ORIGINAL PAPERS

Altered expression of COX-2 and TNF-α in patients with hepatocellular carcinoma

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ABSTRACT

Background and aim: hepatocellular carcinoma is a type of cancer related with inflammation, as 90% of cases develop in a chronic inflammation condition. Excess inflammation can affect tissue homeostasis. Cytokines and inflammatory mediators are immunological components that can influence the functioning of cells and tissues. In addition, the estrogen receptor appears to play an important role in hepatocarcinogenesis. The aim of the study was to evaluate the expression of inflammatory markers and ER in patients with hepatocellular carcinoma.

Methods: data from 143 patients of ISCMPA were analyzed. Immunohistochemistry was performed of cyclooxygenase-2 enzyme (COX-2), nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α) and ER in paraffin-embedded hepatic tissue. The percentage of the stained area, intensity of staining and of the number of ER positive nuclei were evaluated using the ImageJ 1.50 software.

Results and conclusion: there was a significant difference between the groups in terms of the percentage of marked area (p = 0.040) for COX-2 and the intensity of staining of TNF-α (p = 0.030). No significant differences were observed in any of other parameters evaluated. In conclusion, COX-2 and TNF-α are possible markers that should be further studied to determine their immunohistochemical profile and role in HCC development.

Key words: Hepatocelular carcinoma. Immunohistochemistry. Inflammation. Ciclooxigenase-2. Tumor necrosis factor-alpha. Nuclear kappa B factor.

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents 70-80% of primary liver tumors (1,2) and is the sixth most common cancer and the third leading cause of cancer mortality worldwide (1,2). The global incidence is 782,000 new cases per year with a similar mortality of 746,000, according to data from 2012 (2). In Brazil, HCC is the eighth leading cause of cancer death (3). Conditions such as chronic hepatitis and cirrhosis, particularly related to hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol and aflatoxin B1, are the main risk factors for this tumor (1). Diagnosis at an early stage is essential to reduce mortality, which highlights the importance of studying the pathophysiology of HCC (4).

It has been suggested that inflammation may play an important role in promoting conditions that lead to cancer in all of its stages (5). Cytokines and inflammation mediators are directly involved with inflammation and its progression and influence cells and tissues (6,7). Taking this into account, HCC is characterized as a type of cancer associated with inflammation, as around 90% of cases develop from a state of chronic inflammation (8).

The tumor necrosis factor alpha (TNF-α) is one of the mediators that are first produced in the inflammatory response. It promotes different effects on the endothelium and leu-

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kocytes, such as the stimulation of the expression of adhesion factors, stimulation of macrophages and the endothelial cell production of chemokines (9). Primarily, TNF-α was associated with anti-cancer effects, but it is now known that it plays a pro-tumorigenic role. It is involved in angiogenesis, inflammation, the formation of metastases and the activation of tumor survival pathways (10). Increased levels of TNF-α have been reported in patients with tonsil cancer (11), colorectal cancer (12) and liver cirrhosis (13), among others.

The major intracellular mediators of TNF-α are the target genes of nuclear factor kappa B (NF-κB) that are involved in cell proliferation and survival (8). NF-κB is involved in various critical elements of the regulation of immune responses (7). A previous study (14) suggested that NF-κB has a dual role in the development of HCC. In the early stages of tumor growth, its anti-apoptotic effect seems to be desirable as it prevents hepatocyte death. However, in more advanced stages, NF-κB leads to the survival of tumor cells. The activation of NF-κB in non-liver parenchyma cells leads to the persistent activation of the inflammation cascade (14).

Another mediator that has also gained prominence in the relationship between inflammation and cancer is cyclooxygenase-2 enzyme (COX-2). COX-2 is related to the formation of carcinogens, tumor promotion, inhibition of apoptosis, angiogenesis and metastatic processes (15-17). Moreover, elevated levels of COX-2 have been reported in some tumors such as breast, prostate, pancreas, skin, lung, bladder, esophagus and head and neck tumors (17,18). In HCC, it has been reported that COX-2 expression is related to the presence of inflammatory cells, including macrophages (8). In fact, some studies have reported its relevance in tumor differentiation and relation to HBV infection (19-22).

