Original Article

Cyclooxygenase-2 expression is associated with elevated aspartate aminotransferase level in hepatocellular carcinoma

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ABSTRACT

Background: Cyclooxygenase-2 (COX-2), the inducible rate-limiting enzyme of prostaglandins biosynthesis, is involved in the pathogenesis of many chronic inflammation-related human malignancies including hepatocellular carcinoma (HCC). However, its clinical significance in HCC remains obscure. The aim of our study was to evaluate COX-2 expression in HCC and correlate its expression to both clinicopathological parameters and patients survival.

Materials and Methods: The present study was conducted on 17 HCC and 21 adjacent nontumor liver tissues obtained from 22 HCC patients underwent hepatectomy. Eight normal liver tissues taken from normal donors and HepG2 cells were used as controls. Total RNA was extracted and COX-2 mRNA was detected by reverse transcription polymerase chain reaction and correlated to the clinicopathological criteria and to patient's survival.

Results: COX-2 mRNA was detected in 58.8% of the HCC tissues and in 28.6% of the adjacent nontumor liver tissues. COX-2 expression was significantly associated with elevated levels of serum aspartate aminotransferase (AST) with high specificity for disease detection. There was no significance between COX-2 expression and any of the histopathological criteria.

Conclusions: COX-2 expression may be involved in HCC carcinogenesis with high specificity for disease detection. COX-2 expression is significantly associated with elevated AST levels indicating a mechanism that may correlate both markers. However COX-2 expression seems to be an independent factor with no correlation to any of the histopathological data or patient's survival.

KEY WORDS: Aspartate aminotransferase, cyclooxygenase-2, hepatocellular carcinoma, sensitivity, specificity

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor and the third leading cause of cancer related mortality worldwide.[1] Major etiologic factors associated with HCC include infection with hepatitis C (HCV) and hepatitis B (HBV) viruses, excess alcohol intake and aflatoxin B1 exposure.[2,3] HCC, with and without HCV infection, represents a major health problem in Egypt, where the two pathological conditions are integrated in many cases.[4,5]

Hepatocellular carcinoma is a complex and heterogeneous tumor with several genomic mutations and it usually develops in the context of chronic liver damage and inflammation, suggesting that understanding the mechanism(s) of inflammation-mediated hepatocarcinogenesis is essential for the treatment and prevention of HCC.[6]

The resistance of HCC to existing treatments and the lack of biomarkers for early detection make it one of the deadliest cancers. Surgical resection, liver transplantation, and ablation by radiofrequency or ethanol injection are now conventional therapies at early disease stages. However, survival at 5 years is poor due to high incidence of recurrence, a complication that cannot be prevented by existing therapies.[2-4] These poor results of conventional treatments underscore the importance of developing alternative approaches that target molecular events of liver tumorigenesis.

Cyclooxygenase (COX) is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin (PG) H2, the precursor of various compounds including PGs, prostacyclin and thromboxane's which are important inflammatory mediators.[7] At least two COX genes, COX-1 and COX-2, have been identified. COX-1 is constitutively expressed in many tissues and plays important roles in homeostasis. In contrast, COX-2 is an immediate early gene induced by various stimuli such as mitogens, growth factors, cytokines, hormones, and tumor promoters.[8]

Upregulation of COX-2 has been demonstrated in various human carcinomas[9,10] and in many contexts related to liver cell pathologies including, regeneration after partial hepatectomy,[11,12] animal models of cirrhosis,[13] human hepatoma cell lines,[14,15] after chronic hepatitis B or C infection[16,17] and HCC.[18] Nevertheless, recent data reported that COX-2 mRNA levels were significantly higher in the adjacent liver than in HCC[19] and there was an inverse correlation between COX-2 expression and the differentiation grade and poor prognosis of HCC.[20] Therefore, COX-2 down-regulation in advanced HCC may be advantageous and specific for HCC development. However, the mechanisms regulating COX-2 expression at specific stages of HCC development remain unknown.[21]

The aim of the present study was to analyze the expression profile of COX-2 mRNA in HCC patients and correlate this expression profile to their clinicopathological data and survival results.

