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Toll-Like Receptors and Nuclear Factor Kappa-B as a Cross-Bridge between Inflammation and Carcinogenesis in Chronic Viral Hepatitis

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ABSTRACT

Toll-like Receptors (TLRs) are not only crucial for the initiation of immune response, but also play a key role in several inflammatory diseases. This study postulated a potential contribution of TLR3 and TLR9 in the initial triggering of inflammatory ambiance via Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells (NF-κB) activation in Chronic Virus Hepatitis (CVH) with later participation in inflammation induced carcinogenesis. The study was carried out on a 100 of chronic hepatitis Egyptian patients: 50 of virus C (CHC) and 50 of virus B (CHB) etiologies including: 20 and 15 cases with mild liver fibrosis (stages 1 or 2), 10 and 15 cases with liver cirrhosis (stage 4) and 40 cases of virus associated Hepatocellular Carcinoma (HCC), 20 of each etiology, respectively. Fifteen gender and age-matched, individuals were enrolled in the study as healthy controls. Toll-like receptor 3 or 9 expression on peripheral blood monocytes and liver tissue was evaluated in CHC and CHB patients respectively and in control cases. Liver tissue NF-κB expression and serum levels of Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-1β and IL-6 were measured in all cases of the study. The results showed a significant increase of all parameters in CHC and CHB patients compared to controls. Gradual but significant increment was recorded from mild fibrosis to cirrhosis, reaching the highest in HCC groups. Increased expression of TLRs in peripheral blood monocytes and liver tissue was running hand in hand with increased serum levels of TNF-α, IL- and IL-6 favoring the current postulation. This was further supported by the parallel increase in NF-κB expression in liver tissue especially in HCC group suggesting a potential link between chronic hepatic injury, fibrosis and HCC. These findings await further implementation and inclusion in therapeutic research.

Keywords: Chronic viral hepatitis, Hepatocellular carcinoma, Toll-like receptors, NF-κB, TNF-α, IL-1β, IL-6

INTRODUCTION

Toll-like Receptors (TLRs) constitute a highly conserved group of pattern recognition receptors that function as pathogen sensors in vertebrate and invertebrate species. The recognition of specific signature molecules termed Pathogen Associated Molecular Patterns (PAMPs) by TLRs is a cornerstone of the innate immune system, and enables it to rapidly mount protective responses against invading pathogens [1-3].

The human TLR family consists of 10 members, which are structurally characterized by the presence of a Leucine-rich Repeat (LRR) in their extracellular domain and a Toll/interleukin (IL)-1 receptor (TIR) in their intracellular domain [4]. Leucine rich repeated domains may be involved in ligand binding [5]. The existence of a large number of TLRs enables the innate immune system to discriminate between PAMPs that are characteristic of different microbial classes and launch specific defense mechanisms [6].

TLR3 is a member of the TLR family that can recognize Double-stranded RNA (dsRNA), playing an important role in antiviral immunity. It is expressed on parenchymal and non-parenchymal cells of liver as well as on several types of immune cells [7]. Meanwhile, TLR9 recognizes non-methylated CpG-containing DNA from bacteria and viruses.

Toll-like receptor-mediated signals are transduced through two major pathways, the Myeloid Differentiation Primary-response Protein 88 (MyD88-dependent pathway and the MyD88-independent pathway that initiate the transcription of a specific set of genes involved in pro-inflammatory, antiviral and antibacterial responses [4]. Binding of TLR3 and its ligand leads to conformational changes in the TLR3 cytoplasmic tail, followed by subsequent activation of the Mitogen Activated Protein (MAP) kinase pathway, the Nuclear Factor B (NF-B) family of transcription factors and the Interferon (IFN), Interferon Regulatory Factor (IRF) family of transcription factors, which then induce IFN and inflammatory cytokine production [8,9].

In TLR9 signaling, MyD88 induces NF-κB-dependent up-regulation of inflammatory cytokines [10]. Many pro-inflammatory effects of PAMPs are a consequence of TLR-induced secretion of inflammatory mediators such as Tumor Necrosis Factor-α (TNF-α), IL-1β and IL-6 [11]. Accordingly, in addition to their role in the innate immune system, TLRs contribute significantly to other processes including adaptive immune responses, regulation of sterile inflammation, wound healing and promotion of epithelial regeneration [12,13].