Estrogen receptors (ER) belong to a family of nuclear receptors involved in the regulation of a variety of genes (23). Their actions include the induction of cell proliferation and the management of cell growth and cell death programming (23). In animal models, it has been reported that estrogen can promote tumor growth and induce hepatocarcinogenesis (24). In turn, its role in inflammation seems to be related to anti-inflammatory effects via the inhibition of pro-inflammatory cytokines (25). However, it was reported that NF-κB activation in ER-positive tumors is associated with a more aggressive phenotype of breast cancer (26).

Considering the role of inflammation in the development of cancer, mediators such as TNF-α, NF-kB, COX-2 and ER may play an important role. Some studies have already investigated the relationship of those markers (27-29) with the development of chronic liver disease, but not necessarily with HCC in human tissue. The search for new prognostic markers for HCC is essential for the development of new treatment methods and the definition of new criteria for the selection of patients, as prognostic evaluation is an important stage for the management of HCC (4). Therefore, the purpose of this study was to investigate the association of these makers with HCC in a group of south Brazilian patients. These results may contribute to the characterization of a HCC profile in Brazil and provide new data regarding tissue expression of these markers.

METHODS

Sample characterization

In this case-control study, hepatic specimens in paraffin blocks were obtained (KCM laboratory, Porto Alegre, Brazil) from patients with (case group) and without a HCC diagnosis (control group) from ISCMPA, Brazil. The control group did not have HCC but had other hepatic diseases such as non-alcoholic steatohepatitis, hemosiderosis, hemochromatosis, cholelithiasis, autoimmune hepatitis, primary sclerosing cholangitis and primary hyperoxaluria. The number of samples differs for each marker studied due to availability of samples of each patient. Informed consent was previously obtained and data regarding age, gender and etiological and clinical factors were collected from their medical records. Liver specimens were obtained after procedures such as hepatectomy, liver transplantation or liver biopsy. Samples with excess necrosis and an insufficient amount of liver tissue were excluded. The present study was approved by the UFCSPA Ethics Committee (protocol number 1785/2012).

Immunohistochemistry assay

With regard to the immunohistochemistry assay, 4 μm sections of hepatic specimens were used, according to the protocol described by Hsu et al. (30). Slides were consecutively incubated with mouse anti-TNFα monoclonal antibody (1:300 dilution, Santa Cruz Biotechnology, Inc.), mouse anti-NF-κB monoclonal antibody (1:200 dilution, Santa Cruz Biotechnology, Inc.), rabbit anti-COX-2 monoclonal antibody (1:50 dilution, Biocare Medical®) and mouse anti-estrogen receptor monoclonal antibody (1:50 dilution, Abcam®). The primary antibody was replaced by PBS for the negative control.

Image acquisition and analysis

Image acquisition was performed using a camera coupled to an optic microscope (Olympus BX-41, Japan). Four photos of representative fields ("hot spots") were captured by 2,070 x 1,548 resolution and 24 bit, using 200x magnification for each patient. Representative fields included areas with the most intensive positive staining in hepatocytes (modified protocol of Weidner et al. [31]).

Evaluation of ER nuclear staining was performed by counting positive nuclei using the Cell Counter Plugin of the ImageJ 1.50 software. Nuclei were counted when they were inside the previously defined test-area, unless one of the two borderlines of the frame was superimposed over them (forbidden lines), according to the modified protocol described by Mandarim-de-Lacerda (32). Results were subdivided into four categories of the percentage of positive nuclei based on that described in the literature, such as that described by Lin (33).

Two parameters were evaluated in each photo for the markers NF-κB, TNF-α and COX-2; these were the percentage and intensity of the stained area (cytoplasmic staining). The ImageJ 1.50 software was also used for this evaluation. The tools color threshold, color deconvolution (from DAB) and

binarization were used to determine the percentage analysis and color threshold, color deconvolution (from DAB) and the measure of mean gray value for the intensity analysis. In this case, the mean gray value was subcontracted from the 255 pixel value. The protocols were modified based on the techniques described by Chodbury et al. (34), Brey et al. (35) and Ruifrok and Johnston (36). A mean of the four photos was calculated for each patient.

