BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2021, VOL. 78, NO. 1, 18–22
https://doi.org/10.1080/09674845.2020.1778842

Toll-like receptor 7 mRNA is reduced in hepatitis C-based liver cirrhosis and hepatocellular carcinoma, out-performs alpha-fetoprotein levels, and with age and serum aspartate aminotransferase is a new diagnostic index

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ABSTRACT

Background: Hepatitis B and C viruses are leading causes of liver cirrhosis and hepatocellular carcinoma (HCC). Toll-like receptor 7 (TLR-7) has been implicated in the pathogenesis of HCC linked to hepatitis B. We hypothesised a role of leukocyte TLR-7 mRNA in hepatitis C related liver cirrhosis and HCC, using alpha-fetoprotein (AFP) and liver function tests as comparators.
Methods: We recruited 102 patients with HCV-related HCC, 97 with HCV-related liver cirrhosis and 60 healthy controls. Quantification of TLR-7 mRNA was performed using real-time PCR, AFP and routine LFTs by standard techniques.
Results: TLR-7 mRNA levels were significantly lower in HCC patients compared to cirrhotic patients and lower again in healthy controls ($p < 0.001$ for trend). In multivariate analysis, age, aspartate transaminase (AST), AFP, and TLR-7 mRNA were significant predictors of HCC. The ROCC/AUC for age, AST and TLR-7 mRNA were all between 0.64 and 0.78 (all $P < 0.01$), but for AFP was 0.57 (95% CI 0.48–0.65, $P = 0.09$). We derived an index score using age, AST and TLR-7 mRNA for the diagnosis of HCC. The ROCC/AUC for the index was superior to all three root indices in the prediction of HCC. The index linked significantly with the Tokyo and Vienna liver cancer staging systems, but not with those of the CLIP and Okuda systems, in distinguishing HCC from liver cirrhosis.
Conclusion: The combination of TLR-7 mRNA levels with age and AST improves the performance of TLR-7 in HCC diagnosis, out-performs alpha-fetoprotein and predicts early HCC.

ARTICLE HISTORY
Received 14 March 2020
Accepted 1 June 2020

KEYWORDS
Liver cancer; diagnosis; Toll-like receptor-7; index

Introduction

Hepatitis C virus (HCV) is a principal cause of chronic liver diseases and hepatocellular carcinoma (HCC) [1,2]. Although mechanisms of HCC development from HCV infection remain unclear, several cellular changes that drive liver carcinogenesis had been described. The role of non-invasive tools in the early detection of HCC has resulted in the identification and evaluation of several novel markers [1–3]. Early diagnosis is important for improving the HCC survival rate [3]. Alpha-fetoprotein (AFP) and imaging are routinely used in the screening of liver cirrhosis patients every 6–12 months [2], but the sensitivity and specificity of AFP for early diagnosis of HCC are not fully acceptable [3], whilst imaging techniques are influenced by the apparatus model and the expertise of the analyst [4]. The diagnostic performances of imaging techniques are low with 35% sensitivity [5].

In the last decade, novel markers such as cytokines and oncogenes had been assessed as potential tools for HCC diagnosis [6]. Toll-like receptors (TLRs) have an important role in innate immunity and are over-expressed by leukocytes in viral and inflammatory diseases [7]. Ning et al. [8] reported decreased TLR-7 and TLR-9 mRNA in hepatitis B infection, the reduced expression of TLR-7 in hepatitis B infection liver cirrhosis and HCC, and an inverse correlation between TLR-7 protein and serum copies of HBV. Similarly, Kataki et al. [9] reported altered expression of mRNAs for TLR2 and TLR3 in chronic hepatitis B infection, cirrhosis and HCC. These and other data prompted us to extend these data by testing the hypothesis of altered expression of TLR-7 mRNA in these conditions when on a background of HCV infection. We also sought to determine the potential of TLR-7 as a laboratory marker (in comparison with routine markers), and any links with clinical disease staging.

