 21) and cirrhosis (n = 32). Samples of nine histologically normal livers and eight precancerous dysplasias were also analyzed. The level of COX-2 increased from normal liver to chronic hepatitis to cirrhosis. The majority of cirrhotic livers (81%) displayed marked COX-2 expression. In dysplasias, COX-2 expression was mainly moderate or strong (88%). In HCC, 17% of samples displayed a high COX-2 expression, and 37% of samples expressed COX-2 at a moderate level. Concordant results were obtained with reverse transcription-PCR and Western blot analyses. Clinicopathological survey indicated a significant correlation between COX-2 expression and differentiated carcinoma (P = 0.019). Although there was no correlation between COX-2 expression in HCC and prognosis, a striking difference was found between COX-2 expression in nontumor tissue and shorter disease-free survival (P = 0.0132). Moreover, high COX-2 expression in nontumor tissue was significantly correlated with the presence of active inflammation (P < 0.0001). The present findings suggest that COX-2 expression in nontumor tissue may play a positive role in relapse of HCC after surgery.

INTRODUCTION

Primary HCC3 is one of the most common tumors in Southeast Asia and Africa, where the incidence of this disease is ~30 per 100,000 men/year. The prognosis of HCC is generally poor, and the 5-year survival rate is limited to 25–39% after surgery (1). Many investigators have reported a putative link between infection with hepatitis B or C, liver cirrhosis, and the development of HCC. Hepatic cirrhosis is observed in up to 90% of patients with HCC (2). In the United States, it has been estimated that 60–90% of those infected with HCV each year develop chronic hepatitis C, which often leads to cirrhosis. The result is often liver failure or liver cancer. Chronic hepatitis C is the leading cause of liver cancer in the United States and, according to the American Liver Foundation, is the chief reason for liver transplantation. Also in Japan, HCV-based cirrhosis is a serious problem because it develops to HCC at a ratio of 7% per year, and it is found in 70% of HCCs (3).

COX is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, the precursor of various compounds including prostaglandins, prostacyclin, and thromboxanes. Two COX genes, COX-1 and COX-2, have been identified that share >60% identity at the amino acid level (4). COX-1 is constitutively expressed in many tissues and responsible for various physiological functions including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane A2, by platelets. On the other hand, COX-2 is an inducible immediate-early gene originally found to be induced by various stimuli, such as mitogens and cytokines, and growth factors (5–7). Overexpression of COX-2 has been demonstrated in various chronic inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease, ulcerative colitis, and gastritis caused by Helicobacter pylori (8–10).

Recent studies have highlighted the relevance of COX-2 in human carcinogenesis. Epidemiological studies indicate that NSAIDs lead to a regression of colonic polyps in patients with...
COX-2 in Nontumor Tissues Predicts Relapse of HCC

membrane, no bands appeared on the blots (data not shown).

Lanes 7–9. The recombinant COX-2 protein obtained from Cayman Chemical (Ann Arbor, MI) served as a positive control (Lane 10). When preabsorbed antibody, instead of the COX-2 antibody, was applied to the transferred membrane, no bands appeared on the blots (data not shown).

familial adenomatous polyposis (11). NSAIDs are also known to reduce the risk of sporadic colorectal cancers and breast and lung cancers (12–13). Because NSAIDs can inhibit activities of both COX-1 and COX-2, and double knock-out mice for the adenomatous polyposis coli and COX-2 genes showed marked reduction in the size and frequency of intestinal polyps, it is suggested that the antitumor effect of NSAIDs is attributable, at least in part, to inhibition of COX-2 (14, 15). In fact, the expression of COX-2 is significantly increased in various types of carcinoma, including HCC (16–28). There is also sufficient evidence from animal studies indicating that selective COX-2 inhibitors produce effective prevention of carcinogenesis and that these compounds act by induction of apoptosis of various cancer cells (29–38). These findings suggest that COX-2 may be involved in carcinogenesis and/or progression of certain types of human malignancies. In human hepatocellular carcinoma, COX-2 expression has been shown to be associated with differentiated phenotype (16–17). However, COX-2 expression in the premalignant hepatic dysplasia and the impact of COX-2 expression on clinical course have not yet been elucidated.

