ORIGINAL ARTICLE

Retrospective Study

Cyclooxygenase-2 expression is associated with initiation of hepatocellular carcinoma, while prostaglandin receptor-1 expression predicts survival

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Abstract

AIM

To determine whether cyclooxygenase-2 (COX-2) and prostaglandin E1 receptor (EP$_1$) contribute to disease and whether they help predict prognosis.

METHODS

We retrospectively reviewed the records of 116 patients with hepatocellular carcinoma (HCC) who underwent surgery between 2008 and 2011 at our hospital. Expression of COX-2 and EP$_1$ receptor was examined by immunohistochemistry of formalin-fixed, paraffin-embedded tissues using polyclonal antibodies. Possible associations between immunohistochemical scores and survival were determined.

RESULTS

Factors associated with poor overall survival (OS) were alpha-fetoprotein > 400 ng/mL, tumor size $\geq$ 5 cm, and high EP$_1$ receptor expression, but not high COX-2 expression. Disease-free survival was not significantly different between patients with low or high levels of COX-2 or EP$_1$. COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade Ⅰ-Ⅱ) than in poorly differentiated tissues (Edmondson grade Ⅲ-Ⅳ) (P = 0.003). EP$_1$ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue (P = 0.001).

CONCLUSION

COX-2 expression appears to be linked to early HCC events (initiation), while EP$_1$ receptor expression may participate in tumor progression and predict survival.

Key words: Cyclooxygenase-2; Hepatocellular carcinoma; Liver resection; Prognosis; Prostaglandin E1 receptor

Core tip: We retrospectively reviewed the records of 116 patients with hepatocellular carcinoma (HCC) who underwent surgery between 2008 and 2011 at our hospital. Our results suggest that the factors associated with poor overall survival were alpha-fetoprotein > 400 ng/mL, tumor size $\geq$ 5 cm, and high prostaglandin E1 (EP1) receptor expression, but not high cyclooxygenase-2 (COX-2) expression. Disease-free survival did not differ significantly between patients with low or high levels of COX-2 or EP$_1$. COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade Ⅰ-Ⅱ) than in poorly differentiated tissues (Edmondson grade Ⅲ-Ⅳ) (P = 0.003). EP$_1$ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue (P = 0.001).

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most aggressive tumors and the third most frequent cause of cancer-related death in the world[1,2]. Although early diagnosis and treatment of HCC have improved substantially, prognosis remains unsatisfactory. HCC often involves highly malignant tumors that respond poorly or not at all to adjuvant systemic and local therapies. This highlights the need for new approaches to prevent and treat the disease.

Two molecules that may be involved in HCC at different stages, and that therefore may be useful for understanding the pathogenesis and progression of the disease, are cyclooxygenase-2 (COX-2) and prostaglandin E1 receptor (EP$_1$ receptor). These molecules have already been shown to play important roles in the onset of various cancers, including HCC[3-9].

COX-2 inhibits apoptosis and increases proliferation in various type of tumors[10-12]. It also triggers the production of vascular endothelial growth factor and activates metalloproteinases, substantially altering the tumor microenvironment of various cancers[13-15]. The precise role of COX-2 in HCC remains unclear. Its expression decreases with extent of de-differentiation, and it does not appear to be associated with prognosis[16-19]. It may be involved in HCC initiation, although direct evidence of this is lacking.

COX-2 catalyzes the conversion of arachidonic acid to prostaglandin E2, which promotes the progression of various types of tumors by binding to the G-protein-coupled EP1 receptor. This led us to wonder whether EP$_1$ receptor expression might correlate with HCC progression and might even serve as a prognostic indicator of survival. In fact, in a mouse model of chemically induced colon cancer, administration of selective EP$_1$ receptor antagonists or knockout of the EP$_1$ receptor gene led to nearly 60% fewer precancerous lesions and a lower overall colon cancer incidence[20,21]. EP$_1$ receptor antagonists have also been reported to block the progression of other types of tumor[22,23], including HCC[18,24].