Chronic Viral Hepatitis (CVH) is inflammation of the liver, caused by viral infection. Of these, Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) infections account for a substantial proportion of liver diseases worldwide. Approximately 7% and 3% of the world's population are infected with HBV and HCV, respectively **[**14]. Egypt has the highest prevalence of HCV in the world, estimated nationally at 14.7%. More than 8 million people are infected with HCV and the incidence of new infections remains the highest worldwide [15]. Moreover, the prevalence rate of Hepatitis B Surface Antigen (HBsAg) in the Egyptian population is moderately high (10.1%) [16]. HCV is a single-strand RNA virus that infects liver and lymphoid cells causing chronic hepatitis, and HCC [17]. The genome encodes regions of extensive secondary Double-stranded RNA (dsRNA) structure and its replicative machinery yields dsRNA intermediates that are likely exposed to the cell dsRNA-sensing receptors, such as TLR3 [18], using its pathway to evade immune surveillance [19]. Meanwhile, HBV is a DNA virus causing chronic infection [20] and aggressive HCC tumors [21]. Recognition of viral DNA by the vertebrate immune system is based on the presence of un-methylated CG dinucleotide in particular sequence contexts (CpG motifs) by TLR9 [22].

The liver is probably the best example for the link between chronic inflammation and cancer. Almost 80% of HCCs in western world develop as a consequence of chronic inflammation and arise in fibrotic or cirrhotic livers [23] with a high probability that fibrosis-associated HCC is mediated by TLR signaling [24].

The aim of the current study was to evaluate the role of TLRs and NF-κB in inflammation-induced fibrosis, its progression and the subsequent development of HCC in chronic viral hepatitis patients.

SUBJECTS AND METHODS

Subjects

The study was conducted on a 100 of Egyptian patients admitted to Gastro-enterology/Hepatology Department, Theodor Bilharz Research Institute (TBRI) suffering from chronic viral hepatitis disease. Thirty were HCV positive (CHC), 30 were HBV positive (CHB) and 40 patients were complicated with HCC (20 cases on top of each etiology, respectively). Hepatitis patients were further sub-classified according to the histopathological stage of hepatic fibrosis into mild fibrosis (Stage 1 or 2, 20 HCV and 15 HBV) and cirrhosis (Stage 4, 10 HCV and 15 HBV). Chronic HCV was proved by reactive anti-HCV antibodies for more than 6 months (using Murex enzyme immunoassay kit, Dartford, England) and positive HCV-RNA by PCR according to Hodinka [25]. Also, positive HBs antigen was detected in CHB cases for more than 6 months. None of the patients had received antiviral treatment before inclusion in the study to avoid the possible effect of therapy on TLRs expression, such effect has been described by Vollmer et al. [26]. HCC was diagnosed by ultrasonography and confirmed by liver biopsy. Fifteen age and gender matched healthy individuals were included in the study to serve as controls and fifteen control wedge liver biopsies were obtained from cases of laparoscopic cholecystectomy after their consent. The study protocol was approved by the Institutional Review Board (IRB, TBRI) and all patients gave written informed consent before the study.

METHODS

Sampling

Blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) anticoagulated polypropylene vacuum tubes to perform the hemogram and flowcytometric analysis, in citrate anticoagulated plastic vacuum tubes and in serum separator tubes. Citrated blood samples were centrifuged for 15 min at 1000 × g then plasma samples were aspirated and used to measure prothrombin time. Serum separator tubes were allowed to clot for 30 min before centrifugation at 1000 × g for 15 min. Serum samples were aspirated and used to measure bilirubin, transaminases and albumin levels. The rest of samples were aliquoted and stored at -70°C for a period less than 6 months to measure TNF-α, IL-1β and IL-6 levels.

