Tumor cyclooxygenase-2 levels correlate with tumor invasiveness in human hepatocellular carcinoma

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INTRODUCTION
Cyclooxygenase (COX) is a membrane-bound enzyme responsible for the oxidation of arachidonic acid to prostaglandin G2 and its subsequent reduction to prostaglandin H2. COX-2 is expressed in at least two different isoforms, a constitutively expressed form, COX-1, and an inducible form, COX-2. COX-1 is expressed in many normal tissues and is involved in a number of homeostatic body functions, such as hemostasis, vasodilatation in renal vessels, and cytoprotection of the gastric mucosa. The expression of COX-2 is induced by various stimuli, such as growth factors and cytokines, and is involved in chronic inflammatory pathologies such as inflammatory bowel disease. COX-2 was recently found to have angiogenic property. This has attracted strong research interest in its significance in human cancers because of the potential use of COX-2 inhibitors in cancer therapy. Recent studies have highlighted the relevance of COX-2 in human carcinogenesis and cancer progression. There is evidence that COX-2 enhances the angiogenic and metastatic potential of tumor cells. Studies in human colorectal cancer, head and neck cancer, endometrial cancer and breast cancer suggested that COX-2 is involved in the angiogenesis and progression of these cancers. Recent data have indicated that COX-2 stimulates angiogenesis through induction of vascular endothelial growth factor (VEGF). VEGF is known to be a specific stimulator of endothelial cell proliferation in many human cancers, and it is the most potent angiogenic factor for tumor angiogenesis.

Hepatocellular carcinoma (HCC) is a highly malignant tumor characterized by active neovascularization. Angiogenesis plays a significant role in the aggressiveness of HCC. Previous studies from our group have revealed a significant association between VEGF expression and invasiveness of HCC. Recently, a few studies have demonstrated the expression of COX-2 in HCC and they found increased expression of COX-2 by immunohistochemistry in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver. The authors suggested that COX-2 might play a role in the early stages of hepatocarcinogenesis, but not...
in the advanced stages of tumor progression. Kondo et al\textsuperscript{23}, also reported a similar relationship between COX-2 expression and differentiated HCC. In addition, they found a significant correlation between COX-2 expression and active inflammation in the noncancerous liver. A similar finding in the noncancerous liver was reported by Morinaga et al\textsuperscript{24}, who otherwise found no correlation between COX-2 mRNA expression and clinicopathological features in HCC. A more recent study of COX-2 expression in HCC caused by hepatitis B virus (HBV) revealed that COX-2 expression correlated with VEGF expression and microvessel density in HCC\textsuperscript{25}. However, the role of COX-2 in tumor progression of HCC is unclear. Hence, we performed a study to evaluate the relationship between tumor COX-2 expression and clinicopathological features of HCC.

**MATERIALS AND METHODS**

**Patients and samples**

Fresh tumor tissues and adjacent noncancerous liver tissues were obtained immediately from the resected specimens of 100 HCC patients who underwent hepatectomy at the Department of Surgery of the University of Hong Kong at Queen Mary Hospital, Hong Kong between January 1, 1998 and December 31, 2001. Fresh tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C for analysis of cytosolic COX-2 and VEGF protein levels. For immunohistochemistry, tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections of normal liver from 10 living liver donors for transplantation were also collected for immunohistochemical study of COX-2 expression as controls. All clinical samples were collected with informed consent. The study was approved by the Institutional Review Board of our institution.

**Clinicopathological data**

Tissue sections (4-μm thick) were deparaffinized in xylene, rehydrated in graded alcohol solution, and stained with hematoxylin and eosin solution. Histological examination of resected specimens was performed by a pathologist who was blinded to the COX-2 and VEGF expression results. Tumor differentiation was classified into three types according to the criteria proposed by the Liver Cancer Study Group of Japan: well-, moderately-, and poorly-differentiated\textsuperscript{27}. Tumors were staged by using the International Union Against Cancer pathologic Tumor-Node-Metastasis (pTNM) classification\textsuperscript{28}. Histological evidence of cirrhosis in the adjacent noncancerous livers was examined, and the adjacent noncancerous livers were classified into those with active and those without active inflammation according to the presence of lymphocytic piecemeal necrosis with lobular confluent necrosis as previously described\textsuperscript{29}. All patients were regularly followed in a HCC clinic and all clinicopathological and follow-up data were collected prospectively in a computerized database.