Statistics

Statistical analysis was performed using the IBM® SPPS Statistics software, version 20. The Kolmogorov-Smirnov normality test was used for numerical variables. The following tests were used to test differences between the case and control groups: Chi-square test for ER expression categories, Student's t test for COX-2 and NF-κB expression, and Mann-Whitney U test for TNF-α and NF-κB (intensity parameter) expression. The significance level was set at $p < 0.05$.

RESULTS

Patient characterization

Table 1 shows the characterization of samples, which are divided into the case and control groups for all markers. The number of patients for each marker is shown in the respective figure. In total, data from 143 patient biopsies were analyzed and a similar average age was observed between the case and control groups, around the fifth decade of life. A predominance of the male sex was also found in both groups and infection with HCV was the most common etiology, followed by infection with HBV and alcohol consumption. Cirrhosis was also present in the majority of patients in both groups. The same characteristics were found when analyzing each group of patients for each marker. Five samples were lost due to necrosis or lack of tissue.

Table 1. *Characteristics of the biopsy samples*

Variables	Case	Control	p
Biopsies, n	68	75	
Gender, n (%)	.		0.417
Female	21 (30.9)	28 (37.3)	
Male	47 (69.1)	47 (62.7)	
Age (years) - Mean ± SD	55.1 ± 9.3	55 ± 9.5	0.211
Condition, n (%)			
Cirrhosis	49 (72)	63 (84)	0.080
HCV	58 (85.3)	39 (52)	> 0.001
HBV	3 (4.4)	10 (13.3)	0.060
Alcohol	12 (17.6)	26 (34.7)	0.020

HCV: hepatitis C virus; n: number of biopsies. Continuous data are shown as the mean ± standard deviation and categorical values are shown as an absolute frequency (relative frequency). Continuous data were compared using the Mann-Whitney U test and categorical data by the Chi-square test.

ER expression

Immunohistochemistry revealed positive nuclei for case and control liver samples (figure not shown). Table 2 shows the percentage of positive nuclei of samples for the case and control groups. However, there was no significant difference between either group ($p = 0.175$).

COX-2, NF-κB and TNF-α expression

Figure 1 (A and B) shows positive staining of the COX-2 marker in the cytoplasm of cells. When the percentage of the stained area parameter (C) was analyzed, there was a significant increase of protein expression between the case and control groups ($p = 0.040$). However, no significant difference was found for the intensity of the stained area parameter (D) when both groups were compared ($p = 0.798$).

Staining for the NF-κB marker was detected in the hepatocyte cytoplasm (Fig. 2). No significant differences were found for either the percentage ($p = 0.430$) or intensity ($p = 0.890$) parameters that were evaluated for both groups. TNF-α staining was also cytoplasmic (Fig. 3) and a significant difference was observed in the intensity of the stained area parameter between the cases and controls ($p = 0.030$). No significant difference was found for the percentage of the stained area parameter between both groups ($p = 0.368$).

DISCUSSION

HCC is the main type of primary liver cancer worldwide with a higher incidence in males (1,23). Major risk factors for HCC include cirrhosis, hepatitis virus infections and alcohol consumption (1). Previous studies have shown that cirrhosis is present in 80% of HCC cases (1,2), whereas HBV infection is associated with around 50% of cases, mostly in East Asia (1,37). The present study evaluated characteristics such as sex, age and etiology in patients with HCC and other liver diseases in a cohort of patients from south Brazil.

Our results show that the majority of HCC cases are male and in the fifth decade of life. This is consistent with previous findings (23) that indicate a higher incidence in males than in females, with a HCC risk two to seven times higher (23,38).

Table 2. *Percentage of positive nuclei for ER staining in cases and controls*

	ER		
	Case	Control	p
0-25%	20 (54%)	18 (60%)	0.175
25-50%	5 (13.5%)	8 (26.6%)	
50-75%	9 (24.3%)	4 (13.4%)	
> 75%	3 (8.7%)	0 (0%)	

Categorical values are shown as the absolute frequency (relative frequency) and were compared by the Chi-square test.

