Overexpression of cyclooxygenase-2 in noncancerous liver tissue increases the postoperative recurrence of hepatocellular carcinoma in patients with hepatitis B virus-related cirrhosis

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BACKGROUND: Many previous studies have evaluated the histopathological features of tumours as risk factors for postoperative recurrence in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC). However, there have been few large studies investigating the relationship between cyclooxygenase-2 (COX-2) expression in noncancerous regions of the liver and postoperative recurrence in the remnant liver, especially in HBV-related HCC.

OBJECTIVE: To evaluate the significance of COX-2 expression levels in noncancerous liver regions as a prognostic indicator of HCC in patients with HBV-related cirrhosis.

METHODS: A total of 124 patients who underwent curative resection for HCC were reviewed retrospectively. Immunohistochemistry was used to evaluate the expression of COX-2 in noncancerous liver tissue. Clinicopathological variables were compared between patients with high COX-2 expression (n=58 [COX-2-positive group]) and patients with low COX-2 expression (n=66; [COX-2-negative group]). Univariate and multivariate analyses were performed to identify factors that affected disease recurrence.

RESULTS: There was a significant correlation between COX-2 expression and alanine aminotransferase levels and vascular invasion. The recurrence-free survival rates in the COX-2-positive group were significantly lower than the rates in the COX-2-negative group. On multivariate analysis, the overexpression of COX-2 in noncancerous liver regions was found to be an unfavourable prognostic indicator for the recurrence of HCC.

CONCLUSIONS: The results of the current study suggest that overexpression of COX-2 in noncancerous liver regions is an independent and significant indicator predictive of early recurrence of HCC in patients with HBV-related cirrhosis.

Key Words: *Cyclooxygenase-2; Hepatic resection; Hepatocellular carcinoma; Prognosis; Recurrence*

La surexpression de la COX-2 dans les tissus hépatiques non cancéreux accroît la récurrence postopératoire du cancer hépatocellulaire chez les patients atteints de cirrhose liée au VHB

CONTEXTE : De nombreuses études ont évalué les caractéristiques cytopathologiques des tumeurs comme facteurs de risque de récurrence postopératoire du cancer hépatocellulaire (CHC) associé au virus de l'hépatite B (VHB). Toutefois, peu de grandes études ont porté sur le lien entre l'expression de la cyclooxygénase 2 (COX-2) dans les zones non cancéreuses du foie et la récurrence postopératoire dans les tissus hépatiques résiduels, surtout dans le CHC lié au VHB.

OBJECTIF : Évaluer le rôle des taux d'expression de la COX-2 dans les zones hépatiques non cancéreuses comme indicateurs pronostiques du CHC chez des patients souffrant d'une cirrhose liée au VHB.

MÉTHODES : En tout, 124 patients ayant subi une résection curative pour CHC ont été examinés de manière rétrospective. Les auteurs ont utilisé les tests immunohistochimiques pour évaluer l'expression de la COX-2 dans les tissus hépatiques non cancéreux. Ils ont comparé les variables clinicopathologiques des patients présentant une forte expression de la COX-2 (n = 58 [groupe COX-2-positif]) à celles des patients présentant une faible expression de la COX-2 (n = 66 [groupe COX-2-négatif]). Des analyses uni- et multivariées ont permis d'identifier les facteurs ayant influé sur la récurrence de la maladie.

RÉSULTATS : On a noté une corrélation négative entre l'expression de la COX-2 et les taux d'alanine aminotransférase et l'envahissement vasculaire. Les taux de survie sans récurrence dans le groupe COX-2-positif ont été significativement plus bas que dans le groupe COX-2-négatif. À l'analyse multivariée, la surexpression de la COX-2 dans les zones hépatiques non cancéreuses s'est révélée être un indicateur pronostique défavorable à l'égard de la récurrence du CHC.

CONCLUSIONS : Les résultats de la présente étude donnent à penser que la surexpression de la COX-2 dans les zones hépatiques non cancéreuses est un indicateur indépendant et significatif qui permet de prédire la récurrence précoce du CHC chez des patients souffrant d'une cirrhose liée au VHB.