Flowcytometric analysis

Immunophenotype characterization for identification of TLR3 and TLR9 on monocytes of healthy controls, TLR3 on monocytes of HCV and TLR9 on monocytes of HBV patients was performed using anti-TLR3 MAb (PE) and anti-TLR9 MAb (FITC) (Alexis Biochemicals, San Diego, CA, USA). The samples were processed using (COULTER® EPICS® XL™, USA).

Histopathological study

Serial sections (5 µm thick) of formalin-fixed, paraffin-embedded liver biopsies of all studied cases were stained with hematoxylin/eosin and Masson trichrome stains. Control cases were found to be histopathologically unremarkable of any hepatic lesion. The stage of hepatic fibrosis in each chronic viral hepatitis patient was determined using the French METAVIR Scoring System [27]. Accordingly, they were divided into mild fibrosis (Stage 1 or 2) and liver cirrhosis (Stage 4).

Immunohistochemistry study

The 5 µm thick sections from formalin-fixed, paraffin-embedded liver biopsies were collected on microscopic slides coated with 3-amino propyl triethoxysilane (Sigma Chemicals; St. Louis, USA) for proper tissue adherence and to minimize staining artifacts. Following de-paraffinization, washing with Phosphate-buffered Saline (PBS, pH 7.4) and incubation in 3% H_2O_2/methanol for 10 min to block endogenous peroxidase; antigen retrieval was performed by microwaving in 10 mM citrate buffer, pH 6.0 (Dako, Denmark). Non-specific antibody binding was hindered by pre-incubation with 100 µl blocking serum for 30 min at room temperature. Liver sections of all hepatitis C-infected patients and controls were incubated overnight, at 4°C, with the primary anti-human monoclonal antibodies for TLR3 and NF-κB (Alexis Biochemicals, San Diego, CA, USA) while liver sections of all hepatitis B-infected patients and controls were incubated overnight, at 4°C with the primary anti-human monoclonal antibodies for TLR9 and NFκB (Alexis Biochemicals, San Diego, CA, USA) according to manufacturer's guidelines. After thorough rinsing in PBS, the biotinylated secondary antibody was applied followed by Streptavidin peroxidase conjugation.

Peroxidase activity was developed using diaminobenzidine as the chromogen, and Mayer's hematoxylin as the counter stain. PBS was substituted for the primary antibody to serve as a negative control with each setting. TLR3, TLR9 and NF-κB staining was evaluated semi-quantitatively on the basis of the percentage of positive cells and classified as follows: Diffusely positive (+++) when positive cells comprised more than 75% of the total cell population; moderately positive (++) when 26-75% of cells were positive, weakly positive (+) when, 5%-25% of cells were positive and negative when less than 5% or no cells were positive.

Measurement of pro-inflammatory cytokines

TNF-α concentration was measured in serum by Enzyme-linked Immunosorbent Assay (ELISA) technique using Human TNF-α assay kit (Genzyme Diagnostics, San Diego, CA, USA). IL-1β serum concentration was measured by Human IL-1β assay ELISA test kit (BioSource International, California USA). IL-6 serum level was measured by ELISA technique using Human IL-6 Assay kit (BioSource International, California, USA). The procedures given by the manufacturers were followed.

Statistical analysis

Data are expressed as mean values ± SD. Means of different groups were compared using ANOVA test. Comparison between percent positive cases was calculated by Chi-square test. The correlation between variables was assessed by Pearson correlation coefficient except for correlating monocyte and liver tissue expression of TLRs where Spearman rank correlation coefficient was used. Receiver Operating Characteristic (ROC) curve was applied to detect the cutoff points and the correlation between the sensitivity and specificity of monocyte TLRs expression using the corresponding liver tissue expression as reference. The threshold for significance was a P-value ≤0.05. Statistical Package for Social Sciences (SPSS) for Windows (version 18) computer program was used for statistical analysis.

RESULTS

Routine laboratory investigations of liver function tests and hemogram showed significant increase in CHC, CHB and hepatitis associated HCC patients compared to control cases. Pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) of all studied patients and TLR3 or TLR9 expression on peripheral monocytes of all CHC or all CHB patients, respectively, were significantly increased compared to controls. Progressive significant increment was also detected with increased stage of fibrosis up to the development of HCC (Tables 1 and 2).