In an effort to determine the prognostic value of COX-2 expression in both tumor and nontumor tissues, we examined the level of COX-2 mRNA by RT-PCR and extent of COX-2 protein by immunohistochemistry in HCV-infected HCCs and their associated nontumor tissues. A subset of dysplastic lesions was also assessed in this study.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. Three HCC cell lines, HuH7, HLE, and HLF, were obtained from the Japanese Cancer Research Resources Bank. Cells were cultured in DMEM supplemented with 10% FCS at 37°C. Tissue samples were obtained from 61 patients with hepatic tumors, who underwent hepatectomy at the Department of Surgery II, Osaka University Medical School. All patients had HCV infection, but none had hepatitis B viral infection. The mean follow-up period for the patients’ prognosis was 3.4 ± 2.0 years. Various hepatic lesions were immunohistochemically examined for COX-2 expression, including 8 dysplasias and 53 paired HCCs. Nontumor tissues were also examined, which comprised 21 CH-based livers and 32 cirrhotic livers. Prior to hepatectomy for HCC, 15 patients were treated with TAE. Nine histologically normal livers devoid of hepatitis B or C viral infection were obtained from nine patients with liver metastasis of colorectal cancer and served as control.

For immunohistochemistry, tissue samples were fixed in 10% neutral buffered formalin, processed through graded ethanol, and embedded in paraffin. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C for analysis by RT-PCR and Western blotting.

Pathological Examination. Tissue sections (4-μm thick) were deparaffinized in xylene, rehydrated, and stained with H&E solution. Pathological diagnosis of tissues into nontumor and tumor tissues was determined by a skilled pathologist (K.W.), who was blinded to the clinical background. For 53 nontumor tissues, the presence of active inflammation and cirrhotic nodule was examined. Tumor tissues were examined for the following characteristics: cell differentiation (well, moderately, or poorly differentiated), number of tumors, capsular formation, septal formation, capsular invasion, portal vein tumor thrombus formation, and hepatic vein invasion.

Semiquantitative RT-PCR. RNA extraction was carried out with TRIzol reagent in a single-step method, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI), as described previously (39). Semiquantitative analysis for expression of COX-2 mRNA was performed by the multiplex RT-PCR technique, using a housekeeping gene, PBGD, as an internal standard (28, 40–41). To minimize the differences of inter-PCRs, PCR was performed with COX-2 and PBGD primers in an identical tube, under unsaturated conditions. PCR was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 1× Perkin-Elmer PCR buffer, 1.5 mm MgCl₂, 0.8 mm deoxyribonucleotide triphosphates, 0.8 μM of each primer for COX-2, 80 nm each for PBGD, and 1 unit of Taq DNA polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Branchburg, NJ). The PCR primers used for detection of COX-2 and PBGD cDNAs were synthesized as reported previously, and the amplified products were 305 and 127 bp, respectively (42, 43). The condition for multiplex PCR was set up as follows: one cycle of denaturing at 95°C for 12 min, followed by 35–40 cycles of 95°C for 1 min, and 72°C for 1 min, before a final extension at 72°C for 10 min. The electrophoresed PCR products were scanned by densitometry, and the relative value of the COX-2 band to PBGD band was calculated in each sample.

Reagents. Rabbit polyclonal anti-human COX-2 antibody (23, 28) and its blocking peptide that was used as immunogen (17 amino acids; position 251–267) for generation of this antibody were obtained from IBL Co. (Gunma, Japan). Recombinant COX-2 protein was obtained from Cayman Chemical (Ann Arbor, MI) and used as a positive control in Western blot analysis.