The occurence of hepatocellular carcinoma (HCC) after infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is particularly common in Asia and Africa, and its incidence is also increasing in western countries (1). HCC has become the second most common cause of cancer-related death in China, accounting for approximately 137,500 deaths each year (2). Many investigators have reported a putative link between infection with HBV or HCV, liver cirrhosis and the development of HCC (3).

Cyclooxygenase (COX) is a key enzyme involved in the production of a variety of eicosanoid products. Two COX isoforms have been characterized: COX-1 and COX-2. COX-1 is the

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housekeeping isoform of COX, while the inducible form, COX-2, responds to many intracellular and extracellular stimuli, and acts in either a proinflammatory or tumorigenic fashion (4).

Recently, a probable association between increased COX-2 expression and the pathophysiology of chronic viral hepatitis and cirrhosis has been documented. The *COX-2* gene and protein have been shown to be overexpressed in chronically hepatitic or cirrhotic livers of patients with either HBV or HCV infection (5-9). This overexpression correlated with a greater degree of active inflammation, regenerative activities and progression of fibrosis in the livers of these patients (5,7,10).

Cirrhosis represents the most important predisposing factor because 80% of HCC cases develop in a cirrhotic background (11). In patients with HBV-related cirrhosis, the five-year cumulative risk of HCC in high endemic areas is 15%, and 10% in the western hemisphere (12-14). Therefore, effective chemopreventive strategies are needed to benefit individuals at high risk for HCC. HCC in HBV-related cirrhosis could be the result of long-term hepatocellular proliferation associated with active inflammation in the infected livers (15-17). To improve the long-term prognosis after resection of HCC in patients with HBV-related cirrhosis, it is important to prevent postoperative recurrence. To date, many previous studies have evaluated the histopathological features of tumours as risk factors for postoperative recurrence of HCC. However, there have been few large studies investigating the relationship between COX-2 expression in noncancerous regions of the liver and the postoperative recurrence of HCC in the remnant livers of patients, especially with regard to HBV-related HCC.

The present study evaluated the relationship between the postoperative recurrence of HCC and COX-2 expression in noncancerous regions of remnant livers after resection of HCC in patients with HBV-related cirrhosis.

METHODS

Patients

From January 1998 to March 2005, 199 consecutive patients with HBV-related cirrhosis underwent curative hepatic resection for HCC at the Anhui Provincial Hospital (affiliated with the Anhui Medical University), Hefei, People's Republic of China. In the present study, curative hepatic resection was defined as the macroscopic and microscopic removal of all tumours from the liver. Thirty patients were lost to follow-up. Laboratory and clinical data, and noncancerous liver tissue samples were not available for 20 patients; therefore, these patients were excluded from further analysis. Also excluded were three patients who had malignancy other than HCC, 12 patients who died within 30 days after surgery (operative mortality) and 10 patients who underwent transarterial chemoembolization before surgery, which may have affected COX-2 expression levels in noncancerous liver tissues. Therefore, 124 patients with HCC were included in the present retrospective analysis. Follow-up ranged from 41 to 3286 days (median 1432 days). The study was approved by the Ethics Committee of the Anhui Medical University. Informed consent was obtained from each patient. The end of follow-up was defined as either the time of final follow-up or death.

COX-2 immunohistochemistry of noncancerous liver regions

Archival formalin-fixed, paraffin-embedded specimens of HCC and surrounding noncancerous liver tissue were obtained. For immunohistochemical COX-2 staining of noncancerous tissue, formalin-fixed, paraffin-embedded 5 µm tissue sections were deparaffinized by rinsing with xylene and rehydrated in distilled water through graded alcohol, followed by microwave retrieval of antigen according to standard procedures. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min. Nonspecific binding was blocked with 5% rabbit serum (Dako, Denmark), then incubated with antibody to COX-2 (1:100; Santa Cruz Biotechnology, USA) in tris-buffered saline containing 2% rabbit serum and 1% bovine serum albumin for 2 h. Tissues were incubated in the same buffer without the antibody as negative control. This was followed by incubation with biotinylated rabbit antigoat immunoglobulin for 45 min (1:400; Dako, Denmark) and, subsequently, with streptavidin/horseradish peroxidase complex (1:400; Dako, Denmark) for 45 min. The colour reaction was developed in 3,3'-diaminobenzidine substrate solution (Sigma-Aldrich, USA). The sections were then counterstained with Mayer's hematoxylin.