MATERIALS AND METHODS

Materials

Patients and tissue samples

The present study included 17 HCC and 21 adjacent nontumor liver tissues obtained from 22 HCC patients underwent curative hepatectomy during the period from December, 2003 to August, 2005.

Before operation, all patients were subjected to complete blood picture, liver function tests, viral markers (hepatitis B surface antigen and anti-HCV antibody), and preoperative serum alpha-fetoprotein (AFP) as well as radiological evaluation by abdominal U/S and computed tomography scan. Diagnosis was confirmed for all cases by histopathological examination of the removed hepatectomy specimens by two independent pathologists. After surgery, all patients were followed-up for up to 95 months for disease-free survival (DFS) and overall survival (OS).

Control groups

Eight normal liver tissue samples, collected from age and sex matched normal donors for liver transplantation and HepG2 cells were used as a positive control.

Methods

After approval of the Ethical Committee, tissue samples were collected from the resected specimens and divided into two parts, one section was fixed in 10% neutral-buffered formalin solution and sent to the Pathology Department for histopathological examination.

The other part was immediately placed in transport media and directly transferred to the laboratory with the name and hospital number of each patient. Under aseptic conditions, tumor and normal tissue samples were dissected preserved in cryotubes and directly frozen in liquid nitrogen then stored at - 80° till RNA extraction.

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium; (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Sigma), 100 mg/mL streptomycin (Sigma), 100 units/mL penicillin (Sigma) and L-glutamine (Life Technologies) and maintained in a humidified atmosphere at 37°C with 5% CO_2.

Total RNA extraction and reverse transcription polymerase chain reaction

Total RNA from frozen tissues and cultured cells was extracted using SV total RNA isolation system (Promega, Cat. number Z3100), according to the manufacturer's protocol and stored at−80°. Reverse transcription polymerase chain reaction (RT-PCR) was performed in Mx 3000P QPCR Thermocycler (Stratagene) by using Qiagen one step RT-PCR kit (Catalog no. 210212). The following set of primer sequences for COX-2 and β-2 microglobulin (β2M) were used: COX-2 forward, 5'-CAG CAC TTC ACG CAT CAG TT-3'; COX-2 reverse, 5'-TCT GGT CAA TGG AAG CCT GT-3'. β2M forward, 5'-CAC CCC CAC TGA AAA AGA TGA-3'; β2M reverse 5'-CAT CTT CAA ACC TCC ATG ACG-3'. The RT-PCR cycles were as follows: One cycle of reverse transcription at 50°C for 30 min, one cycle of initial PCR activation at 94°C for 15 min. followed by 35 cycles of amplification as follows denaturation for 30 s at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C, Followed by one step of final extension for 10 min at 72°C.

The amplified PCR products were electrophoresed on 2% agarose gel (Sigma) stained with ethidium bromide (50 μg/μl) and visualized under ultraviolet illumination. The desired fragments of COX-2 (756 bp) and β2M (116 bp) were identified using molecular weight marker of 100 bp DNA ladder (Promega Corp, Cat. number G2101) [Figure 1].

Statistical analysis

Statistical Package for the Social Sciences for Windows (version 12.0, SPSS, Chicago, IL, USA) was used for data management. Chi-square and Fisher's exact test were used for testing proportion independence. Nonparametric *t*-test compared means of two groups. Kaplan–Meier was used for estimating survival and log-rank test compared the curves. Receiver-operator characteristic curve was used to define cut-off level for quantitative variables according to COX-2 results. Data were presented as mean ± standard deviation (or median) and frequencies (%). *P* value is significant at 0.05 levels.

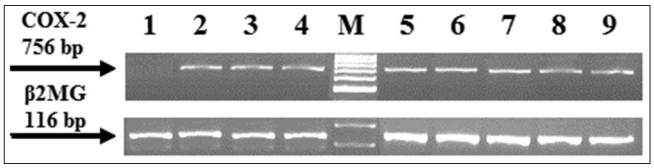

Figure 1: 2% Agarose gel stained with ethidium bromide showing reverse transcription polymerase chain reaction products for cyclooxygenase-2 (756 bp) and β-2 microglobulin (116 bp). Lane 1: normal control (N), Lane 2–4: Adjacent non tumor liver (NT), Lane M: 100 bp DNA ladder and lane 5–9: Hepatocellular carcinoma tissues (T)

RESULTS

Clinicopathological features of hepatocellular carcinoma patients

The histopathological and clinicopathological profiles of the studied HCC patients are shown in Tables 1 and 2.