Immunohistochemistry. After deparaffinization, heat antigen retrieval was performed as described previously (44). The slides were then processed for immunohistochemistry on the TeckMate Horizon automated staining system (Dako, Carpinteria, CA; Refs. 28 and 45), using the Vectastain ABC-peroxidase kit (Vector Labs, Burlingame, CA; Ref. 44). In the
step of primary antibody reaction, the slides were incubated with the COX-2 antibody (final concentration, 5 μg/ml) for 1 h at room temperature. For the positive controls, sections of colon cancer expressing COX-2 protein were included in each staining procedure. For negative controls, nonimmunized rabbit IgG (Vector Labs) or TBS (Tris-buffered saline) was used as a substitute for the primary antibody to verify the possibility of false-positive responses from nonspecific binding of IgG or from the secondary antibody. In addition, absorption tests were performed on tissue sections.

Evaluation of COX-2 Immunostaining. All immunostained sections were evaluated in a blinded manner without knowledge of the clinical and pathological parameters. For each section, the intensity of staining was scored on a scale from 0 to 2, where 0 represented negative or faint staining; 1, moderate; and 2, strong staining. COX-2 expression was very faint or undetectable in the vascular epithelium, whereas epithelial cells of the bile ducts devoid of significant inflammation generally expressed moderate levels of COX-2. Accordingly, the latter level of staining was used as an inner control within the sample, which was designated arbitrarily as intensity level 1. COX-2 expression was generally homogeneous in each sample, except in 14 HCCs, in which tumor tissues were composed of more than two different histological types. In these cases, the carcinoma type that constituted the major volume of the tumor was selected as the representative type for evaluation. Staining was repeated at least twice to avoid possible technical errors, but similar results were obtained. All slides were interpreted by two investigators (H. Y. and Y. I.) on different occasions. The results of interpretation were similar except for <10% of the samples. The final diagnosis in the latter samples was determined by using a multihead microscope.

Western Blot Analysis. Western blot analysis was performed as described previously (46). Briefly, 100 μg of the total protein from tissues and 2.5 μg/ml COX-2 were used for this assay.

Statistical Analysis. Statistical analysis was performed using the Statview J-4.5 program (Abacus Concepts, Inc., Berkeley, CA). The χ² test and Fisher’s exact probability test or the log-rank test were used to examine the association between COX-2 expression and clinicopathological parameters or prognosis. P < 0.05 denoted the presence of a statistically significant difference.
RESULTS

Western Blot Analysis for COX-2. To confirm the specificity of COX-2 antibody, a small set of tissue samples (three paired nontumor and tumor tissues and three normal livers) were subjected to Western blot analysis (Fig. 1). COX-2 antibody yielded a strong band to the control purified COX-2 protein (Fig. 1, Lane 10). All three nontumor tissues with liver cirrhosis expressed clear bands for the COX-2 protein (Fig. 1, Lanes 1, 3, and 5) and one well-differentiated HCC expressed COX-2 at a modest level (Lane 2). In contrast, COX-2 expression was not detected in two of poorly differentiated HCCs and three normal livers (Lanes 4, 6, and 7–9, respectively). When preabsorbed antibody was applied to the transferred membrane instead of the COX-2 antibody, no bands appeared on the blots (data not shown).

Immunohistochemical Analysis for COX-2. Immunohistochemical staining of positive control sections of colon cancer expressing the COX-2 protein displayed a marked staining for COX-2 (Fig. 2A). In contrast, no staining was observed when the primary antibody was substituted with nonimmunized rabbit IgG or TBS (data not shown). Preabsorbed antibody with excess amount of immunogenes abolished staining on the sections (data not shown), indicating that the COX-2 antibody was highly specific to the COX-2 protein on tissue sections. Immunohistochemical assays were performed on a series of 53 paired HCCs and their matched nontumor tissues, 8 dysplasias, and 9 normal livers. The level of COX-2 showed a wide spectrum. Specifically, in normal livers, COX-2 expression was not detected, or moderate at the most, but CH-based hepatocytes expressed a moderate to strong COX-2 protein in all samples. The majority of cirrhotic livers (26 of 32; 81%) displayed a marked COX-2 expression. These results are summarized in Table 1, and representative cases of each stage are shown in Fig. 2, B–D. The difference in proportion of cells expressing a high level of COX-2 (intensity 2) between normal and cirrhotic livers was significant ($P < 0.0001$) as was that between CH and cirrhosis ($P = 0.0001$; Table 1). In tissue samples with dysplasia, COX-2 expression was mainly moderate or strong (7 of 8; 88%; Fig. 2E). In carcinoma tissues, 46 of 53 HCCs were evaluated because 7 HCCs were found to be completely necrotic, probably because of preoperative TAE treatment. In approximately one-half of examined cases (21 of 46; 46%), little or no staining was observed, whereas 17% of tissue samples (8 of 46) displayed a marked COX-2 expression (Fig. 2F), and 37% of cases (17 of 46) expressed moderate levels of COX-2 protein.