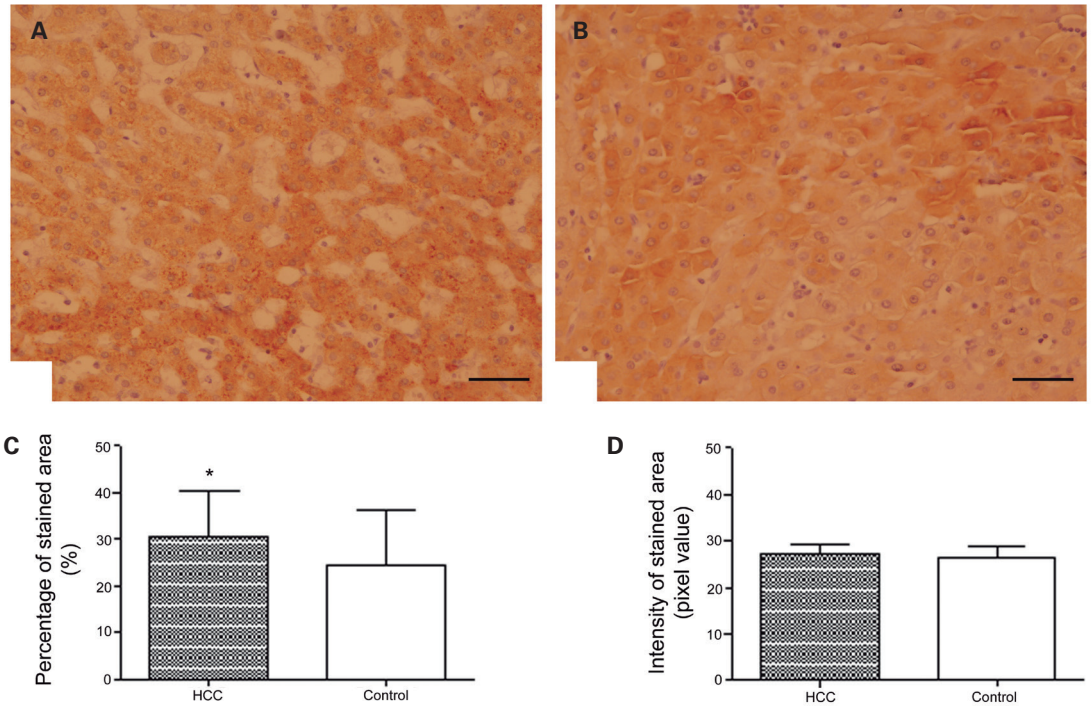

Fig. 1. *Digitized photomicrograph of immunohistochemical cytoplasmic staining of the COX-2 marker in case (A) and control (B) liver samples. Mean (± SD) percentage (C) and intensity (D) of the stained area for the COX-2 maker in cases (HCC; n = 25) and controls (non-HCC; n = 25). The Student's t test was used to compare groups, at a significance level of p < 0.050. *Indicates a significant difference between groups. Scale bar = 50 μm.*