To begin to clarify the potential roles of COX-2 and EP$_1$ receptor in HCC, and to determine the potential prognostic value of EP$_1$ receptor expression, we examined relative expression levels in tissues taken from HCC patients treated at our hospital, and correlated these levels with survival.

MATERIALS AND METHODS

This research was approved by the Ethics Committee of the Tumor Hospital of Guangxi Medical University,

and patients provided informed consent for their data and tissue to be used for research purposes when they were admitted for treatment at our hospital.

Patients

This study was a retrospective analysis of HCC patients treated by curative hepatectomy at the Tumor Hospital of Guangxi Medical University between May 2008 and May 2011. To be included in our study, patients needed to have pathology-confirmed HCC and no history of antitumor therapies before hepatic resection. They also needed to satisfy the following curative hepatectomy criteria: (1) the tumor removed by hepatectomy was solitary; (2) the surgery margin was greater than 1 cm; (3) there was no residual tumor[25], portal tumor thromboses[26] or extrahepatic metastases based on post-surgical imaging; and (4) patients with high levels of alpha-fetoprotein (AFP) before surgery had normal levels within two months after surgery. Patients were excluded from the study if they had multiple tumors, extrahepatic metastases or macroscopic intrahepatic metastases adjacent to the primary tumor.

Follow-up

All HCC patients were followed up 1 mo after re-section, then at 3-mo intervals in the first year, and then at 3-6 mo intervals thereafter until 60 mo after resection or death. During each follow-up visit, routine investigations including AFP level, liver function, chest X-ray, ultrasound, CT or MRI were conducted.

Immunohistochemistry of COX-2 and EP₁ receptor

Tumor specimens were fixed in 10% formalin, embedded in paraffin, cut into 3-μm sections, deparaffinized with xylene and rehydrated by decreasing concentrations of ethanol. Antigen retrieval was performed for 10 min at 95 ℃ in citrate buffer (pH 6.0) in a microwave oven. Sections were immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidases, then incubated at 37 ℃ for 1 h with rabbit anti-human COX-2 polyclonal antibody (1:400; Abcam, United Kingdom) or rabbit anti-human EP₁ receptor polyclonal antibody (1:200; Abcam). The sections were rinsed in phosphate-buffered saline (PBS), incubated with biotinylated anti-rabbit immunoglobulin for 20 min at room temperature, and then rinsed again with PBS. The sections were incubated with anti-horseradish peroxidase conjugate for 10 min, rinsed with PBS, and incubated with diaminobenzidine for 10 min. Finally, the sections were counterstained with hematoxylin. As a negative control, tissues were treated as described above, except they were incubated with PBS instead of primary antibodies.

Immunohistochemical staining results were independently evaluated by three authors (Hao-Jie Yang, Zhe Guo and Yu-Ting Yang) and an experienced hepatopathologist (Chun-Jun Li) from the Department of Pathology of Guangxi Tumor Hospital. The per-centages of cells positive for COX-2 or EP₁ receptor as well as relative staining intensity were determined. Percentages of positive cells were categorized as follows: 0 (no positive tumor cells), 1 (1%-25% positive), 2 (26%-50% positive), 3 (51%-75% positive), and 4 (76%-100% positive). Staining intensity was categorized as follows: 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellow-brown), and 3 (strong, brown)[27]. The scores for positive cell percentages and for staining intensity were multiplied together to yield a single immunohistochemical staining index from 0 to 12. Sections with an index of 0-5 were defined as showing low expression, while those with an index of 6-12 were defined as showing high expression.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM, United States). Inter-group differences in categorical variables were assessed for significance using the χ^2 test; differences in continuous variables were assessed using the Mann-Whitney *U* test or *t*-test. OS and DFS were analyzed using the Kaplan-Meier method, and differences between curves were assessed for significance using the log-rank test. Multivariate Cox proportional hazards modeling was used to identify independent prognostic factors. The threshold for significance was defined as $P < 0.05$.