Cyclooxygenase-2 mRNA expression in tumor and adjacent nontumor liver tissues

Cyclooxygenase-2 mRNA was detected in 10 out of 17 HCC tissues (58.8%) and in 6 out of 21 adjacent nontumor liver tissues (28.6%). The difference in COX-2 expression between both tissue types did not reach the level of significance ($P = 0.060$) [Table 3].

Correlation between cyclooxygenase-2 mRNA expression and the clinicopathological parameters of hepatocellular carcinoma patients

Cyclooxygenase-2 mRNA expression in HCC tissues was correlated to the different clinical and histopathological parameters. A statistically significant association was found between elevated levels of serum aspartate aminotransferase (AST) and COX-2 mRNA expression ($P = 0.007$). However, there was no significant correlation between COX-2 mRNA expression and any of the other characteristics of the patients [Table 4].

Sensitivity and specificity of cyclooxygenase-2 mRNA expression in hepatocellular carcinoma

By studying the sensitivity and specificity of COX-2 mRNA expression for diagnosis (tumor vs. normal) and prognosis (tumor vs. nontumor distant tissue) of HCC, we found that COX-2 expressions was 100% specific and 58.8% sensitive for HCC diagnosis. And it was 71.4% specific and 58.8% sensitive for disease prognosis [Table 5].

Correlation between cyclooxygenase-2 mRNA expression and patients' disease free survival

There was no difference in DFS between patients expressing COX-2 and those who are negative for COX-2 expression (37 vs. 38 months) [Figure 2]. However we could not study the OS as there were only two cases died throughout the study.

Sensitivity and specificity of cyclooxygenase-2 mRNA expression in hepatocellular carcinoma according to serum aspartate aminotransferase level

Receiver-operator characteristic curve describes the sensitivity and specificity of COX-2 according to serum AST level. The curve results show that at AST level over 50 IU/L, we expect the sensitivity and specificity of COX-2 will be 80% and 100% respectively [Figure 3].

Table 1: The histopathological profile of the studied HCC patients

Patient characteristics	Number	Percentage
Mean age		
56.72±10.47	22	100
Sex		
Male	18	81.8
Female	4	18.2
Histological grade		
Grade II	11	50
Grade III	10	45.5
Unavailable	1	4.5
Liver cirrhosis		
Cirrhotic	19	86.4
Noncirrhotic	3	13.6
Mean tumor size (cm)		
8.4±4.9 cm	22	100
Safety margin		
Negative tumor	19	86.4
Positive tumor	3	13.6
Chronic active hepatitis		
Positive	14	63.6
Negative	2	9
Unavailable	6	27.3
Vascular invasion		
Invasive	9	40.9
Noninvasive	6	27.3
Unavailable	7	31.8
Lymph node involvement		
Negative	16	72.7
Positive	2	9.1
Unavailable	4	18.2

HCC=Hepatocellular carcinoma

Table 2: The clinicopathological profile of the studied HCC patients

Parameter	Number	Mean
sAFP	19	13759.38±47181.07
TLC	20	6.2830±2.02766
Albumin	16	4.175±1.116
sGPT	20	80.37±89.906
sGOT	20	79.58±90.401

HCC=Hepatocellular carcinoma, sGOT=Serum glutamic oxaloacetic transaminase, sGPT=Stable glutamate-pyruvate transaminase, sAFP=Serum alpha fetoprotein, TLC=Thin layer chromatography

Table 3: Cyclooxygenase-2 mRNA expression in HCC and adjacent nontumor liver tissues in the 22 HCC patients

COX-2 RT-PCR (%)	HCC tissues (*n*=17)	Adjacent nontumor liver tissues (*n*=21)	*P*
Positive	10 (58.8)	6 (28.6)	0.060
Negative	7 (41.2)	15 (71.4)	