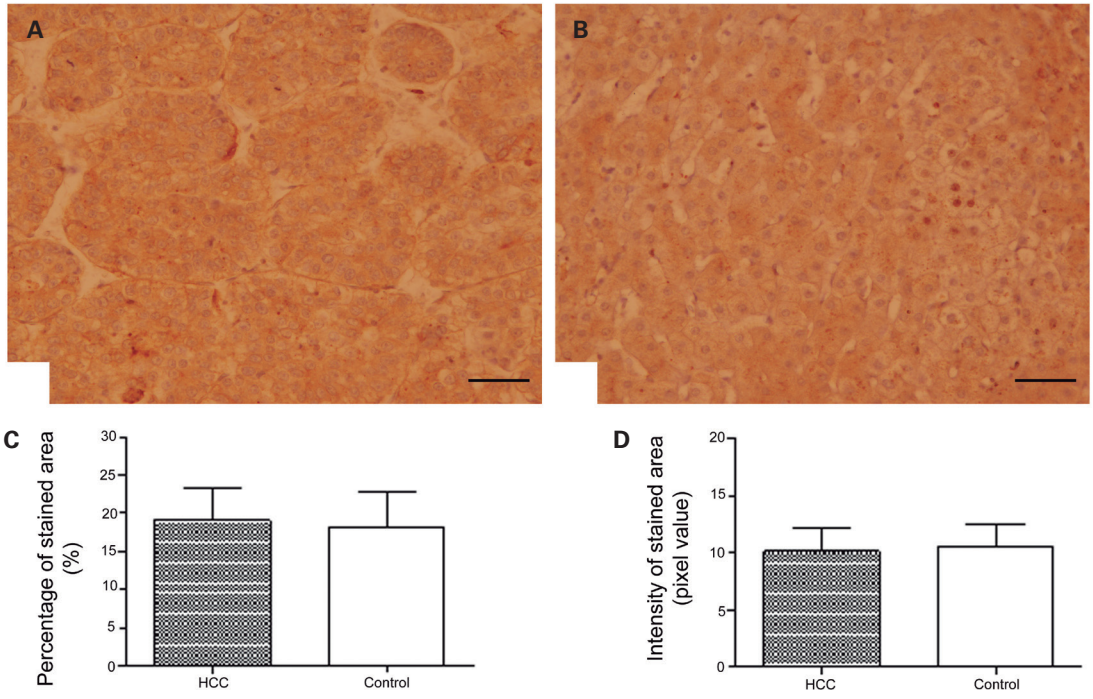

Fig. 2. *Digitized photomicrograph of immunohistochemical cytoplasmic staining of the NF-kB marker in case (A) and control (B) liver samples. Mean (± SD) percentage (C) and intensity (D) of the stained area for the NF-kB marker in cases (HCC; n = 23) and controls (non-HCC; n = 29). The Student t-test was used to compare the percentage parameter between the groups and the Mann-Whitney test was used for the intensity parameter, at significance level of p < 0.050. Scale bar = 50 μm.*