Table 1: TLR3 monocyte expression and serum levels of TNF-α, IL-1β and IL-6 in controls, CHC and HCC patients

Correlations	Controls	CHC Stage 1 and 2 (Mild fibrosis)	CHC Stage 4 (Cirrhosis)	HCC on top of CHC
TLR3 monocyte expression (%)	29.27±6.51	62.76 ± 6.57[a]	83.29 ± 4.58[ab]	93.51 ± 4.41[abc]
TNF-α (pg/ml)	5.94 ± 1.17	12.18 ± 1.85[a]	24.56 ± 5.28[ab]	30.25 ± 5.87[abc]
IL-1β (pg/ml)	57.22 ± 10.71	87.09 ± 9.95[a]	117.8 ± 15.33[ab]	158.9 ± 39.89[abc]
IL-6 (pg/ml)	8.55 ± 2.78	46.88 ± 9.74[a]	76.3 ± 11.4[ab]	89.86 ± 8.95[abc]

CHC: Chronic Hepatitis C, HCC: Hepatocellular Carcinoma; TLR3: Toll-like receptor 3; TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin-1β, IL-6: Interleukin-6; a: Significant difference compared to control group P<0.05; b: Significant difference compared to mild fibrosis group P<0.05; c: Significant difference compared to cirrhosis group P<0.05

Table 2: TLR9 monocyte expression and serum levels of TNFα, IL-1β and IL-6 in controls, CHB and HCC patients

Correlations	Controls	CHB Stage 1 and 2 (Mild fibrosis)	CHB Stage 4 (Cirrhosis)	HCC on top of CHB
TLR9 monocyte expression (%)	35.75 ± 6.55	66.51 ± 9.73[a]	80.23 ± 8.35[ab]	86.95 ± 6.1[abc]
TNF-α (pg/ml)	5.94 ± 1.17	11.91 ± 3.37[a]	23.49 ± 7.33[ab]	31.53 ± 6.31[abc]
IL-1β (pg/ml)	57.22 ± 10.71	93.37 ± 19.84[a]	148.67 ± 25.88[ab]	189.75 ± 33.7[abc]
IL-6 (pg/ml)	8.55 ± 2.78	44.56 ± 8.57[a]	61.8 ± 8.92[ab]	88.16 ± 7.46[abc]

Correlation analyses (Tables 3 and 4) revealed significant direct correlation between either TLR3 or TLR9 monocyte expression and each of TNF-α, IL-1β or IL-6, between TNF-α and each of IL-1β or IL-6 and also between IL-1β and IL-6 in all CHC and all CHB patients, respectively.

Table 3: Significant correlations in CHC and HCC groups

Correlations	CHC Stage 1 and 2 (Mild fibrosis)	CHC Stage 4 (Cirrhosis)	HCC on top of CHC
TLR3 monocyte expression *vs.* TNF-α	0.947**	0.845**	0.839**
TLR3 monocyte expression *vs.* IL-1β	0.933**	0.832**	0.884**
TLR3 monocyte expression *vs.* IL-6	0.668**	0.645*	0.793**
TNF-α *vs.* IL-1β	0.945**	0.869**	0.836**
TNF-α *vs.* IL-6	0.732**	0.811**	0.712**
IL-1β *vs.* IL-6	0.731**	0.825**	0.753**

*Correlation is significant at 0.05 levels; **Correlation is significant at 0.01 levels

Table 4: Significant correlations in CHB and HCC groups

Correlations	CHB Stage 1 and 2 (Mild fibrosis)	CHB Stage 4 (Cirrhosis)	HCC on top of CHB
TLR9 monocyte expression *vs.* TNF-α	0.572*	0.912**	0.621**
TLR9 monocyte expression *vs.* IL-1β	0.665**	0.853**	0.665**
TLR9 monocyte expression *vs.* IL-6	0.626*	0.915**	0.757**
TNF-α *vs.* IL-1β	0.555*	0.782**	0.988**
TNF-α *vs.* IL-6	0.958**	0.906**	0.922**
IL-1β *vs.* IL-6	0.537*	0.788**	0.952**

TLR3 hepatic expression was mildly (+) positive in 20% (3/15) of normal control cases, in 35% (7/20) of cases with mild hepatic fibrosis, in 40% (4/10) of cirrhotics and in 55% (11/20) of complicated HCC cases. TLR3 expression in HCC was significantly higher compared to cirrhosis, mild fibrosis and normal liver tissue. It was also significantly increased in stage 4 CHC fibrosis compared to mild fibrosis cases (Table 5; Figures 1 and 2).