**Immunohistochemical study of COX-2 expression**

Tumor and adjacent noncancerous liver sections were deparaffinized and rehydrated by standard procedures of histology. Slides were then immersed in 3% hydrogen peroxide for 15 min at 22 °C to quench endogenous peroxidase activity. The slides were then microwaved in 10 mmol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval. After washing the slides in phosphate buffer saline for 10 min at 4 °C, the specimens were preblocked for 10 min at room temperature in biotin blocking reagent (DAKO, Produktionsvej, Denmark). The biotin solution was washed off with phosphate buffer saline thrice before application of the first antibody. Then the slides were incubated overnight with mouse antihuman COX-2 monoclonal antibody (dilution, 1:100; Cayman Chemical, Ann Arbor, MI) at 4 °C. After three washes in phosphate buffer saline, the sections were incubated with rabbit biotinylated antimouse immunoglobulin G (Zymed Lab Inc., San Francisco, CA) for 60 min at 37 °C, and then incubated with streptavidin-biotin complex/horseradish peroxidase (DAKO, Produktionsvej) at room temperature for 30 min. The slides were stained with 3,3'-diaminobenzene tetrahydrochloride/hydrogen peroxide solution and counterstained with hematoxylin after washing with phosphate buffer saline. COX-2 staining was compared with the corresponding tissue slide from the same patient without primary antibody as negative control. Staining of COX-2 was defined as negative when there was undetectable or minimal (less than 10% of the tumor cells or hepatocytes) staining for COX-2; and positive when >10% of the tumor cells or hepatocytes were stained for COX-2.

**Measurement of cytosolic COX-2 and VEGF levels in tumor and adjacent noncancerous liver tissues**

Cytosol was obtained by sonication of tumor and adjacent noncancerous liver tissues. In brief, frozen tissues were sonicated in four volumes of RIPA buffer (25 mmol/L Tris, pH 7.4, 0.15 mol/L KCl, 1% NP-40, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 0.1% SDS). Sonication was performed for five cycles of 30-s bursts at 1-min intervals on ice. The supernatant was collected after 5 min of centrifugation at 15 000 g at 4 °C and assayed for the COX-2 and VEGF protein levels immediately. The total protein concentration in the supernatants was measured using Bio-Rad total protein assay (Bradford, Hercules, CA). Measurement of COX-2 and VEGF levels in the tissue cytosol was performed by two separate investigators who were not aware of the results of each other or the clinicopathological data of the patients.

The COX-2 protein level in tissue cytosol was determined by using a commercial enzyme immunometric assay (EIA) kit (Assay Designs, Inc., Ann Arbor, MI). The procedure of the EIA provided by the manual was followed. Briefly, 100 µL of recombinant human COX-2 standard and test samples were pipetted into a microtiter plate coated with monoclonal antibody specific for human COX-2, and then incubated for 1 h at 37 °C. After washing off any unbound substances, 100 µL of a rabbit polyclonal antibody to human COX-2 conjugated with the enzyme horseradish peroxidase was added to the wells to sandwich the COX-2. Finally, substrate solution was added for color development. The intensity of color developed, which was in proportion to the quantity of COX-2 bound, was measured by reading optical absorbance at 490 nm. The COX-2 level was
The VEGF content of tissue cytosolic samples was determined from a standard curve generated for each set of samples assayed. The VEGF content of tissue cytosolic samples was quantified by an enzyme-linked immunosorbent assay (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN). The assay employs the quantitative sandwich enzyme immunoassay technique using recombinant human VEGF and antibodies raised against the recombinant protein. Details of the assay have been described elsewhere.[21,30] The assay exhibits no significant cross-reactivity with other angiogenic factors. All samples were assayed in duplicate.

Statistical analysis
Continuous variables were expressed as median and range, and compared between groups using the Mann-Whitney U test. Categorical variables were compared between groups using the \( \chi^2 \) test with Yates’ correction (or the Fisher’s exact test where appropriate). Correlation of continuous variables was examined using the Spearman correlation test (\( \rho \)). Survival was computed using the Kaplan-Meier method and compared between groups using the log-rank test. The prognostic influence of tumor COX-2 levels and tumor pathological features on survival was studied by a multivariate analysis using the Cox regression model. All statistical analyses were performed using a statistical software (SPSS/PC, SPSS Inc., Chicago, IL). A \( P \) value <0.05 was considered statistically significant.

RESULTS
Among the 100 patients with HCC studied, there were 82 men and 18 women, and the median age was 55 years (range 16-79 years). Eighty-five patients (85%) tested positive for HBV surface antigen, 4 (4%) tested positive for anti-HCV antibody, and the other patients were seronegative for both HBV and HCV. Forty-seven patients (47%) had associated cirrhosis according to histological examination. The median size of the tumors was 5.7 cm (range 1.5-20.0 cm).