Immunohistochemical evaluation

The immunohistochemical evaluation was independently performed by two authors (X-QW and KC) who were blinded to the clinical data. The COX-2 immunoreactivity levels of each patient were assessed semiquantitatively under light microscope by calculating the average signal intensity (based on a scale from 0 to 3) and the proportion of cells showing a positive cytoplasmic stain (0=none; 0.1=less than one-tenth; 0.5=less than one-half; and 1=greater than one-half). The intensity and proportion scores were then multiplied to give an H-score according to the published literature (18-20). The rare cases with discordant scores were re-evaluated and scored on the basis of consensus opinion. A priori, the median value of all cases' H-scores was chosen to be the cut-off for distinguishing COX-2 positive from COX-2 negative in noncancerous tissue samples according to the published literature (20).

Determination of inflammatory activities in noncancerous liver regions

Modified gradings of inflammatory activity in adjacent liver tissue were measured according to the histological activity index (21-23). The histological activity index consists of four separate scores for different lesion components: periportal necrosis with or without bridging necrosis; intralobular degeneration and focal necrosis; portal inflammation; and fibrosis. The first three categories were measured with a total score range of 0 to 18; the first three categories represent grading, whereas the fourth is the method of staging. From this total score, values of 1 to 8 were defined as inactive inflammation, with values of 9 to 18 defining active inflammation.

Parameters

Recurrence-free survival rates were compared between the two groups using the following clinicopathological variables: host factors were age, sex and liver function tests (such as alanine aminotransferase [ALT, 10 U/L to 40 U/L], albumin [35 g/L to 55 g/L] and total bilirubin [0 µmol/L to 17 µmol/L]); tumour factors were alpha-fetoprotein (AFP) level, maximal tumour dimension, number of tumours and histological findings such as capsular formation, vascular invasion (including vascular invasion or tumour thrombi in the portal or hepatic vein) and

TABLE 1
Relationship between cyclooxygenase-2 (COX-2) expression in noncancerous liver tissue and clinicopathological parameters in patients with hepatocellular carcinoma

Parameter	Patients	COX-2 expression		P
		Positive	Negative	
Age, years				0.081
≥65	18	5	13	
<65	106	53	53	
Sex				0.182
Male	108	53	55	
Female	16	5	11	
Alanine aminotransferase, U/L				<0.05
≥80	36	30	6	
<80	88	28	60	
Albumin, g/L				0.421
≥35	102	46	56	
<35	22	12	10	
Total bilirubin, μmol/L				0.296
≥34	21	46	57	
<34	103	12	9	
Alpha-fetoprotein, μg/L				0.025
≥400	51	30	21	
<400	73	28	45	
Tumour size, cm				0.016
≥5	85	46	39	
<5	39	12	27	
Tumours				0.065
Solitary	81	33	50	
Multiple	43	25	16	
Capsular formation				0.995
Present	77	36	41	
Absent	47	22	25	
Vascular invasion				<0.05
Present	57	40	17	
Absent	67	18	49	
Intrahepatic metastases				0.026
Present	41	25	16	
Absent	83	33	50	
Perioperative blood infusion				0.136
Yes	66	35	31	
No	58	23	35	
Type of resection				<0.05
Minor	78	29	49	
Major	46	29	17	

Data presented as n unless indicated otherwise

intrahepatic metastases; multiple tumours were classified as intrahepatic metastases if they were multiple satellite nodules surrounding a main tumour, or a single nodule with a similar or poorer degree of cell differentiation than the main tumour; and operative factors were perioperative blood infusion and type of resection. Transfusions were performed with packed red blood cells or allogeneic blood. Perioperative blood infusion was defined as intraoperative blood infusion and/or postoperative blood infusion within one week after surgery. A resection was considered to be 'major' if three or more liver segments were removed, the remainder were considered to be 'minor' according to the classification proposed by Couinaud (24).