Table 5: TLR3 expression in liver tissue of controls, CHC and HCC patients

Histopathological diagnosis	Number of cases	Site of staining	Number of positive cases (%)	Intensity of TLR3 expression		
				+ Number (%)	++ Number (%)	+++ Number (%)
Controls	15	Cytoplasmic	3 (20)	3 (20)	0	0
CHC Stage 1 and 2 (Mild fibrosis)	20	Cytoplasmic	7 (35)[a]	1 (5)	2 (10)	4 (20)
CHC Stage 4 (Cirrhosis)	10	Cytoplasmic	4 (40)[a,b]	1 (10)	1 (10)	2 (20)
HCC	20	Membranous/Cytoplasmic	11 (55)[a-c]	1 (5)	4 (20)	6 (30)

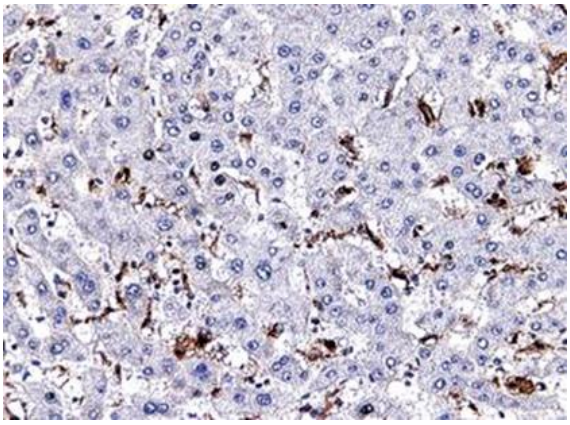

Figure 1: TLR3 expression in hepatic stellate cells and Kupffer cells of a case of CHC infection (immunohistochemistry X20)

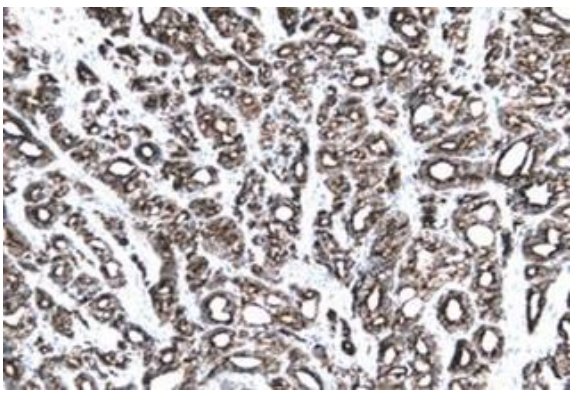

Figure 2: Membranous and cytoplasmic hepatocyte expression of TLR3 in a case CHC associated HCC, acinar pattern (Immunohistochemistry X10)

Regarding TLR-9, it was positively expressed in 13.3% (2/15) of normal liver tissue, in 53.3% (8/15) of mild fibrosis cases, in 80% (12/15) of cirrhosis cases and in 95% of HCC cases (19/20). TLR-9 liver tissue expression was significantly higher in all diseased cases compared to controls, in HCC compared to CHB patients and in cirrhosis compared to mild fibrosis (Table 6; Figures 3 and 4).