RESULTS

Patient characteristics

During the study, 748 patients with HCC were scheduled for hepatectomy in my center. Of these, 221 (29.5%) were excluded as they had received initial HCC treatment in other hospitals. Of the remaining 527 patients, 161 (30.5%) had solitary nodular tumors without portal tumor thromboses or extrahepatic metastases. We excluded 33 (20.4%) because they underwent only transarterial chemoembolization, local ablation therapy, or ethanol injection, and we excluded 12 (7.4%) as they lacked complete follow-up data. In total, 116 (72%) patients were included in the final analysis (93 men, 23 women) with a median age of 67 (range, 39-83) (Table 1). Immunohistochemistry showed low COX-2 expression in 62 patients (53.4%) and low EP₁ receptor expression in 73 (62.9%).

EP₁ receptor expression is a prognostic predictor of OS

OS was significantly lower among patients with high expression of EP₁ receptor than among those with low expression (Figure 1). OS did not differ significantly between patients with low or high COX-2 expression. DFS did not differ significantly between patients with low or high expression of COX-2 or EP₁ receptor.

The Cox hazards model showed 3 independent predictors of poor OS: tumor size ≥ 5 cm, high expression of EP₁ receptor and AFP ≥ 400 ng/mL (Table 2).

Table 1 Clinicopathological characteristics of 116 hepatocellular carcinoma patients treated by curative resection, stratified by COX-2 and EP₁ receptor expression

Variable	COX-2		P value	EP₁ receptor		P value
	Low expression (*n* = 62)	High expression (*n* = 54)		Low expression (*n* = 73)	High expression (*n* = 43)	
Gender, M/F	48/14	45/9	0.426	60/13	33/10	0.477
Age (yr)	46.9 ± 10.8	47.9 ± 11.5	0.308	46.6 ± 11.1	47.3 ± 11.2	0.486
HbsAg						
Negative	10	10	0.734	11	9	0.420
Positive	52	44		62	34	
Liver cirrhosis						
No	10	10	0.734	14	6	0.472
Yes	52	44		59	37	
AFP (ng/mL)						
< 400	41	44	0.062	56	29	0.276
≥ 400	21	10		17	14	
Edmondson grade						
I - II	26	37	0.004	46	17	0.014
III–IV	36	17		27	26	
Child-Pugh class						
A	43	48	0.773	60	31	0.201
B	11	14		13	12	
Tumor capsule						
Complete	37	34	0.717	44	27	0.788
Incomplete	25	20		29	16	
Tumor size (cm)	5.7 (3-7)	6.6 (4-8.6)	0.134	5 (3.5-7)	6 (3.7-8.5)	0.207
Albumin (g/L)	41.1 ± 4.4	40.4 ± 4.6	0.434	41.5 ± 4.3	40.2 ± 4.5	0.134
Platelet count (10⁹/L)	167.9 (107.3-205.3	186.6 (136.3-230)	0.136	178.9 (107.3-205.3	186.6 (136.3-230)	0.833
AST (U/L)	56.6 (28.3-61)	55.6 (27-60)	0.924	38 (27-60)	44 (30.5-61.5)	0.737
ALT (U/L)	54.8 (28-56)	62.7 (24.8-57.9)	0.539	37(27-56)	38(27-55)	0.917
Total bilirubin (μmol/L)	14.08 (8.9-18.4)	14.3 (9.4-15.3)	0.912	11.9(9.35-16.6)	13.4(9-17.7)	0.580

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antigen.

Table 2 Multivariable analysis to identify predictors of overall survival in 116 hepatocellular carcinoma patients

Factors	Hazard ratio	95%CI	P value
AFP ≥ 400 ng/mL	1.691	1.094-2.614	0.018
Incomplete tumor capsule	0.979	0.659-1.454	0.915
Tumor size ≥ 5 cm	1.582	1.027-2.438	0.038
Edmondson grade III–IV	1.149	0.663-1.992	0.621
EP1 receptor expression	2.318	1.190-4.516	0.014

AFP: Alpha-fetoprotein.