Table 1 Immunohistochemical analysis of COX-2 expression

<table>
<thead>
<tr>
<th>Intensity</th>
<th>No. of cases</th>
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<tr>
<td>0</td>
<td>6</td>
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<tr>
<td>1</td>
<td>3</td>
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<tr>
<td>2</td>
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Table 2 Relationship between COX-2 expression and clinicopathological parameters in cases with HCC

<table>
<thead>
<tr>
<th>COX-2</th>
<th>No. of cases</th>
<th>Low</th>
<th>High</th>
<th>$P$</th>
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<tbody>
<tr>
<td></td>
<td>Age</td>
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<tr>
<td>0.019</td>
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</tr>
<tr>
<td></td>
<td>Gender</td>
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<tr>
<td></td>
<td>Tumor size</td>
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<tr>
<td></td>
<td>Histological grade</td>
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<td></td>
<td>Hepatic vein invasion</td>
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<tr>
<td></td>
<td>No. of tumors</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Septal formation</td>
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<tr>
<td></td>
<td>Capsular formation</td>
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<td></td>
<td>TAE</td>
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<tr>
<td>$P &lt; 0.0001$</td>
<td>$P = 0.0001$</td>
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\[ ^a \] NS, not significant.
\[ ^b \] Well, well differentiated; mod., moderately; poor, poorly; undiff., undifferentiated.
\[ ^c \] Statistically significant.
\[ ^d \] This category includes both intrahepatic metastasis and tumors generated by multicentric carcinogenesis.

Correlation between COX-2 Expression and Clinicopathological Parameters. The correlation between level of COX-2 expression in 46 HCCs and various clinicopathological features was examined (Table 2). For statistical analysis, cases were divided into two groups, high COX-2 expressors (intensity 2) and low COX-2 expressors (intensities 0 and 1). Inconsistent with previous reports (16, 17), there was a significant difference
in COX-2 expression with regard to histological grade (Table 2). Thus, high COX-2 expression was associated with well- and moderately differentiated carcinomas \((P = 0.019; \text{Table 2})\). The correlation between COX-2 expression in HCC and disease-free period or overall survival rate was examined, but no significant relation was noted (data not shown).

We also analyzed the relationship between COX-2 expression in nontumor tissues (21 chronic hepatitis and 32 cirrhosis) and disease-free survival time, because the nontumor tissues of liver are usually not normal but are pathological lesions. As shown in Fig. 3, a striking difference in the disease-free survival time was found between high COX-2 expressors and low COX-2 expressors \((P = 0.0132)\). To examine possible involvement of inflammation in COX-2 induction, 53 nontumor tissues were divided into two groups by pathological survey on their extent of inflammation (by K. W.). Active inflammation was noted in 3 of 21 CH-based livers and 29 of 32 cirrhosis. A significant correlation was found between high COX-2 expression in nontumor tissues and inflammation \((P < 0.0001; \text{Table 3})\).