Table 6: TLR9 expression in liver tissue of controls, CHB and HCC patients

Histopathological diagnosis	Number of cases	Site of staining	Number of positive cases (%)	Intensity of TLR3 expression		
				+ Number (%)	++ Number (%)	+++ Number (%)
Controls	15	Membranous	2 (13.3)	2 (13.3)	0	0
CHB Stage 1 and 2 (Mild fibrosis)	15	Mostly membranous	8 (53.3)[a]	0	6 (40)	2 (13.3)
CHB Stage 4 (Cirrhosis)	15	Mostly cytoplasmic	12 (80)[a,b]	2 (13.33)	4 (26.67)	6 (40)
HCC	20	Cytoplasmic	19 (95)[a-c]	3 (15)	6 (30)	10 (50)

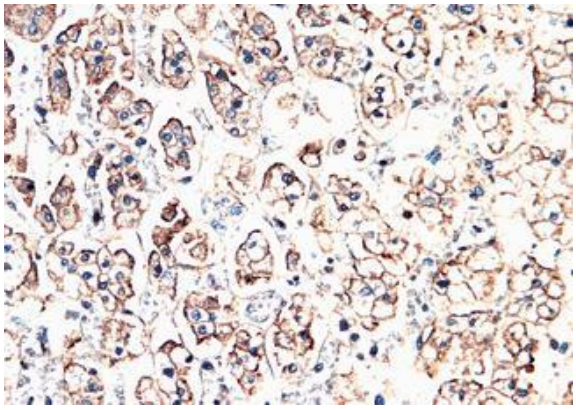

Figure 3: Membranous hepatocyte expression of TLR9 in a case of CHB (Immunohistochemistry X10)

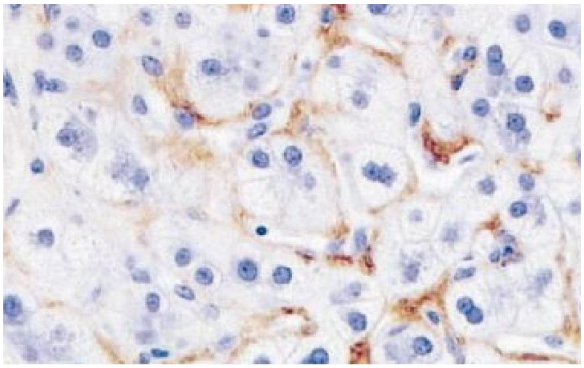

Figure 4: TLR-9 expression in hepatic stellate cells of a case of HBV induced cirrhosis (immunohistochemistry X40)

Hepatic expression of TLR3 and TLR9 was directly correlated to the corresponding peripheral monocyte expression in mild fibrosis, cirrhosis and hepatitis-associated HCC patients (Table 7). The ROC curve for monocyte expression of TLR3 using the liver tissue expression as reference showed that at cutoff value 52.8 the sensitivity was 50% and the specificity was 79% while for TLR9 monocyte expression at cutoff value 82.0 the sensitivity was 100% and the specificity was 43%.

Table 7: Correlation between TLRs monocyte and liver tissue expressions in mild fibrosis, cirrhosis and HCC groups

	Stage 1 and 2 (Mild fibrosis)	Stage 4 (Cirrhosis)	HCC
TLR3	0.743**	0.645**	0.739**
TLR9	0.723**	0.765**	0.712**

The NF-κB positive material was represented as fine particles localized to the cytoplasm and/or the nucleus of hepatocytes and bile ductless. The NF-κB-positive cells were detected in 75% (15/20) of CHC patients and in 66.6% (10/15) of CHB patients with mild fibrosis. In cirrhosis, it was positive in 80% of either CHC or CHB patients (8/10 and 12/15, respectively). All HCC patients (100%) were positive for NF-κB expression. NF-κB expression was significantly increased in HCC patients compared to chronic hepatitis patients, and in cirrhosis patients compared to mild fibrosis (Table 8; Figures 5 and 6).

Table 8: NF-κB expression in liver tissue of controls, CHC, CHB and hepatitis associated HCC patients

Histopathological diagnosis	Number of cases	Site of staining	Number of positive cases (%)	Intensity of NF-κB expression		
				+ Number (%)	++ Number (%)	+++ Number (%)
Controls	15		0	0	0	0
CHC Stage 1 and 2 (Mild fibrosis)	20	Cytoplasmic	15 (75)[a]	4 (20)	9 (45)	2 (10)
CHC Stage 4 (Cirrhosis)	10	Cytoplasmic	8 (80)[a,b]	2 (20)	3 (30)	3 (30)
HCC on top of CHC	20	Nuclear/Cytoplasmic	20 (100)[a-c]	0	15 (75)	5 (25)
CHB Stage 1 and 2 (Mild fibrosis)	15	Cytoplasmic	10 (66.6)[a]	2(13.3)	5(33.3)	3(20)
CHB Stage 4 (Cirrhosis)	15	Nuclear/Cytoplasmic	12 (80)[a,b]	1(6.67)	5(33.33)	6(40)
HCC on top of CHB	20	Nuclear/Cytoplasmic	20 (100)[a-c]	2(10)	5(25)	13(65)