HCC=Hepatocellular carcinoma, COX-2=Cyclooxygenase-2, RT-PCR=Reverse transcription polymerase chain reaction, *P* value is significant at ≤ 0.05

DISCUSSION

Cyclooxygenase-2 has been shown to be up-regulated in a variety of human cancers including colon, gastric, esophagus, pancreas, lung and HCCs, despite the fact that COX-2 is undetectable in most normal tissues.[12,22-25] Recent studies showed that COX-2 expression is high in early stages of HCC, but low in advanced stages. These findings suggest that COX-2 plays an important role in the early stages of hepatocarcinogenesis.[26] Thus, COX-2 may be related to HCC dedifferentiation, an early event in hepatocarcinogenesis.

Cyclooxygenase-2 is responsible for at least three tumor-related processes. First, it affects angiogenesis through an acceleration

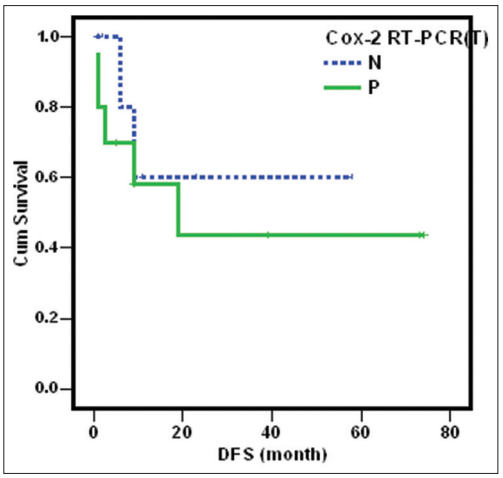

Figure 2: Kaplan-Meier survival curve for DSF of HCC patients based on Cox-2 mRNA expression in HCC tissues (*n*=17) (*P* = 0.52)

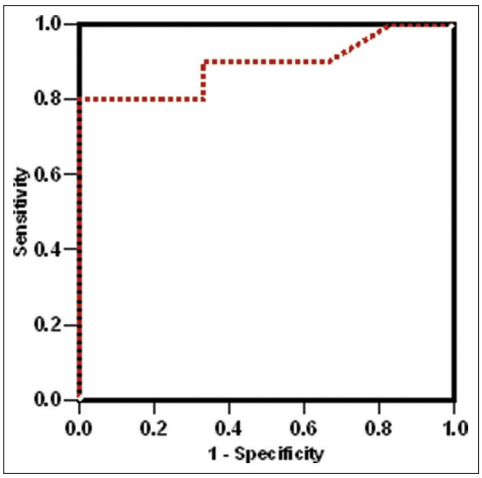

Figure 3: Receiver Operating Characteristic (ROC) curve analysis showing the sensitivity and specificity of COX-2 expression according to s AST levels in HCC tissues

of production of both vascular endothelial growth factor, and of PGs.[8] Second, it affects anti-apoptosis factors mediated by Bcl-2 and protein kinase B signaling.[27,28] Third, it has a strong effect on invasiveness via the action of matrix metalloproteinases.[29]

In the present work, 58.8% of the HCC tissues expressed COX-2 mRNA, while in the adjacent nontumor liver tissues only 6 were expressing COX-2 (28.6%).

The expressions of HBx and COX-2 were higher in HBV-related HCC.[30] Furthermore, COX-2 was shown to be highly expressed in the HCC tissue where 84.3% of the tumor tissue samples were positive for COX-2 expression.[7] In a third study, COX-2 expression was observed in 79% of the HCC cases.[8] The frequency of COX-2 expression in these two studies was relatively higher than our study. This may be due to the difference in the method of detection.

Recent studies suggested many roles for COX-2 in HCC which include tumor angiogenesis,[8] inhibition of apoptosis dependent on the intrinsic mitochondrial pathway where PGs produced by COX-2 expression protected the liver against fas-mediated apoptosis.[9] And inhibition of apoptosis and cell cycle progression.[31] This is in addition to stimulating the production of angiogenic factors, which are in favor of tumor growth and metastasis.[32,33]

Our study showed that 28.6% of the adjacent nontumor tissues are expressing COX-2. The expression of COX-2 in these areas may suggest a role of COX-2 in precancerous lesions and accordingly in liver carcinogenesis.