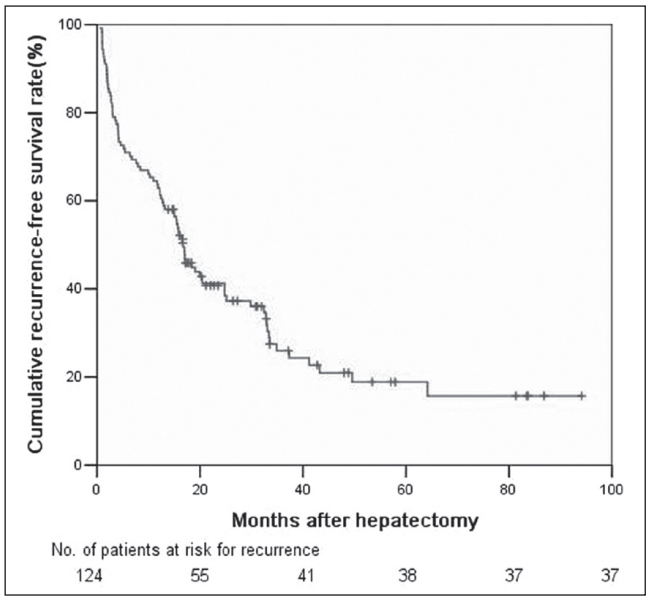

Figure 1) *Recurrence-free survival rates of 124 hepatocellular carcinoma patients with hepatitis B virus-related cirrhosis after hepatectomy*

Follow-up study

After curative surgery, patients were assessed monthly by physical examination, liver function tests, complete blood cell count and AFP levels. Ultrasonography was performed every three months, and computed tomography (CT) or magnetic resonance imaging was used every six months to detect recurrence. The recurrence of HCC was further diagnosed by additional imaging examinations including helical dynamic CT, lipiodol CT and angiography, together with evaluation of serum AFP levels.

Statistical analysis

Clinicopathological data according to COX-2 status were compared in univariate analyses with the use of χ^2 tests, and in a multivariate logistic model including all variables with $P<0.05$. The recurrence-free survival rates were estimated using the product-limit method of Kaplan-Meier. Any significant differences in the recurrence-free survival rates were determined using the log-rank test. All variables were entered into a Cox proportional hazard model to identify independent variables that were closely related to the recurrence-free survival rates. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc, USA). A two-sided $P<0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics

Clinicopathological characteristics of the study cohort are summarized in Table 1. The patients ranged in age from 19 to 78 years (median age 50 years). Of the 124 patients, 108 were men (87.1%) and 16 (12.9%) were women. The one-, three- and five-year recurrence-free survival rates were 62.9%, 25.95% and 18.87%, respectively (Figure 1).

Immunohistochemical assessment of COX-2 expression

Figure 2 shows COX-2 expression localized mainly to the cytoplasm in noncancerous hepatocytes. Bile duct epithelium,