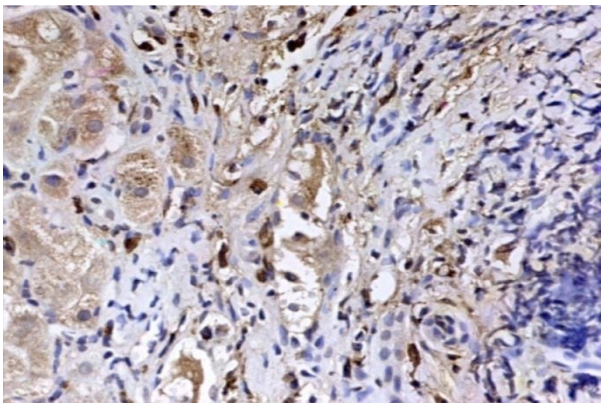

Figure 5 Cytoplasmic hepatocyte expression of NF-KB in a case of HCV-induced cirrhosis (Immunohistochemistry X40)

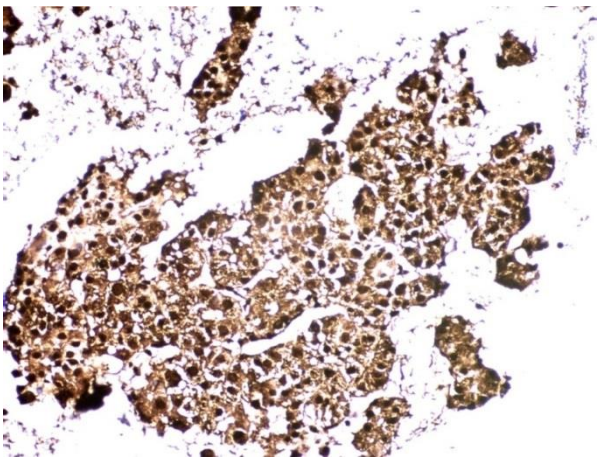

Figure 6: Both cytoplasmic and nuclear expression of NF-kB in a case of CHB associated HCC (Immunohistochemistry X10)

DISCUSSION AND CONCLUSION

Inflammation has for long been recognized as a localized protective tissue reaction to diverse stimuli with the ultimate aim of serving the body welfare. Recently, argument has risen around the connection of inflammation to a wide variety of diseases including cancer [28], indicating that it can act as a double-edged sword. The long-term prognosis of HCC is still unsatisfactory, thus further understanding of its molecular carcinogenic mechanisms is important [29]. Within the intricate circle of interacting participants, TLRs presented themselves as a highly conserved group of pattern recognition receptors that can rapidly mount protective responses against invading pathogens [3] and their detailed role points to their involvement in carcinogenesis [13]. The setting of liver disease was chosen to elaborate TLRs implication since they play a major role in liver patho-physiology due to liver`s anatomic association with the intestine and its exposure to a relatively large amounts of intestinally derived PAMPs in both health and disease states. On the other hand, monocytes are among the representative populations of innate and adaptive immunity and their cooperation is required to recognize, limit and eliminate invading microorganisms [30,31].

Following liver injury, hepatic stellate cells undergo an activation process and become the predominant extracellular matrix-producing cell type in the liver [32]. As raised hepatic stellate cell expression of TLR3 and TLR9 was detected in chronic hepatitis C and B cases of the current study compared to controls and increased gradually with disease progression, reaching the maximum in complicated HCC cases, we can suggest that tumor cells evade or overcome immune surveillance by multiple mechanisms, one of which is the activation of these two TLR signaling.