Figure 1 Overall survival of hepatocellular carcinoma patients stratified by low or high expression of EP₁ receptor.

COX-2 and EP₁ receptor expression correlates with tumor differentiation

COX-2 immunoreactivity was significantly higher in well-differentiated HCC tissues (Edmondson grade I - II) than in poorly differentiated tissues (Edmondson grade III-IV) (*P* = 0.003). EP₁ receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue (Figure 2; *P* = 0.001). Figure 3 shows representative examples of different staining results in tissues of different histology grade.

DISCUSSION

HCC is one of the most aggressive tumors and has a poor prognosis. Some patients can undergo curative resection, but this trea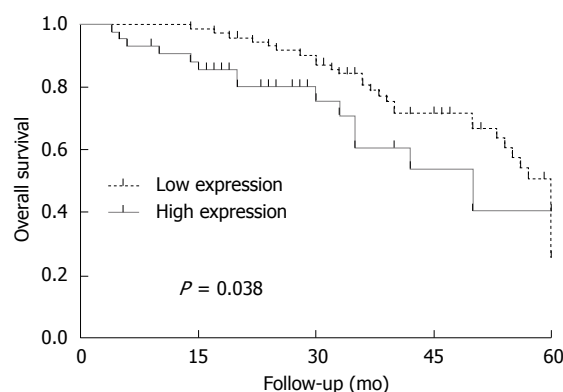tment is associated with a high rate of intrahepatic recurrence. Most patients with HCC are ineligible for resection because their disease has already reached an advanced stage by the time it is diagnosed. These patients are treated with local or systemic adjuvant modalities that provide only short-term regression, stabilization, or symptomatic control. Therefore, new therapeutic strategies are needed to improve long-term survival.

Our results indicate that the EP₁ receptor is involved in HCC progression, suggesting that it may be a reasonable therapeutic target. High expression of the EP₁ receptor was associated with poor prognosis in our
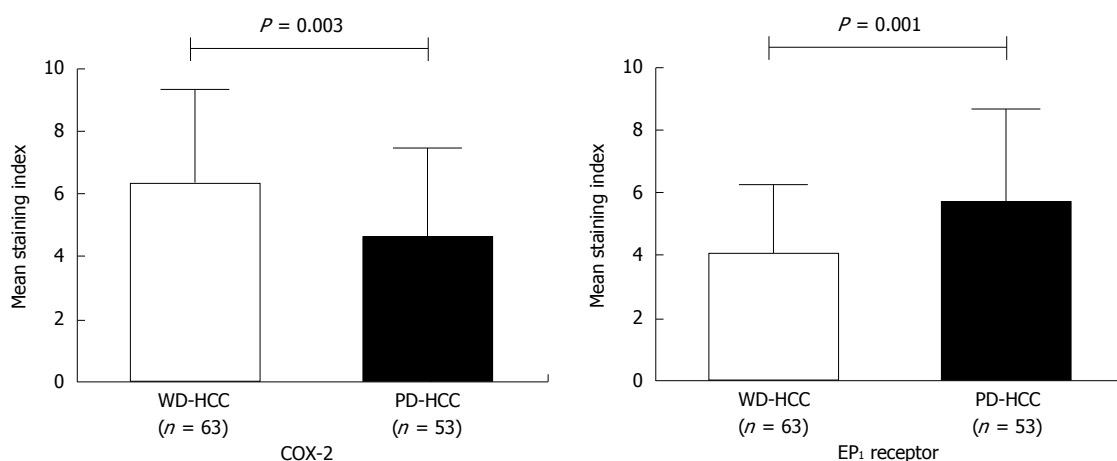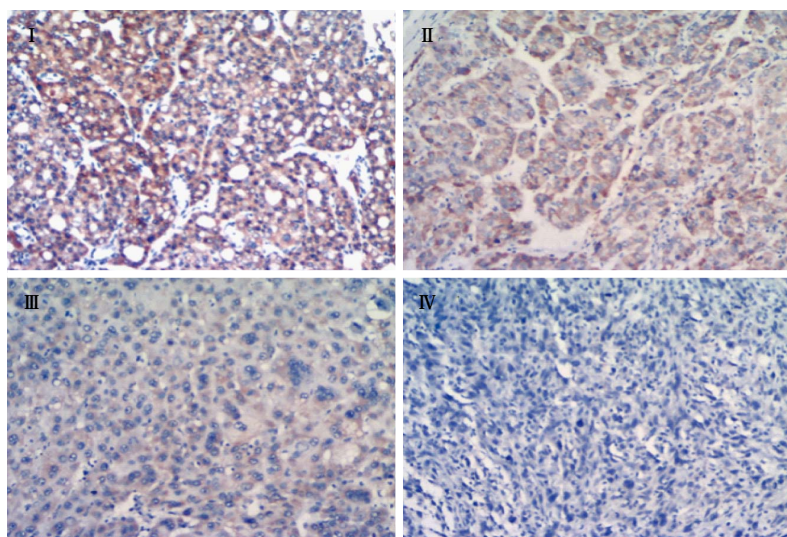
Figure 2 Cyclooxygenase-2 and prostaglandin E1 receptor immunoreactivity scores in hepatocellular carcinoma tissues at different histological grades. Cyclooxygenase-2 (COX-2) expression was higher in well-differentiated tissue (Edmondson grade I - II), while EP$_1$ receptor expression was higher in poorly-differentiated tissue (Edmondson grade III-IV). WD: Well-differentiated; PD: Poorly differentiated; HCC: Hepatocellular carcinoma.