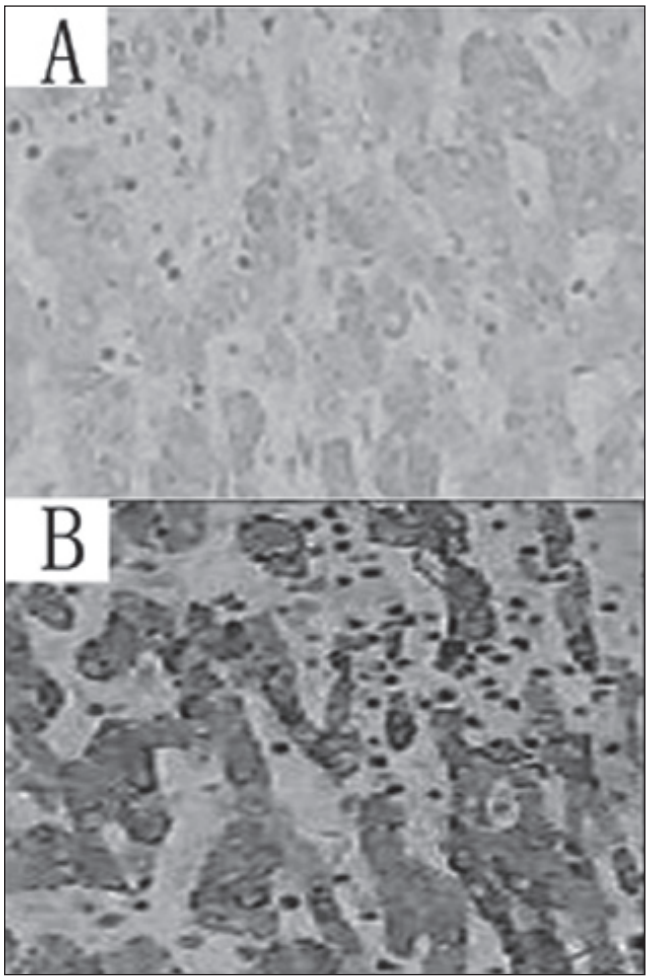

Figure 2) A *Cyclooxygenase-2-negative noncancerous tissue (original magnification ×200).* **B** *Cyclooxygenase-2-positive noncancerous tissue (original magnification ×200)*

hepatic artery vascular endothelium, portal vein and sinusoids, Kupffer cells and inflammatory cells did not show significant COX-2 expression. The median percentage of hepatocytes with cytoplasm that stained positive for the monoclonal antibody was 55% (range 0% to 100%), whereas the median value of the H-scores in the cohort was 1.5. Noncancerous hepatocytes with an H-score exceeding 1.5 (ie, noncancerous tissues with a staining intensity score of 2 and with 50% or more positive cytoplasm, or with a staining intensity score of 3 and 50% or more positive cytoplasm) were considered to be COX-2 positive. Of the 124 noncancerous tissue samples, 58 (46.8%) were COX-2 positive. Table 1 compares the host, tumour and operative factors according to COX-2 expression in a univariate analysis. A multivariate stepwise logistic model based on a backward elimination procedure for model selection showed that the expression of COX-2 was significantly associated with ALT level (P<0.05) and vascular invasion (P<0.05).

To examine the possible involvement of inflammation in COX-2 induction, the 124 noncancerous tissue samples were divided into two groups (by X-QW and KC) by pathological survey according to their extent of inflammation. Active inflammation was noted in 48 of 58 COX-2-positive livers and in eight of 66 COX-2-negative livers. A significant correlation was found between high COX-2 expression in noncancerous tissue and inflammation (r=0.708; P<0.05) (Table 2).

TABLE 2
Relationship between cyclooxygenase-2 expression and inflammation status in noncancerous liver tissue

	Cyclooxygenase-2 expression		
Inflammation status	Positive	Negative	P
Active	48	8	<0.05
Inactive	10	58	

Data presented as n unless indicated otherwise

Significant factors regarding recurrence-free survival

The recurrence-free survival rate in the COX-2-positive group was significantly lower compared with the rate in the COX-2-negative group (Figure 3) (P<0.05). The one-, three- and five-year recurrence-free survival rates were 46.6%, 13.4% and 4.5%, respectively, in the COX-2-positive group and 77.3%, 35.5% and 29.8%, respectively, in the COX-2 negative group.

In the univariate analysis of recurrence-free survival, significant differences were observed in the following 11 variables: COX-2 expression level (P<0.05), intrahepatic metastasis (P<0.05), ALT level (P<0.05), total bilirubin level (P=0.037), albumin level (P=0.016), capsular formation (P=0.032), vascular invasion (P<0.05), AFP level (P<0.05), number of nodules (P<0.05), maximal tumour dimension (P=0.020) and blood infusion (P=0.012). The results of the multivariate analysis are shown in Table 3. COX-2 expression level (P<0.05), vascular invasion (P<0.05) and intrahepatic metastasis (P<0.05) were shown to be independent and significant predictors of recurrence-free survival.