Immunohistochemical study of COX-2 expression in tumors and adjacent noncancerous liver tissues
Immunohistochemical staining of COX-2 in the tumors showed that COX-2 was expressed predominantly in the cytoplasm of tumor cells (Figure 1A). Of the tumor specimens, 56 cases were classified as positive and 44 cases as negative for COX-2 by immunostaining.

In the adjacent noncancerous liver tissues, COX-2 was expressed to a variable extent, predominantly in the hepatocytes (Figure 1B). Positive staining was also observed in endothelial cells and stromal cells in both tumor and adjacent noncancerous liver tissues. Of the noncancerous liver tissue specimens, 43 cases were classified as positive and 51 cases as negative for COX-2. In contrast, immunostaining for COX-2 was negative in all 10 specimens of normal liver obtained from living liver donors.

Tumor and noncancerous liver cytosolic COX-2 levels and correlation with cytosolic VEGF levels
The median cytosolic COX-2 concentration in the tumors was 0.350 ng/mg total protein (range 0.044-1.971 ng/mg total protein), which was not significantly different from that of the corresponding adjacent noncancerous livers (median 0.378 ng/mg total protein, range 0.053-1.885 ng/mg total protein; \( P = 0.216 \)). In contrast, the cytosolic VEGF concentration in the adjacent noncancerous livers (median 22.8 pg/mg total protein, 12.7-37.6 ng/mg total protein) was significantly lower than that in the tumors (median 46.3 pg/mg total protein, 23.0-143.6 ng/mg total protein; \( P <0.001 \)). Positive correlation was observed between tumor COX-2 and VEGF levels (\( r = 0.469, P<0.001 \)), and between noncancerous liver COX-2 and VEGF levels (\( r = 0.230, P = 0.021 \)). A detailed study of cytosolic VEGF concentration in tumor and noncancerous adjacent tissues and their association with clinicopathological parameters has been reported in a previous publication from our group.[30]

Relationship between tumor cytosolic COX-2 levels and clinicopathological features
Table 1 shows the relationship between tumor cytosolic COX-2 levels and tumor pathological features. There was a significant association between high tumor COX-2 levels and the presence of multiple tumors (\( P = 0.027 \)), venous invasion (\( P = 0.030 \)), microscopic satellite lesions (\( P = 0.037 \)), and advanced pTNM stage (\( P = 0.008 \)). Figure 2 shows the boxplots of tumor COX-2 levels in early stage (I/II) vs advanced stage (III/IVA) HCCs. There was no significant association between tumor COX-2 levels and tumor grade or tumor size (\( \leq 5 \) cm in diameter). When correlated as continuous variables, there was no significant relationship between tumor COX-2 levels and tumor size (\( r = 0.040, P = 0.694 \)). Tumor COX-2 levels did not have a significant correlation with serum alpha fetoprotein levels either (\( r = 0.046, P = 0.654 \)).
**Relationship between noncancerous liver cytosolic COX-2 levels and clinicopathological features**

There was no significant difference in the noncancerous liver cytosolic COX-2 levels between patients tested positive for HBV and those tested negative for HBV (0.370 [0.065-1.885] vs 0.387 [0.053-1.128] ng/mg total protein, P = 0.527), nor was there any significant difference in the noncancerous liver cytosolic COX-2 levels between patients with cirrhosis and those without cirrhosis (0.401 [0.070-1.885] vs 0.370 [0.053-1.128] ng/mg total protein, P = 0.386).

There was no significant correlation between cytosolic COX-2 levels in the noncancerous liver tissues and serum alanine aminotransferase levels (r = 0.108, P = 0.283), aspartate aminotransferase levels (r = 0.004, P = 0.972), albumin levels (r = -0.124, P = 0.218) or bilirubin levels (r = 0.030, P = 0.765). No significant difference was observed in the cytological COX-2 levels in the noncancerous livers between those patients with active inflammation (n = 48) and those without active inflammation in the livers (n = 52) (0.405 vs 0.387; P = 0.116).