Recent findings found that the up-regulation of COX-2, Wnt/β-catenin activation is implicated in various stages of hepatic tumorigenesis, including the dysplastic foci, hepatic adenoma, hepatoblastoma and HCC.[7,8,10,34] In addition, COX-2 is overexpressed in liver cirrhosis where COX-2 production could be a contributor to HCC development in cirrhosis.[27]

Table 4: Correlation between cyclooxygenase-2 mRNA expression and the clinicopathological characteristics of HCC patients

Patients characteristic	COX-2 RT-PCR (T)		*P*
	Positive (*n*=10) (%)	Negative (*n*=7) (%)	
Gender			
Male	9 (60)	6 (40)	1.00
Female	1 (50)	1 (50)	
Mean age (years)	59.1±10.6	57.7±7.5	0.60
Mean TLC	7.2±2.1	5.6±1.65	0.22
Mean PLT	207±84.7	153±22.4	0.18
Mean SALT (IU/L)	71.2±61.3	49.57±14.12	0.56
Mean SAST (IU/L)	107.8±115.8	36.25±13.5	0.007**
Mean albumin	4.0±1.0	4.7±1.2	0.22
Mean sAFP (ng/mL)	27073.38±70336.47	4790.2±11075.4	0.39
Histological grade			
Grade II	5 (50)	5 (50)	0.62
Grade III	5 (71.4)	2 (28.6)	
Liver cirrhosis			
Cirrhotic	8 (53.3)	7 (46.7)	0.33
Noncirrhotic	2 (100)	0 (0.0)	
Mean tumor size (cm)	8.0±6.3	7.6±4.1	0.74
Safety margin			
Negative	9 (60)	6 (40)	1.00
Positive	1 (50)	1 (50)	
Chronic active hepatitis			
Positive	5 (41.7)	7 (58.3)	0.46
Negative	1 (100)	0 (0)	
Vascular invasion			
Invasive	5 (62.5)	3 (37.5)	1.00
Noninvasive	3 (75)	1 (25)	
Lymph node			
Negative	8 (61.5)	5 (38.5)	0.48
Positive	1 (50)	1 (50)	
Metastasis			
Metastatic	3 (75)	1 (25)	0.60
Nonmetastatic	7 (53.8)	6 (46.2)	
Recurrence			
Recurrent	5 (71.4)	2 (28.6)	0.62
Nonrecurrent	5 (50)	5 (50)	
Mean DFS (months)	38	37	0.52

HCC=Hepatocellular carcinoma, COX-2=Cyclooxygenase-2, RT-PCR=Reverse transcription polymerase chain reaction, TLC=Thin layer chromatography, PLT=Platelet, SALT=Serum alanine aminotransferase, SAST=Serum aspartate aminotransferase, sAFP=Serum alpha fetoprotein, DFS=Disease-free survival, **=*P* value is significant at ≤ 0.05

Table 5: Sensitivity and specificity of COX-2 mRNA for diagnosis and prognosis of HCC

Sensitivity for diagnosis (COX-2 positive/total tumor)	Specificity for diagnosis (COX-2 negative/total normal)	Sensitivity for prognosis (COX-2 positive/total tumor)	Specificity for prognosis (COX-2 positive/total adjacent nontumor)
10/17×100 (58.8%)	8/8×100 (100%)	10/17×100 (58.8%)	6/21×100 (71.4%)

COX-2=Cyclooxygenase-2, HCC=Hepatocellular carcinoma

In our study, we did not find any significant difference between COX-2 expression and the histopathological parameters. This means that COX expression seems to be an independent parameter.

