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ORIGINAL ARTICLE

Prognostic impact of toll-like receptors 2 and 4 expression on monocytes in Egyptian patients with hepatocellular carcinoma

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Abstract

Toll-like receptors (TLRs) have a role in chronic inflammation. Still, little is known about the expression of TLRs in hepatocellular carcinoma (HCC). Herein, we tried to assess the prognostic value of TLR2 and TLR4 expression on circulating monocytes in HCC patients and correlate their levels with some clinical, laboratory data, and treatment outcomes. Forty patients with hepatic focal lesions diagnosed radiologically as HCC by triphasic multislice CT pelviabdominal and chest, and in some patients MRI diffusion and 38 age and sex matching healthy controls were enrolled in the study. Subjects were evaluated for liver functions, alpha-fetoprotein (AFP), imaging, response to different treatments, and overall survival. TLR2 and TLR4 expression by monocytes was detected by flow cytometry. The expression of both TLR2 and TLR4 on monocytes was significantly increased in HCC patients than the controls, in patients with more progressive HCC than those with lower progression and in patients with poor response to treatment than patients with better treatment response. Moreover, their levels showed positive correlations with ALT, AST, and AFP and inverse correlations with the overall survival of HCC patients. The results of the current study suggest that increased expression ofTLR2 and TLR4 on peripheral monocytes might reflect the development and progression of HCC and can be used to indicate poor prognosis. In addition, high expression of TLR2 correlated significantly with poor response to treatment, while high expression of both TLR2 and TLR4 were associated with poor survival. Our findings will help to design more studies on the role of TLRs in HCC pathogenesis and prognosis which may provide new therapeutic targets for HCC.

Keywords TLR · TLR2 · TLR4 · Hepatocellular carcinoma · Inflammation · Overall survival

Abbreviations

TLRs	Toll-like receptors
HCC	Hepatocellular carcinoma
PAMP	Pathogen-associated molecular patterns s, FLR, future liver remnant
TACE	Transarterial chemoembolization

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common primary malignancy of the liver in men and the seventh most common cancer in women. Chronic HBV or HCV infection is one of the leading risk factors for HCC globally beside, non-

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alcoholic steatohepatitis, and exposure to alcohol and/or aflatoxin [1–5]. The distribution of the risk factors of HCC is highly variable according to geographic region and race or ethnic group [1]. HCC is usually associated with asymptomatic progression to advanced stages and a high rate of recurrence even after curative treatments of the primary tumors [6].

Toll-like receptors (TLRs) include a family of receptors that distinguish diverse ligands, including pathogen-associated molecular patterns (PAMPs) [7, 8]. The human TLR is made up of 11 members, possessing a leucine-rich repeat domain in their extracellular domain and a Toll/interleukin (IL)-1 receptor domain in their intracellular domain [9, 10] which is responsible for recognizing different bacterial, viral, and fungal structures [9–11].

Each TLR has specific ligands, which allow the host to sense a wide diversity of pathogens [12]. Ligand binding by TLR initiates the recruitment of adapter proteins that activate specific signaling pathways such as TANK-binding kinase 1and IL-1 receptor-associated kinase family. The recruitment of these adapters initiate pathways leading to the activation of their respective transcription factors, for the induction of inflammatory cytokine genes with subsequent release of proinflammatory cytokines such as IL-6 and tumor necrosis factor which are excellent targets for inflammatory diseases [13].

The crucial role of TLR depends on initiating two arms of the immune response, namely, the innate and the adaptive immune responses that work together to fight infections [14]. Monocytes express all TLRs, of which TLRs 1, 2, and 4 are present at high levels [15]. Additionally, T cells express all TLRs at low levels except TLR5, which is present abundantly [15, 16].

Emerging evidences points to the important role of TLR in immune and inflammatory diseases. TLR-activated tumor cells may provide sufficient signals that act as a molecular link between inflammation and cancer [17]. Previously, it was found that mice deficient in TLRs develop less inducible tumors in experimental models [18, 19].

Hepatocellular carcinoma is a good example for the link between chronic inflammation and cancer [20, 21]. The continued exposure of the liver to gut-derived bacterial products, viral infection, alcohol, or other products via portal vein may result in chronic liver damage [22–27], so increasing the risk for HCC. Subsequently, TLRs play a major role in liver physiology and pathophysiology, due to their role in the innate immune system and their significant contribution to several biological processes such as regulation of inflammation, wound healing, induction of adaptive immune responses, promotion of epithelial regeneration, and carcinogenesis [28]. Activation of TLRs was reported to be involved in the progression of the inflammation-fibrosis-HCC axis [29–31].

So far, little is known about the expression of TLRs in HCC patient. Therefore, we aimed in this study to assess the prognostic value of TLR2 and TLR4 expression on circulating monocytes in HCC patients and correlate their levels with clinical and laboratory data.

Materials and methods

This study was a case-control study. The study population was 40 patients diagnosed with HCC. The patients were recruited to Assiut University Hospitals and South Egypt Cancer Institute. In addition, 38 healthy individuals of comparable age and sex were enrolled as controls.