### Table 3

<table>
<thead>
<tr>
<th>COX-2</th>
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<th>Low</th>
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<tbody>
<tr>
<td>Active</td>
<td>30</td>
<td>2</td>
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<tr>
<td>Inactive</td>
<td>2</td>
<td>19</td>
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Semiquantitative RT-PCR. To examine COX-2 mRNA levels, RT-PCR assay was performed in three HCC cell lines (HuH7, HLE, and HLF), two histologically normal livers, and six paired nontumor and tumor tissues. HuH7 and HLF cell lines expressed 305-bp bands for COX-2 with constitutive expression of PBGD, whereas the HLE cell line did not express a band for COX-2 (Fig. 4, upper panel). In tissue samples, the relative value of COX-2 band to PBGD band was calculated for each lane (Fig. 4, lower panel). In the normal livers, relatively weak levels of COX-2 mRNA were detected. In four of six paired samples (67%), nontumor tissues expressed higher levels of COX-2 mRNA than did the corresponding HCCs (cases 1, 3, 4, and 6), whereas one HCC expressed a higher COX-2 mRNA compared with the corresponding nontumor tissue (case 2). Tissue samples from one patient displayed scarce levels of COX-2 mRNA in both nontumor and tumor tissues (case 5). To avoid saturated PCR conditions, these assays were repeated with lower cycles of amplification, and similar results were obtained. Furthermore, the results of immunostaining and Western blot analysis were in agreement with those obtained by RT-PCR assay in each sample, suggesting that the expression of COX-2 protein is regulated at the transcription level (data not shown).

### DISCUSSION

The present results showed that nontumor liver tissues often expressed high levels of COX-2 protein and mRNA relative to those of carcinoma tissues (Figs. 1, 2, and 4; Table 1). These findings are contrary to those described in other organs such as colon, stomach, esophagus, and lung. It was reported that the COX-2 level was higher in these tumors compared with nontumor tissues (18–28). It is likely that the different results are attributable to the type of control tissue. In cases with liver, nontumor tissues were mostly pathological because of HCV-infected CH or cirrhosis. In support of this explanation, a low level of COX-2 mRNA was shown previously in the normal liver (42), and we found that normal liver tissues devoid of viral infection showed little or no expression of COX-2.

In nonneoplastic liver tissues, we found a wide spectrum of COX-2 expression from normal liver to cirrhosis (Table 1). It appears from the present analysis that induction of COX-2 may be attributable to active inflammation in CH and cirrhotic livers (47). There is evidence that hypoxia can induce COX-2 (48), and COX-2 facilitates angiogenesis via the enhanced release of angiogenic growth factors such as vascular endothelial growth factor (49). Indeed, vascular endothelial growth factor level is increased in cirrhosis (50).

It was of interest from a clinical point of view that an increase in COX-2 expression in the nontumor tissues was significantly associated with relapse of HCC (Fig. 3). The
prognosis of HCC is generally unfavorable. Although primary tumors are curatively resected, 50–60% of patients suffer from the same disease within 5 years (51). This could be because of either newly established tumor from the remnant liver, a process termed multicentric carcinogenesis (52), or recurrence of the original tumor. One possible mechanism for a link between COX-2 and disease relapse is that high expression of COX-2 in the remnant liver might contribute to carcinogenesis, as proposed in other types of human carcinoma (29–34). Alternatively, COX-2 may enhance micrometastatic carcinoma cells to survive and spread after hepatectomy. The latter hypothesis is supported by several studies. For example, introduction of COX-2 cDNA into colon carcinoma cells facilitated growth (49). COX-2 appears to prevent apoptosis because selective COX-2 inhibitors induced apoptosis in various cell culture systems (36–38). Furthermore, it was shown that COX-2 promoted angiogenesis in malignant cells (49).