This concurs with the findings of Maeda [33] and was supported by a recent study of Mohamed et al. [34], showing that TLR 9 was up-regulated in human HCC tissue, and its inhibition by cloroquine led to significant reduction in tumor cell proliferation and inhibited tumor growth. Moreover, it was found in other series that TLRs promote tumor progression by achieving increased tumor cell-endothelial cell adhesion, tumor cell-extracellular matrix adhesion and tumor cell-extracellular matrix invasion through NF-κB mediated up-regulation of β_1 integrin. Additionally, TLR signaling pathways play a key role in activating stem cell/ progenitor proliferation and conversion to cancer-stem-cell-based liver tumor formation [35,36].

Peripheral blood monocytes expression of TLR3 and TLR9 reported in this study was significantly increased in a gradual rising pattern from control to mild fibrosis, cirrhosis, and HCC, also, supports the previous implication. This was in accordance with the findings of Dolganiuc et al. [37] and Xu et al. [38]. Furthermore, Schwabe et al. [39] highlighted the abundant expression of TLR3 and TLR9 among other TLRs in monocytes and tissue macrophages and linked this significant expression to diverse liver disease.

In agreement with the results obtained in the present study, there was increasing evidence that TLRs as well as other critical regulators of cytokines such as NF-κB promote liver tumorigenesis [40]. Engagement of TLRs with viral PAMPs activates NF-κB and induces the production of inflammatory cytokines such as TNF-α, IL-1 and IL-6 [41]. NF-κB is a transcription factor that consists of homodimers and heterodimers. Once activated, NF-κB dimers translocate into the nucleus and stimulate transcription of various genes, such as those encoding inflammatory cytokines, matrix metalloproteinases and antiapoptotic factors [42]. HCV core and HBx proteins are the most potent signal inducers for NF-κB [33].

In this study, NF-κB expression was totally absent in control liver biopsies but positive in 75% and 66.6% of CHC and CHB cases, respectively suffering of mild fibrosis, in 80% of cirrhosis and 100% of HCC patients. As activation and survival of hepatic stellate cells and hepatic myofibroblasts is regulated by NF-κB, its role in cell death regulation, inflammation, wound healing and carcinogenesis can be explained considering it as an important modulator of hepatic disease progression. This was, also, in accordance with Luedde and Schwabe [43].

Moreover, it was suggested that NF-κB activation is directly associated with TNF-α up regulation, which has an accelerator effect on cell proliferation and induces inflammatory mediators and proteases that orchestrate inflammatory responses and acts as an endogenous tumor promoter [44]. This is comparable with the results of the current work that revealed an increase of the inflammatory cytokines in all studied patients relevant to controls and its significant gradual rising with disease progression. TLR3 and TLR9 monocyte expression was positively correlated with TNF-α, IL-1β and IL-6 in all diseased cases pointing to the association of inflammation with fibrosis and cancer development. It was found that secretion of IL-1β into the tumor milieu induces tumor growth through hyper neo-vascularization, while IL-6 acts as a paracrine growth factor for tumors [26].

Despite the significant correlation detected between TLR3 and TLR9 expression on both liver tissue and monocytes, yet the ROC curve analysis proved that monocyte expression of these two receptors could not be a reliable substitute solid enough to replace evaluation of their expression on liver tissue.

The present work has succeeded to identify the role played by TLR3 and TLR9 in initially triggering an inflammatory ambiance via NF-κB activation and later by participating in inflammation induced fibrosis and carcinogenesis. Thus, the role of NF-κB and its inhibitors as potential anti-cancer therapy and TLRs as possible signal initiators for NF-κB activation for inflammation-induced carcinogenesis should be intensively investigated and assessed.

It could be concluded that inhibitors of TLRs and anti-inflammatory agents that suppress NF-κB or NF-κB regulated products might represent a promising strategy to intercept the potentially fatal fibrosis–carcinogenesis sequence. In fact, TLRs and cancer biology is a rich opportunity to discover paradigms and develop research in the field of diagnosis and therapy. Exploring the TLRs signaling pathway for cancer immunotherapy and vaccines may be a very promising step towards cancer prevention and management.

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