Materials and methods

We tested our hypothesis in a cohort of 102 patients with HCV-related HCC, 97 with HCV-related liver cirrhosis free

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of HCC and healthy controls, who were enrolled from August 2018 to October 2019. The patients were recruited from the Endemic Medicine Department, Cairo University Hospitals, Cairo, Egypt; and the Oncology Department, Ismailia Teaching Hospital, Ismailia, Egypt. All participants signed informed consents and they were fully aware of the used diagnostic tools. The number of men was 41 (68.3%), 54 (55.7%) and 70 (68.8%) in the healthy controls, liver cirrhosis cases and HCC cases, respectively (chi-squared $P = 0.13$). The mean [SD] age of studied groups was 44.7 [17.2], 54.8[10.1] and 60.2 [8.1] for the healthy controls, liver cirrhosis patients and HCC patients ($P < 0.01$). All patients were positive for HCV markers (antibody and HCV-RNA).

HCC diagnosis was by the American Association of the Study of Liver Disease (AASLD) Practice Guidelines. HCC tumour size was evaluated using triphasic computed tomography (CT) and/or dynamic MRI. Clinical staging of HCC was performed according to the Cancer of Liver Italian Program (CLIP), Tokyo, Okuda and Vienna systems [10]. Diagnosis of liver cirrhosis was based on non-invasive methods including laboratory and ultrasonography, and MELD (model for end-stage liver disease) scores were calculated. Patients with viral infections other than HCV (HAV, HBV, HIV), bilharzial infection, autoimmune liver, patients with chronic kidney diseases, cardiovascular diseases, rheumatoid arthritis, patients with malignancies other than HCC and patients undergoing chemotherapy and radiotherapy prior to blood sampling collection were excluded. Healthy controls were not suffering from diabetic or hypertensive disease, were negative for HBV and HCV viral markers and their abdominal ultrasound examinations were free of pathology.

After clinical diagnosis and before antiviral therapy, fasting blood samples were collected from all subjects for liver function tests, prothrombin time (as INR) and alpha-fetoprotein (AFP). Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll density centrifugation and sedimentation. RNA was separated from PBMCs using QIAamp viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany). Quantification of TLR-7 mRNA (Hs01933259_s1); Human Catalogue number: 4453320 was performed using TaqMan Gene Expression (Applied Biosystems Inc, Foster City, CA, USA). β-actin was the reference gene in three cohorts for each sample. Fractional threshold cycles (C_T) expressed the initial concentration of the target sequence. Relative mRNA quantification was calculated using the arithmetic formula $2^{-\Delta C_T}$, where $^{\Delta C}{}_T$ is the difference between the C_T of a given target cDNA and an endogenous reference cDNA. Thus, this value yielded the amount of the target normalized to an endogenous reference [11]. The study protocol followed the ethical guidelines of the 1975 Helsinki Declaration.

Using SPSS software (V22), clinical and laboratory data were statistically analysed. Data are presented as mean with SD or numbers (%). The chi-square test or Fisher's exact test was used for category variables, differences according to disease severity by linear trend estimation. Tukey's post-hoc test, Student's t-test or Mann–Whitney U test was used for between-group comparisons. A two-sided P value <0.05 was an indicator of significant differences. Univariate and multivariate logistic regression analyses were used to choose the best candidate markers for HCC diagnosis. Significant variables ($p < 0.05$) at multivariate logistic regression were used to create a novel index aiming at diagnosis of HCC. The diagnostic performances of variables were evaluated by Receiver-operator characteristic (ROC) area under the curve (AUC) with 95% confidence intervals (CIs). These selected the best cut-off value, from which sensitivity, specificity and positive and negative predictive values were calculated.