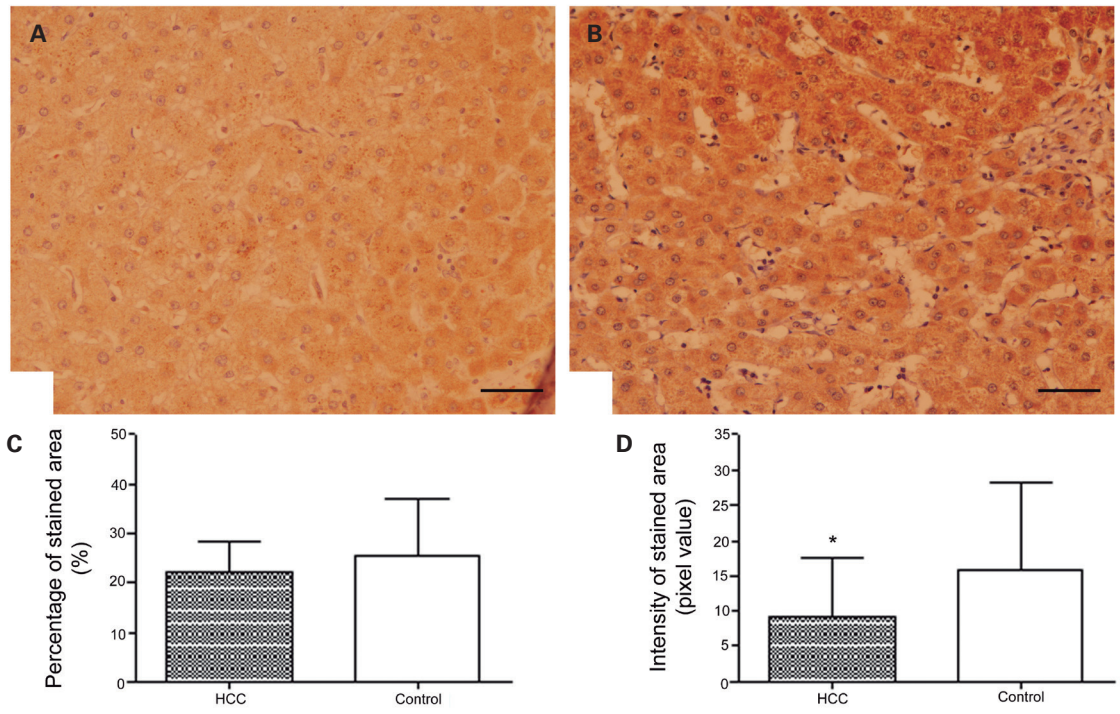

Fig. 3. *Digitized photomicrograph of immunohistochemical cytoplasmic staining of the TNF-α marker in case (A) and control (B) liver samples. Mean (± SD) percentage (C) and intensity (D) of the stained area for the TNF-α marker in cases (HCC; n = 26) and controls (non-HCC; n = 28). The Mann-Whitney test was used to compare groups, at significance level of p < 0.050. *Indicates a significant difference between the groups. Scale bar = 50 μm.*

Possible explanations for this gender disparity include the hypothesis that males are more exposed to HCC risk factors and that estrogen and testosterone may have a role in promoting hepatocyte proliferation (23,38). This divergence in the incidence between males and females emphasizes the importance of studies that associate the effect of sex hormones with the development of HCC.

The main etiology found in our sample (around 80% of cases) was not HBV infection but HCV infection. This is different from the general incidence. In fact, no significant difference was found between the groups with regard to HBV infection. Worldwide, HBV infection is associated with at least 50% of HCC cases (1,2), which differs from our results, which showed a higher frequency of HCV infection related to HCC. However, our findings are consistent with epidemiologic data of viral hepatitis in south Brazil that show a higher frequency of HCV cases compared to HBV (39). The study of Appel-da-Silva et al. (40) evaluated HCC incidence in south Brazil and the ten-year cumulative incidence was 28.8%. In addition, most cases (46.7%) presented HCV infection as the main etiology and HBV infection was only responsible for 2.7% of cases (40). Other studies in the same region of Brazil showed that HCV is responsible for 63% of HCC cases whereas HBV corresponds to approximately 13% of cases (41).

Nevertheless, the higher frequency of HCV infection found in our study is also consistent with the frequency reported in other countries such as USA, Japan and European countries (1,2). Our findings may aid the characterization of a part of the Brazilian population with a major a risk factor that is different from some regions of the world. Therefore, it is necessary to highlight the need to enhance different strategies of health promotion and prevention against HCV and the significance of new studies that associate the role of HCV in the development of conditions that lead to liver cancer.

Cirrhosis was also an important finding in the majority of HCC patients, which is also consistent with the literature. However, no significant difference was found between the frequency in cases and controls. Therefore, we cannot definitely associate the process as a pre-malignant condition, as the mechanism may occur by the promotion of liver susceptibility (42). In fact, studies suggest that the tumor microenvironment can contribute to its progression due to the continuous cycles of the destructive-regenerative process. Cirrhosis is a regenerative process that can influence the microenvironment. Thus, supporting tumor development rather than acting as a risk factor itself (43).

Alcohol consumption is another risk factor for HCC, with a minor contribution compared to viral hepatitis (2). Nevertheless, a significant difference was found between the groups with a higher frequency of alcohol consumption in control patients in our study. This finding may indicate that alcohol consumption is associated with liver diseases other than HCC. In fact, this highlights that the contribution of alcohol to HCC is involved in the progression of fibrosis, a process that is also associated with other hepatic diseases (2).