DISCUSSION

The prognosis of HCC is generally unfavourable. Although primary tumours are curatively resected, 50% to 60% of patients experience recurrence within five years (25,26). All patients in the present study had HBV-induced chronic liver cirrhosis, and it is important to note this background prevalence when considering the 81% recurrence rate of HCC observed. This caused loss of hepatic reserve and subsequently limited the extent of resection for HCC patients in whom there was frequent microscopic intrahepatic metastasis, even at an early tumour stage. This also increased the risk of missing undetected minute lesions – even after an apparently complete resection. Cirrhosis may represent a field of cancerization associated with the development of a new metachronous HCC after resection (27).

It is believed that the main cause of disease recurrence is intrahepatic metastasis (28) or metachronous, multicentric carcinogenesis (29,30). However, adequate techniques to clarify the clonality between multicentric occurrence and intrahepatic metastasis have yet to be established. Therefore, an investigation of both noncancerous and cancerous regions is required to evaluate postoperative recurrence including multicentric occurrence.

In the present study, we demonstrated that COX-2 overexpression in the presence of background liver cirrhosis was significantly associated with the recurrence or new development of HCC, which may be an independent and significant prognostic indicator in patients with HBV-related HCC after curative hepatic resection. The recurrence-free survival rate

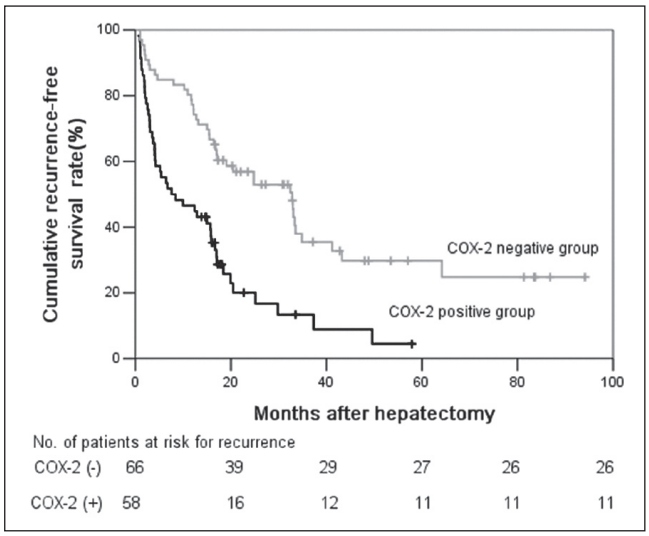

Figure 3) *Recurrence-free survival rates after curative hepatic resection. The recurrence-free survival rate in the cyclooxygenase-2-positive group (COX-2) (n=58) was significantly lower compared with the rate in the COX-2-negative group (n=66; P<0.05). No Number*

in the COX-2-positive group was significantly lower than the rates in the COX-2-negative group. Among the factors investigated in the current study, COX-2 expression level proved to be one of the most powerful indicators. To the best of our knowledge, the present study is the first large report from the Chinese mainland to reveal the prognostic significance of COX-2 overexpression in the livers of HCC patients with HBV-related cirrhosis.

Data from the current study partly confirm the observation by Kondo et al (5), who reported a significant association between COX-2 expression and remnant liver recurrence in patients with HCV infection. However, their study was performed on patients with either chronic hepatitis or cirrhosis, and the cirrhotic patients had a higher expression of COX-2 in the liver. Therefore, Kondo et al could not rule out the possibility that the difference found in the disease-free survival rate might be due to the development of cirrhosis. In contrast, all of the patients in the present study were diagnosed with cirrhosis by pathological examination. Thus, it could be said that the overexpression of COX-2 in diseased liver is associated with recurrence of HCC in patients with HBV-related cirrhosis.