Results

Laboratory results are shown in Table 1. Unsurprisingly, with the exception of total bilirubin, all routine markers varied in a linear trend with that of disease severity, i.e. healthy control – liver cirrhosis – HCC (all $P < 0.001$). Falling TLR-7 mRNA levels also followed this linear trend. In between-group analyses, in all cases, levels of each index were significantly different between healthy controls and HCC patients (all $P < 0.001$), and between healthy controls and cirrhotic patients (all $P < 0.01$). There were significant differences in alanine aminotransferase (ALT) aspartate aminotransferase (AST), the platelet count, AFP and TLR-7 mRNA between the patient groups (all $P < 0.05$). These differences remained when the healthy controls and liver cirrhosis were combined (Figure 1A). The MELD scores for liver cirrhosis and HCC were 17.2 [8.5] and 19.2 [9.5], respectively ($P = 0.134$). Age and indices with a significant trend were fed into a multivariate logistic regression analysis, which found that age, AST, AFP, and TLR-7 were all independent predictors of liver cirrhosis and HCC (p = 0.03–0.0001). ROC analysis was used to assess

Table 1. Laboratory findings in the three groups.

Variables	Healthy controls ($n = 60$)	Liver cirrhosis ($n = 97$)	HCC ($n = 102$)
Aspartate aminotransferase (U/L)	33 [8]	84 [70]	110 [70]
Alanine aminotransferase (U/L)	31 [5]	47 [4]	65 [32]
Platelet count (10^9/L)	235 [74]	145 [72]	120 [54]
Albumin (g/dL)	38 [2]	34 [8]	29 [6]
Total bilirubin (μmol/L)	14 [4]	97 [14]	96 [9]
INR	1.0 [0.1]	1.4 [0.6]	1.5 [0.3]
α-Fetoprotein (U/L)	6 [2]	215 [48]	908 [202]
Toll-like receptor-7	18.3 [4.2]	8.8 [0.99]	3.0 [0.23]

Data mean [SD]. INR: international normalized ratio. All indices except total bilirubin show significant linear trend (all $P < 0.01$). See text for inter-group differences.

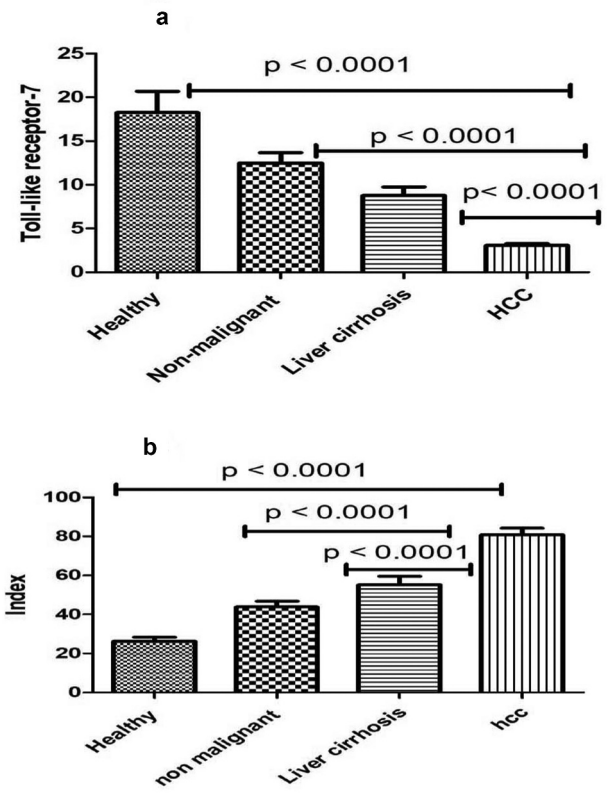

Figure 1. (a) Level of Toll-like receptor7 mRNA in different group studied. Error bar is SD. HCC = hepatocellular carcinoma. (b) Level of index in different group studied. Error bar is SD. HCC = hepatocellular carcinoma.

the diagnostic power of significant variables. The AUCs for age, AST and TLR-7 mRNA were all able to differentiate the three groups (Table 2), but although AFP was able to discrimination of HCC from the healthy controls (AUC 0.68 (95% CI 0.61–0.65), $P < 0.001$) it was unable to differentiate liver cirrhosis from HCC (AUC 0.57 (0.48–0.65), $P = 0.09$).