**Table 1 Relationship between COX-2 levels in tumor cytosol and pathological features of HCC**

| Tumor characteristics               | Tumor COX-2 cytosolic level (ng/mg total protein) | P    |
|--------------------------------------|---------------------------------------------------|------|
| Tumor size: ≤5 cm (n = 44)          | 0.385 (0.084-1.971)                                | 0.567|
| >5 cm (n = 56)                      | 0.350 (0.044-1.690)                                |      |
| Tumor number: solitary (n = 72)     | 0.305 (0.044-1.971)                                | 0.027|
| multiple (n = 28)                   | 0.423 (0.086-1.690)                                |      |
| Tumor grade: well-differentiated (n = 36) | 0.376 (0.084-1.539)                                | 0.799|
| Moderately- or poorly-differentiated (n = 64) | 0.349 (0.044-1.971)                                |      |
| Venous invasion: absent (n = 60)    | 0.295 (0.083-1.325)                                | 0.030|
| present (n = 40)                    | 0.410 (0.044-1.971)                                |      |
| Microscopic satellite lesions: absent (n = 78) | 0.310 (0.093-1.971)                                | 0.037|
| present (n = 22)                    | 0.495 (0.044-1.690)                                |      |
| Tumor encapsulation: absent (n = 71) | 0.351 (0.044-1.971)                                | 0.587|
| present (n = 29)                    | 0.372 (0.084-1.325)                                |      |
| pTNM stage: I and II (n = 50)       | 0.286 (0.083-1.325)                                | 0.008|
| III and IV (n = 50)                 | 0.410 (0.044-1.971)                                |      |

1Includes 60 moderately-differentiated and 4 poorly-differentiated HCCs.

**DISCUSSION**

Since HCC is one of the most vascular human malignancies, angiogenesis plays an important role in this tumor. However, relatively little is known of the role of various angiogenic factors in HCC compared with other common human cancers[31]. Several recent reports on COX-2 expression in other cancers show that COX-2 can induce angiogenesis and is related to tumor growth, invasion, and metastasis[7,11-14,32,33]. Although COX-2 is also known to have an antiapoptotic effect on tumor cells[34], its angiogenic property is believed to play a major role in its relationship with cancer growth and progression[35]. A few studies have evaluated the expression of COX-2 in HCC[22,23,26,36]. However, unlike the studies in other cancers, these studies in HCC have not demonstrated a significant correlation between COX-2 expression and the pathological features of cancer invasion or progression. Instead, some of these studies suggested that COX-2 may be involved in the early stage of hepatocarcinogenesis because of the finding of higher COX-2 expression in well-differentiated HCC[22,23,34]. Other studies suggested that [0.065-1.885] vs 0.368 [0.053-1.130] ng/mg total protein, P = 0.831).
COX-2 may be involved in the inflammatory activity in the adjacent noncancerous liver of HCC patients. A correlation between COX-2 expression and tumor angiogenesis in HCC has been demonstrated in two previous studies, but these studies did not demonstrate any correlation between COX-2 expression and pathological features of tumor invasiveness.

To our knowledge, this is the first study that demonstrated a significant correlation between increased tumor COX-2 expression and tumor invasiveness in HCC. Previous studies that failed to show a significant relationship between COX-2 expression and features of HCC invasiveness used immunohistochemical study alone in the evaluation of COX-2 expression, which is a crude semiquantitative method. This study shows that high levels of tumor cytosolic COX-2 measured by an EIA were significantly associated with tumor multiplicity, presence of venous invasion, presence of microscopic satellite lesions and advanced tumor stage. These were all features indicative of tumor invasiveness and progression. The absence of correlation between tumor cytosolic COX-2 levels and tumor size suggests that COX-2 level is not simply related to tumor growth in size but is specifically related to tumor invasiveness. This is the only study thus far that has quantitatively measured the tissue cytosolic COX-2 protein levels in HCC. Previous studies that employed a similar method to quantify VEGF levels in other cancers have shown that this method provided a reliable quantitative evaluation of tumor expression of the angiogenic factor, which correlated well with tumor invasiveness and progression.

The difference in the results between this study and previous studies by Japanese groups may also be partly ascribed to different patient populations, as the current study population consists of predominantly (85%) patients with HCC related to HBV infection, whereas the majority of patients in the Japanese studies had HCV-related HCC. Differences in the clinicopathological characteristics of HCCs related to HBV infection and those related to HCV infection have been well documented. It is possible that COX-2 may have a different role in HCCs caused by different viral etiologies. Such a speculation is supported by a recent study that demonstrated induction of COX-2 expression by HBV X protein, which is often the only viral protein expressed by transformed hepatocytes in HBV caused by HBV infection. The study showed that HBV X protein could induce HCC cell invasion through the COX-2 pathway, thus contributing to the metastatic spreading and recurrence of HBV-related HCC.