COX-2

EP$_1$ receptor

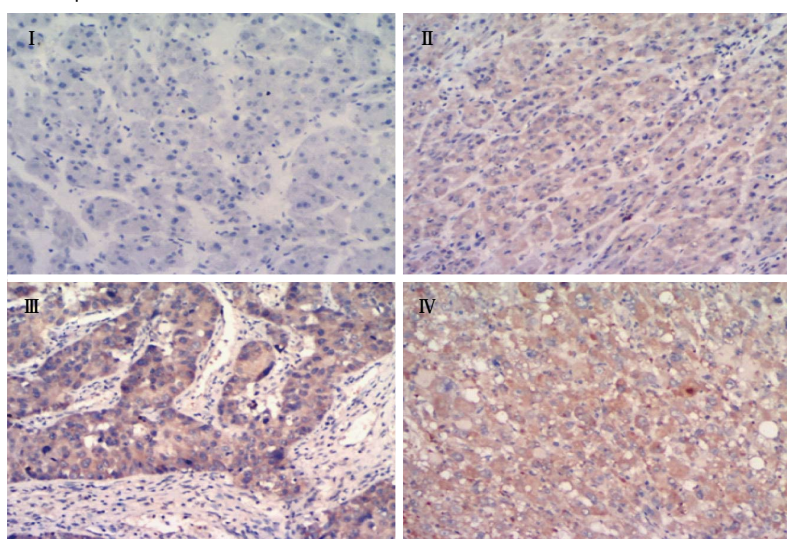**Figure 3 Representative micrographs showing different intensities of immunohistochemical stain against cyclooxygenase-2 and prostaglandin E1 receptor in tissues with different histological grades (Edmondson grade I -IV).** Magnification, × 100. Cyclooxygenase-2 (COX-2) expression level decreased with lower grade of differentiation, while EP$_1$ receptor expression increased with lower grade of differentiation.

patients, and expression was significantly higher in poorly differentiated tissue than in well-differentiated tissue. The observed correlation between higher expression and poorer differentiation is consistent with a previous study[18]. These results suggest that targeting the EP1 receptor may provide a more selective approach to treating HCC than using COX inhibitors to block prostaglandin E2 synthesis, which increases the risk of cardiovascular events[28].