We hypothesised that combining the three most significant indices (age, AST, TLR-7 mRNA) would provide superior discrimination, and modelled an index based on TLR-7 mRNA for early diagnosis of HCC. This modelling generated an index from Age (years) x 0.526 + AST (U/L) x 0.464 – TLR-7 x 0.699. The levels of the index increased with the severity of liver diseases. The mean [SD] of the index in healthy controls was 26.2 [16] versus 55.3 [40] in liver cirrhosis patients and 80.8 [33.3] in those with HCC (linear trend $P < 0.001$). The index correlated with age (r = 0.33), TLR-7 mRNA (r = 0.46) and AST ($r = 0.64$)(all $P < 0.001$). Table 2 shows the performance of the index compared to each component part. In each group comparison, the index gave the highest AUC. Figure 1B shows levels of the index in the three groups, and a fourth of the non-malignant groups combined.

Table 3 shows the ability of the index to identify the stage of the HCC compared to liver cirrhosis according to the CLIP, Okuda, Tokyo and Vienna staging systems. The AUC increased in a linear manner with the Tokyo and Vienna stages, but not the CLIP or Okuda staging systems.

Discussion

Hepatocarcinogenesis is a multistage process, and aberrant TLR signalling may contribute to HCC development and progression. Like others, we found differences in many laboratory indices in a cross-sectional analysis of HCC and liver cirrhosis, and to this, we add decreasing levels of TLR-7 mRNA as the disease spectrum deteriorates. Age and AST were significantly higher in our HCC patients. It is well known that DNA repair decreases with age and this can lead to HCC development and progression [11] whilst hepatic damage is associated with higher AST levels due to reduced clearance of AST [12,13]. Moreover, Omran et al. [14] found that having AST serum level >64 IU/L was associated with an increase in the risk of HCC development.

Toll-like receptors (TLRs) are a class of pattern recognition receptors that play an important role in the host innate immunity [15] as well as in viral infections including HCV [16]. The TLR 3 and 4 expression in patients with liver diseases is associated with HBV and HCV [17], and the association of TLR 3 and 7

Table 2. Performances of candidate markers for predicting hepatocellular carcinoma.

Variable	AUC (95% CI)	Cut-off	Sensitivity %	Specificity %	Accuracy %	PPV %	NPV %
Hepatocellular carcinoma vs. liver cirrhosis							
Age	0.64 (0.55–0.71)[a]	57	59	64	61	63	60
Aspartate aminotransferase (U/L)	0.74 (0.63–0.84)[b]	70	68	63	65	65	69
Toll-like receptor-7 mRNA	0.67 (0.59–0.75) [b]	2.2	62	59	61	62	59
Index	0.79 (0.73–0.85) [b]	55	81	70	76	75	77
Hepatocellular carcinoma vs. non-malignant cases							
Age	0.68 (0.62–0.75) [b]	57	59	67	64	54	71
Aspartate aminotransferase (U/L)	0.84 (0.79–0.88) [b]	70	68	77	74	66	79
Toll-like receptor-7 mRNA	0.72 (0.67–0.78) [b]	2.2	62	65	63	54	72
Index	0.87 (0.83–0.91) [b]	55	81	71	75	65	85
Hepatocellular carcinoma vs. healthy controls							
Age	0.75 (0.66-.0.85) [b]	57	59	72	57	78	42
Aspartate aminotransferase (U/L)	0.74 (0.63–0.84) [b]	70	68	100	72	100	54
Toll like receptor-7 mRNA	0.78 (0.72–0.88) [b]	2.2	62	73	59	79	44
Index	0.99 (0.97–1.0) [b]	50	81	98	88	99	76

AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value. [a]$P<0.01$, [b]$P< 0.001$.

Table 3. Performance of the index according to the CLIP, Okuda, Tokyo and Vienna staging systems.