These results are in agreement with a Turkish study where they evaluated COX-2 expression in hepatitis B and hepatitis C related HCC and in HCC patients with an unknown etiology. They found that COX-2 expression is independent of disease's characteristics.[35] On the contrary, other studies found that COX-2 overexpression in HCC correlated statistically with high histological tumor differentiation and early stage.[36]

Regarding the DFS, we did not find significant difference between COX-2 positive and COX-2 negative cases. This result disagrees with other studies that found COX-2 expression significantly correlate with poor DFS in HCC patients.[26,28] Other study showed that the combined negative expression of nitric oxide synthase and COX-2 had a significant impact on patient survival.[37] The contradiction of the results between our study and the other studies may be related to the difference in the etiology of the disease where our cases were mostly HCV positive HCC. The relatively lower number of cases may also be a factor.

By studying the sensitivity and specificity of COX-2 we found that COX-2 expression is 100% specific with lower sensitivity (58.8%) for diagnosis, while it was 71.4% specific and 58.8% sensitive for disease prognosis.

Studies on COX-2 in HCC found that overexpression of COX-2 constitute an independent prognostic factor.[36]

Serum AFP is recognized as a marker for HCC more than 45 years ago. Although AFP is routinely used for diagnosis and prognosis of HCC, but it has many problems where its level is normal in about 30% of patients at the time of diagnosis and usually remains low even at disease progression.[38] Although the specificity of AFP is near from 100%, the sensitivity falls below 45%.[39] Furthermore, its positive predictive value is low, ranging from 9% to 32%.[40]

If we compare the sensitivity and specificity of COX-2 for the diagnosis of HCC to those of AFP, we may conclude that COX-2 has nearly the same specificity (100%) and higher sensitivity.

Other study found that the sensitivity for serum GP73 and AFP was 76% and 70% respectively, while the specificity of both markers was 86% and 89% respectively. Authors concluded that serum GP73 has a comparable accuracy to AFP for HCC diagnosis.[41] In addition, serum clusterin yielded (90%) sensitivity and (87%) specificity for predicting HCC.[42] Alpha fucosidase was examined on 40 patients with HCC and 40 patients with chronic liver disease as well as 40 healthy controls, the sensitivity and specificity of AFU was 90% and 97.5%, respectively.[43]

Glypican 3 (GPC3), heat shock protein 70 (HSP70) and glutamine synthetase (GS) single and in combination were studied in liver biopsies for diagnosis of very early HCC. The sensitivity and specificity of the three markers for HCC diagnosis was as follows: GPC3 57.5% and 95%, HSP70 57.5% and 85%, GS 50% and 90%, respectively. The sensitivity and specificity of the different combinations were: GPC3 + HSP70 40% and 100%; GPC3 + GS 35% and 100%; HSP70 + GS 35% and 100%; GPC3 + HSP70 + GS 25% and 100%.[44]

By comparing COX-2 in the present study to markers in the other studies, we may conclude that COX-2 has superior specificity and comparable sensitivity to most of them for diagnosis and prognosis of HCC.

In order to reach a final conclusion regarding the use of COX-2 as a marker for HCC, we need to study COX-2 mRNA expression on larger number of cases and on more HBV positive HCC cases.

Among the clinicopathological parameters, only AST serum glutamic oxaloacetic transaminase (sGOT) had a significant correlation with COX-2 mRNA expression (P - 0.007).

In 1977, the activity of five serum enzymes was studied in both HCC and hepatic cirrhosis cases. Serum GOT changes were more closely correlated with HCC growth and sGOT was probably accounted for in part by tumor-derived GOT. Furthermore, sGOT was significantly increased in HCC group against relatively stable glutamate-pyruvate transaminase (sGPT).[45] Another study found that HCC group had higher sGOT/sGPT ratio than cirrhosis group.[46]

Our results showed that the elevation of AST was significantly associated with COX-2 mRNA expression, which may indicates a mechanism that may be directly or indirectly correlate serum AST to COX-2 in HCC. However, we cannot guarantee that serum AST is more closely related to tumor growth where serum AST may be elevated in conditions other than HCC.

CONCLUSION

Cyclooxygenase-2 expression may be involved in HCC carcinogenesis with high specificity for the detection of

the disease. COX-2 expression is significantly associated with elevated AST levels indicating a mechanism that may correlate both markers. However COX-2 expression seems to be an independent factor with no correlation to any of the histopathological data or patient's survival.

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