The present study also demonstrated a significant correlation between tumor cytosolic COX-2 and VEGF levels in HCC. This finding is in agreement with those of studies in other human cancers. VEGF is a highly specific angiogenic factor, and high tumor or serum VEGF levels have been shown to be related to venous invasion, intrahepatic metastasis and advanced tumor stage in patients with HCC in our previous studies. Several investigators have demonstrated that COX-2-derived prostaglandins facilitate angiogenesis by upregulation of VEGF expression, and the increased levels of VEGF can be reversed by using COX-2 inhibitor such as Celecoxib, suggesting that COX-2 regulates VEGF expression and angiogenesis in cancer. The current study suggests that COX-2 enhances the invasiveness of HCC probably through its angiogenic effect via the VEGF pathway. Venous invasion and advanced tumor stage are important risk factors of tumor recurrence after resection of HCC. The association between high COX-2 levels and venous invasion as well as advanced tumor stage explains its negative impact on long-term patient survival.

In this study, COX-2 expression was also detected to a variable degree in the noncancerous liver tissues, which were affected by cirrhosis or chronic hepatitis in most cases. In contrast, COX-2 was not expressed in normal liver tissue taken from living liver donors. In addition to its effect on angiogenesis, COX-2 is a mediator of chronic inflammation. Uregulated expression of COX-2 in the adjacent noncancerous liver have been demonstrated in previous studies of HCV-related HCC. Other studies have also shown that there is little or no expression of COX-2 in normal liver without HBV or HCV infection. However, while previous studies in patients with HCV infection showed a significant relationship between COX-2 expression and inflammatory activity in the noncancerous liver, our data did not reveal a significant association between cytosolic COX-2 levels and the inflammatory activity in the adjacent noncancerous liver in HBV-related HCCs. There was no significant correlation between COX-2 expression in the noncancerous liver tissues and serum transaminase levels, which indirectly reflect hepatitis activity in the liver. Our results are in agreement with a recent study that reported elevated COX-2 expression in chronic hepatitis in patients with HBV infection, but the COX-2 expression level did not correlate with inflammatory activity in that study either.

The role of COX-2 in the noncancerous liver of HCC patients remains to be clarified. A recent study suggested that COX-2 may be involved in liver fibrosis. Another study showed that VEGF expression is increased in cirrhosis and may mediate angiogenesis in cirrhotic liver in HCC patients. The present study demonstrated for the first time a significant correlation between COX-2 and VEGF in the noncancerous liver tissues, suggesting that COX-2 may also be involved in the angiogenesis in chronic liver disease through the VEGF pathway. COX-2 may play a specific role in the chronic liver disease induced by HBV. HBV X protein has recently been found to induce VEGF expression and angiogenesis in HBV-infected noncancerous liver, which may contribute to hepatocarcinogenesis. HBV X protein has also been shown to induce COX-2 expression. Together with our finding of positive correlation of COX-2 and VEGF levels in the noncancerous liver, it is reasonable to speculate a role of COX-2 in the angiogenesis and hepatocarcinogenesis induced by HBV X protein. However, the relationship between COX-2 and HBV X protein in HBV-infected liver needs clarification with further studies.

The elucidation of the significance of COX-2 in the invasiveness and progression of HCC is of potential clinical importance as it provides insight into the feasibility of using COX-2 inhibitors as an antineoplastic therapy. Nonsteroidal anti-inflammatory drugs have been shown to exhibit antineoplastic activity in a number of malignancies because of their ability to inhibit COX-2. Recently, specific COX-2 inhibitors have been developed and shown to possess...
antitumor activity through inhibition of angiogenesis[48]. COX-2 is an important therapeutic target in tumor angiogenesis, and COX-2 inhibitors are being intensely investigated as a novel anticancer therapy[48]. A recent experimental study has demonstrated that COX-2 inhibitors could inhibit angiogenesis and growth of prostate cancer[49]. It has been suggested that COX-2 inhibitors may be an effective strategy of chemoprevention of HCC[49]. The data from this study provide the rationale for future studies to evaluate the use of COX-2 inhibitors not only for chemoprevention but also as a therapy for established invasive HCC. Currently, we are conducting a study on the effect of a COX-2 inhibitor, Celecoxib, on human HCC xenotransplant in a nude mice model. Our preliminary unpublished data show that Celecoxib could inhibit the growth of HCC xenotransplant. COX-2 inhibition may be useful in the treatment of inoperable HCC, either as a monotherapy or in combination with other therapies, and it may also be useful as an adjuvant therapy to prevent recurrence after resection of HCC. This is an attractive approach worthy of further investigation in view of the current lack of effective chemotherapy for HCC[49].

In conclusion, this study shows that tumor cytosolic COX-2 levels correlate with VEGF levels and pathological features of tumor invasiveness in HCC. The possible involvement of COX-2 in the angiogenesis and invasiveness of HCC represents a novel finding that not only provides clues for a better understanding of the tumor biology of HCC, but also unveils a potential novel target for therapy against this aggressive malignancy.

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