	Hepatocellular carcinoma vs. liver cirrhosis					
Classification	AUC (95% CIs)	Sensitivity	Specificity	PPV	NPV	Accuracy
CLIP stage (no)						
Early (n = 13)	0.80 (0.69–0.92)	81	70	27	97	72
Intermediate (n = 53)	0.70 (0.62–0.78)	84	70	55	80	69
Advanced (n = 36)	0.92 (0.86–0.97)	100	70	55	100	79
Okuda stage (no)						
Stage I (n = 17)	0.80 (0.73–0.85)	85	70	37	100	75
Stage II (n = 38)	0.67 (0.58–0.76)	60	70	43	81	66
Stage III (n = 47)	0.87 (0.81–0.93)	94	70	60	96	78
Tokyo stage (no)						
Stage I (n = 45)	0.60 (0.57–0.75)	62	70	49	80	67
Stage II (n = 26)	0.83 (0.75–0.91)	92	70	45	97	75
Stage III (n = 31)	0.93 (0.87–0.98)	100	70	52	100	77
Vienna stage (no)						
Stage I (n = 48)	0.70 (0.62–0.79)	67	70	53	81	68
Stage II (n = 34)	0.79 (0.79–0.87)	92	70	52	96	75
Stage III (n = 20)	0.97 (0.93–0.99)	100	70	41	100	75

AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value.

expression with HCV infection has been evaluated [18]. TLR 7 is a sensor for viral single-stranded RNA (ssRNA), and down-regulation of TLR-7 expression in a hepatoma cell line, due to RNA instability, directly correlates with HCV replication, providing useful cell biology support for our data [19].

AFP is probably the gold standard laboratory marker for HCC and possible liver cirrhosis. An AFP result <400 ng/mL has been considered as an indicator for HCC; however, in one study, only around 18–40% of HCC patients had a high level of AFP, the remainder having a low level of AFP despite suffering from HCC [20]. Moreover, high AFP serum levels are not common in patients with small HCC (<5 cm) and only 30% of HCC patients have AFP serum levels >100 ng/mL, and, furthermore, up to 20% of HCC patients do not produce AFP [21]. Accordingly, there is a need for tumour markers other than AFP, and it is therefore notable that we found that TLR-7 mRNA proved to be a better discriminant of liver disease. Other potential biomarkers include AFP lectin fraction, des-γ-carboxyprothrombin, Glypican-3, squamous cell carcinoma antigen, cytokeratin, epithelial membrane antigen, nuclear matrix protein, fibronectin, thioredoxin, c-Myc, p53, fibromarkers, genetic and epigenetic markers as well as miRNAs [1,12,22–29]. However, to date, no blood markers have shown suitably high diagnostic performances for HCC.

Several researchers used the combination of several biomarkers aiming at improving HCC diagnosis because the stage of HCC dictates the therapeutic modality, making the early diagnosis a primary aim. In the current study, we developed an index based on TLR-7 mRNA, age and AST that has a sensitivity of 81% for discriminating HCC from liver cirrhosis. This value is more than that of previously studied tumour markers; AFP (61%), DCP (39%); AFP-L3 (37%); osteopontin (47%) and dickkopf-1 (69%); respectively [27]. The TLR-7-based index has high diagnostic performance in the diagnosis of early HCC according to CLIP and Okuda staging systems with an AUC of 0.8 and with a sensitivity of 81% and 85%, respectively. This is higher than AFP (70%), combined AFP and ultrasound (63%), CT (63%) but lower than simplified HCC-ART (82%) [29] and MRI imaging (84%) [30]. Nevertheless, it is notable that our index links directly with increasing stage in the Tokyo and Vienna system and so may be a viable clinical tool.

We acknowledge the limiting factors of relatively small sample sizes and that we failed to recruit a group with liver disease without HCC. However, this work represents an advance in biomedical science because it provides a three-marker index (TLR-7 mRNA with age and AST) that could improve the diagnosis of HCV-related liver cirrhosis and HCC.

Summary table

What is known about this topic

- Toll-like receptors play a crucial role in immunity against hepatic viruses
- TLR-7 may have a role in hepatitis B virus-mediated liver cancer

What this work adds:

- TLR-7 mRNA out-performs AFP in discriminating liver cirrhosis and HCC
- An index using age, AST and TLR-7 mRNA is superior to each component alone in predicting HCC.
- The index discriminates early-stage HCC from liver cirrhosis in four different cancer staging systems

Disclosure statement

The authors declare that they have no competing interests.

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