Our immunohistochemical analysis indicated that COX-2 level was often high in cirrhotic livers; therefore, one may suppose that our striking data on prognosis might be because of the cirrhosis itself but not to COX-2 induction. We cannot rule out this possibility at present. However, of particular interest was that COX-2 expression was highly correlated with the presence of active inflammation ($P < 0.0001$). Furthermore, active inflammation in nontumor tissues has been shown to be associated with relapse of HCC (53–55). Although administration of NSAIDs to patients with hepatitis or cirrhosis is generally not accepted because of a possible side effect of renal failure, it was demonstrated recently that a selective COX-2 inhibitor did not impair renal function in a rat model with liver cirrhosis (56). Taken together, it would be important to examine whether selective COX-2 inhibitors may suppress inflammation and lead to better prognosis after hepatectomy for HCC.

Because there is direct evidence that dysplastic nodules (also designated as adenomatous hyperplasia) undergo neoplastic transformation (57), these lesions are considered premalignant. In this study, seven of eight hepatic dysplasias (88%) expressed the COX-2 protein (Table 1). COX-2 overexpression in precancerous liver lesions is a finding similar to that seen in other tissues. For example, COX-2 is induced in colonic polyps, atypical adenomatous hyperplasia of the lung, and Barrett’s esophagus (18, 22, 23). Furthermore, it has been demonstrated that selective COX-2 inhibitors prevent carcinogenesis in animal models (29–36). These findings strongly suggest that COX-2 may be involved in carcinogenesis of these organs. Recently, Denda et al. (58) demonstrated that administration of NSAIDs suppressed cirrhosis and subsequent formation of HCC in the choline-deficient, L-amino acid-defined rat model. To clarify whether COX-2 is a principle enzyme involved in liver carcinogenesis, in vivo animal studies are currently under way using a specific COX-2 inhibitor in our laboratory.

Our results in carcinoma tissues demonstrated a variable level of COX-2 expression among tissue samples. In 46 samples of HCCs, strong and moderate expressions of COX-2 were detected in 8 (17%) and 17 cases (37%), respectively. Among these patients, 28 (61%) presented with active inflammation, whereas in patients with low-grade inflammation, the corresponding figures were only 8 (17%) and 6 (13%), respectively. These results are consistent with the hypothesis that COX-2 expression is induced by inflammation. As shown in Table 1, all of the patients with high COX-2 expression demonstrated active inflammation in nontumor tissues. Furthermore, a higher rate of COX-2 expression was observed in nontumor tissues from patients with active inflammation (53–55). Although administration of NSAIDs to patients with hepatitis or cirrhosis is generally not accepted because of a possible side effect of renal failure, it was demonstrated recently that a selective COX-2 inhibitor did not impair renal function in a rat model with liver cirrhosis (56). Taken together, it would be important to examine whether selective COX-2 inhibitors may suppress inflammation and lead to better prognosis after hepatectomy for HCC.

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Our results in carcinoma tissues demonstrated a variable level of COX-2 expression among tissue samples. In 46 samples of HCCs, strong and moderate expressions of COX-2 were detected in 8 (17%) and 17 cases (37%), respectively, indicating that approximately one-half of HCC samples expressed the COX-2 protein (Table 1). From a therapeutic point of view, this finding is potentially important because several mechanistic...
studies indicated that carcinoma cells overexpressing COX-2, but not those cells lacking COX-2, are sensitive to COX-2 inhibitors (26, 49, 59). On the other hand, a clinicopathological survey indicated that COX-2 expression was associated with low histological grade, i.e., differentiated phenotype (P = 0.019; Table 2). This result is consistent with those of recent studies indicating that COX-2 is induced during differentiation of human endometrial stromal cells and human keratinocytes, as well as HCCs (16, 17, 60, 61). It is known that early HCC is usually a differentiated carcinoma and that it gradually changes to poorly differentiated phenotype during tumor progression (62). In this context, it is suggested that COX-2 may be associated with the early process of the progression of HCCs. This might be a reason for no correlation between COX-2 expression in HCC and patients’ prognoses.

In conclusion, we report here that COX-2 expression in nontumor was closely related to the postoperative relapse of HCC. This aspect is of clinical importance because there is a possibility that pharmaceutical inhibition of COX-2 activity might improve patients’ prognosis in the future, although further analysis of functional significance is essential.

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