Estrogen seems to have an important role in carcinogenesis of the liver and plays a part in promoting and inducing hepatocarcinogenesis, as shown in animal models (24). On the other hand, a review by Shizumu et al. (44) reported

that estrogen, which is a potent antioxidant, may have a protective effect. HCC frequency is higher in males and postmenopausal women, who have lower estrogen levels. In a previous study by Vizoso et al. (45), ER expression in HCC samples was studied by tissue microarray and immunohistochemistry techniques and an elevated expression of the protein was reported. Hong et al. (46) also reported an increased susceptibility to DEN-induced HCC initiation and progression in estrogen receptor-alpha knockout mice. However, we did not find a significant difference in ER immunohistochemistry expression between case and control groups, thus, highlighting the need to further study greater numbers of patients in a subset analysis in order to clarify the contribution of ER expression in HCC. It is important to note that one limitation of our study was the fact that the hepatic specimen slides were made from paraffin blocks already stored in the laboratory of origin, which could have caused difficulties in tissue staining.

Another important point to be discussed is inflammation, which seems to have a significant role in promoting conditions that lead to cancer (5). COX-2, as an inflammation mediator, is related to the formation of carcinogens, tumor promotion and also plays other roles in carcinogenesis (15,16). In the present study, COX-2 expression in patients with and without HCC was evaluated. HCC patients had a significantly different COX-2 cytoplasmic staining, with a higher expression when compared to controls. This finding is consistent with other studies in the literature that evaluated HCC samples as well as other liver diseases such as nonalcoholic steatohepatitis (NASH), chronic hepatitis and liver cirrhosis (19,27,28).

Our findings emphasize that the study of COX-2 inhibitors could lead to a better understanding of the role of COX-2 in hepatocarcinogenesis and the effects that inhibition may cause in the development of disease. In fact, some studies have already demonstrated that Celecoxib® (a COX-2 inhibitor) alone caused growth inhibition of human hepatocellular carcinoma cells *in vitro* and *in vivo* (47), in cell lines and animal models, as well as by synergistic use with Sorafenib® *in vitro* (48).

Another important inflammatory marker is NF-κB, which appears to have a dual role in the development of HCC due to its anti-apoptotic effect. This protein can either prevent early hepatocyte death or enhance tumor cell survival, as reported in the review by Szabo and Lippai (14). This study analyzed the expression of NF-κB in patients with HCC and no significant difference was found between cases and controls. These result differ from a previous study by Jin et al. (29), which analyzed malignant and benign tissues of 305 patients with HCC. Different results may be due to the sample size, highlighting the need for further studies of this marker. NF-κB is a nuclear protein factor that stays in an inactive form in the cytoplasm and is transported to the nucleus when active (8). Our findings indicate the use of different intracellular pathways for the inflammation processes that are independent of NF-κB as a transcription factor in the liver.

TNF-α is a pro-inflammatory cytokine known for its anti-carcinogenic effects, but is also associated with pro-tumorigenic properties (10). Our findings showed a significant difference in the staining intensity of cells but not in the percentage of stained area in the control *vs* case groups. Wang et al. (49) found that TNF-α was highly expressed in HCC in *in vitro* cell lines and HCC tumor tissues. This study also showed a shorter survival time of these patients compared to low TNF-α expressing cases. Our results differ from those found by Wang et al. (49). On the other hand, the study of Dong et al. (50) in male Sprague-Dawley rats showed that TNF-α had a dual role in CCl4-induced liver injury. In that case, both high and low doses of TNF-α showed an increase in the serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) enzymes. This indicates that both high and low levels of TNF-α can have a damaging effect in the liver. This dose-related effect of TNF-α could be a possible process by which HCC patients in our study had a lower expression of TNF-α cytokine compared to the control group. Thus, indicating that HCC is not necessarily associated with high levels of the cytokine.

Our results provide more information about the Brazilian population with HCC, indicating a higher prevalence of HCV in relation to other common etiologies in other regions of Brazil and the world, thus contributing to the characterization of the disease profile in the region. Furthermore, this study provides new information regarding the expression of inflammatory and estrogenic makers in human liver tissue analyzed by immunohistochemistry that may contribute to new studies in the area. An inherent limitation to our study is the small sample size, which may not have sufficient power to detect an association of all markers, as well as a difficulty in performing further analysis. Furthermore, the difficulty to obtain samples from healthy patients as controls was another limitation of our study that should be highlighted.

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