Previous studies of HBV-infected patients did not reveal a significant relationship between COX-2 expression level and inflammatory activity in noncancerous liver regions (31,32). However, two studies (5,7) in patients with HCV infection showed a significant relationship between COX-2 expression and inflammatory activity in noncancerous liver regions. Our data also revealed a significant association between COX-2 expression and inflammatory activity in the noncancerous regions of cirrhotic livers of patients with HBV-related HCC. The results of the present analysis suggest that induction of COX-2 may be attributable to active inflammation in cirrhotic livers (Table 3). This is reasonable, given the fundamental action of COX-2 as a mediator of inflammation. COX-2 is a rate-limiting enzyme in the conversion of arachidonic acid to bioactive prostaglandins, such as prostaglandin E2, which

TABLE 3
Multivariate analyses of recurrence-free survival in patients with hepatocellular carcinoma who underwent hepatic resection

Variable	Coefficient	HR	95% CI	P
COX-2 (positive versus negative)	0.845	2.327	1.465–3.697	<0.05
Vascular invasion (present versus absence)	0.657	1.931	1.224–3.040	<0.05
Intrahepatic metastases (present versus absent)	0.636	1.890	1.195–2.985	<0.05

COX-2 Cyclooxygenase-2

are able to efficiently suppress T cell immune responses in a regulatory manner and elicit an inhibitory immune profile (33). However, T cell failure was significantly associated with HBV viral replication level (34). Persistent HBV replication may increase the incidence of recurrence after surgery. There was also significant correlation between COX-2 expression in noncancerous liver tissues and serum ALT levels, which has been recognized as a serological marker reflecting necroinflammatory processes in chronic liver diseases (21). These results suggest that COX-2 is related to background necroinflammatory and regenerative activity, and these pathological changes may result in the recurrence of HCC.

The other explanation for COX-2 contribution to recurrence or new development of HCC is that COX-2 in cirrhotic livers acts as a 'landscaping' tumour promoter (4,35). Recent experimental data (4,36) have shown that COX-2 derived from the stromal component may promote tumour growth by producing bioactive prostaglandins, which act angiogenetically or immunosuppressively, and affect carcinoma cells in a paracrine fashion. Therefore, according to the landscaper hypothesis (35), COX-2 derived from cirrhotic liver may promote the growth of newly developed or metastatic malignant cells. The present study demonstrated a significant correlation between tumour vascular invasion and COX-2 in noncancerous liver tissues, suggesting that COX-2 may also be involved in angiogenesis, and portal and hepatic vein invasion in HCC in a paracrine pattern. COX-2 may play a specific role in HBV-induced chronic liver disease. HBV X protein has recently been found to induce vascular endothelial growth factor expression and angiogenesis in HBV-infected noncancerous liver tissue, which may contribute to hepatocarcinogenesis (37). HBV X protein has also been shown to induce COX-2 expression (38,39). Together with our finding of a positive correlation between vascular invasion and COX-2 levels in noncancerous liver tissue, it is reasonable to suggest a role for COX-2 in angiogenesis, portal and hepatic vein invasion and hepatocarcinogenesis. However, the exact role of COX-2 in HCC is less clear. Further investigation to more clearly delineate the role of COX-2 in chronic liver disease and HCC recurrence and/or development is needed.

A noteworthy limitation of the present study was that we could not evaluate the significance of baseline HBV DNA levels as a prognostic indicator in HCC patients with HBV-related cirrhosis because HBV DNA was not detected in some of the patients. It is now recognized that the baseline HBV DNA level before hepatic resection is a predictive factor for HCC recurrence and/or development (40-43).

SUMMARY

The results of the present study showed that the overexpression of COX-2 in the presence of background liver cirrhosis is associated with early recurrence and/or new development of HCC after curative surgery in HBV-related cirrhotic patients. This is of clinical importance because pharmacological inhibition of COX-2 activity may improve patient prognosis, although further analysis of the functional significance of COX-2 is required.

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