Although studies have associated COX-2 expression with differentiation, invasion and metastasis in HCC[3,18,24], it has not been linked with survival. In the present study, we failed to find an association between COX-2 expression and survival when patients were dichotomized into groups with low or high expression. While it is possible that a more quantitative approach may identify associations between COX-2 levels and survival, we believe it is more likely that expression of COX-2 may be important only during initiation of HCC, whereas the EP1 receptor, which is the downstream target of prostaglandin E2 generated by COX-2, may be involved in disease progression. This may explain why we observed a different relationship between the expression of COX-2 and EP1 receptor: patients with high expression of one showed low expression of the other. This may also explain why we found that expression of the EP1 receptor, but not the COX-2 receptor, predicted OS in our cohort.

Several factors may help to explain why high expression of the EP1 receptor predicts poor survival. The receptor enhances tumor cell proliferation, invasion and migration[4,6,29,30], as well as adaptation to hypoxic conditions[6,31]. The receptor has also been reported to inhibit immune function and promote tumor progression[32]. The EP1 receptor can even induce prostaglandin E2 production by binding to the receptor of Fas ligand[32-34].

This study has some limitations. Firstly, the authors used only an HE method, therefore more accurate and quantitative methods, such as Western blot, polymerase chain reaction *etc.*, should be applied in future studies. Secondly, it is known that other cytokines, except COX-2 and EP1, have been reported to be strongly associated with HCC. These cytokines may be involved in different aspects of the pathogenesis of HCC and should be explored as a network pattern in future studies.

In conclusion, our results suggest that COX-2 expression correlates with an early event during the initiation of HCC, while EP1 receptor expression plays an important role in tumor progression and predicts OS.

COMMENTS

Background
Hepatocellular carcinoma (HCC) is one of the most aggressive tumors and the third most frequent cause of cancer-related death in the world. Although early diagnosis and treatment of HCC have improved substantially, prognosis remains unsatisfactory. HCC often involves highly malignant tumors that respond poorly or not at all to adjuvant systemic and local therapies. This highlights the need for new approaches to prevent and treat the disease.

Research frontiers
Two molecules that may be involved in HCC at different stages, and that therefore may be useful for understanding the pathogenesis and progression of the disease, are cyclooxygenase-2 (COX-2) and prostaglandin E1 receptor (EP1 receptor). These molecules have already been shown to play important roles in the onset of various cancers, including HCC.

Innovations and breakthroughs
The authors retrospectively reviewed the records of 116 patients with HCC who underwent surgery between 2008 and 2011 at their hospital, and found that COX-2 expression appears to be linked to early HCC events (initiation), while EP1 receptor expression may participate in tumor progression and predict survival.

Applications
These results suggest that targeting the EP1 receptor may provide a more selective approach to treating HCC than using COX inhibitors to block prostaglandin E2 synthesis, which increases the risk of cardiovascular events.

Terminology
Percentages of positive cells were categorized as follows: 0 (no positive tumor cells), 1 (1%-25% positive), 2 (26%-50% positive), 3 (51%-75% positive), and 4 (76%-100% positive). Staining intensity was categorized as follows: 0 (no staining), 1 (weak, light yellow), 2 (moderate, yellow-brown), and 3 (strong, brown). The scores for positive cell percentages and for staining intensity were multiplied together to yield a single immunohistochemical staining index from 0 to 12. Sections with an index of 0-5 were defined as showing low expression, while those with an index of 6-12 were defined as showing high expression.

Peer-review
The paper is a good study on COX-2 and EP1 receptor immunoreactivity in patients with HCC. The investigators shown that COX-2 immunoreactivity was higher in well-differentiated HCC tissues and EP1 receptor immunoreactivity was significantly higher in poorly differentiated tissue than in well-differentiated tissue.

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