The study was approved by the institutional review board and all patients provided written informed consent before enrollment.

All cases were diagnosed on clinical basis, with imaging and pathological confirmation of the tumor (if needed).

All patients and controls were subjected to complete blood count (CBC) that was done by fully automated blood counters (Celltac E automated hematology analyzer, Tokyo, Japan). Also, serum albumin and liver function tests were evaluated using Cobas Integra 400 Chemistry Analyzer (Roche Diagnostics GmbH, Mannheim, Germany). In addition, alpha-fetoprotein (AFP) was done for all patients by Access 2 (Beckman Coulter, USA).

Flow cytometric detection of monocyte subsets and their TLR2 and TLR4

One hundred microliters of blood sample was stained with 10 μl of each of fluoroisothiocyanate (FITC)-conjugated CD282 (TLR2), phycoerythrin (PE)-conjugated CD284 (TLR4), and peridinium-chlorophyll-protein (Per-CP)-conjugated-CD14 (all from Becton Dickinson (BD) Biosciences, San Jose, CA, USA, except CD284 from eBiosciences, San Diego, CA, USA). After incubation for 20 min at 4 °C in the dark, red blood cells lysis was done. The cells were suspended in phosphate buffer saline (PBS) and analyzed by FACSCalibur flow cytometer with Cell Quest software (BD Biosciences, USA). About 20,000 events were acquired. Anti-human IgG isotype-matched negative control was used with each sample. Scatter was used to define the monocytes population. Then, monocytes were gated using the expression of CD14. The expression of TLR2 and TLR4 were assessed on monocytes as a geometric mean of fluorescence intensity (MFI) (Fig. 1).

Diagnosis and treatment plans for HCC patients in the study

The diagnosis of HCC depends mainly on triphasic multiphasic CT or MRI of the abdomen with contrast for nodules ≥ 10 mm using Liver Imaging Reporting and Data System (LI-RADS) criteria that include arterial phase enhancement,

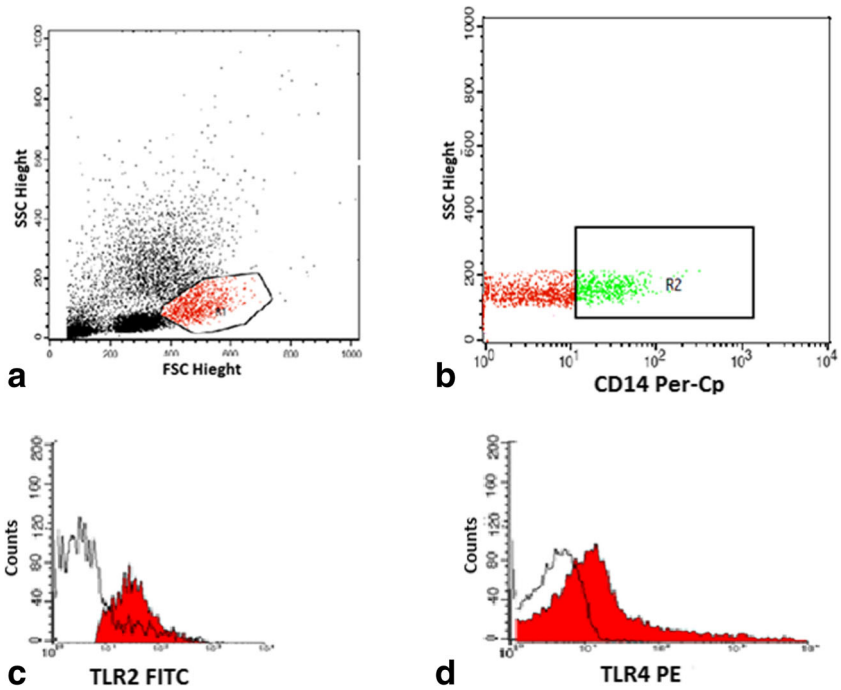

Fig. 1 Flow cytometric detection of TLR2 and TLR4expressionon monocytes. **a** Forward and side scatter histogram was used to detect monocytes population (R1). **b** Then, the expression of CD14 was assessed on monocyte population and then gated for further assessment ofTLR2 and TLR4. **c**, **d** The expression of TLR2 and TLR4 on $CD14^{+}$monocytes.The positivity was defined as fluorescence (red histogram) higher than that of the isotype control (open histogram)

venous or delayed porto-venous phase washout appearance, enhancing capsular appearance, and threshold growth. Hepatic nodules that did not fulfill these criteria underwent biopsy.

All patients were evaluated by abdominal U/S, triphasic multislice CT pelviabdominal and chest, and in some patients MRI diffusion abdomen, PET/CT scan (because of limited sensitivity of PET/CT, it was not used for diagnosis but for metastatic workup), and bone scan for proper metastatic work up and proper volumetric study for remnant liver tissue were done.

All HCC patients enrolled in the study were clinically and radiologically evaluated for the possibility of surgical resection based on adequate liver function; Child-Pugh class A without portal hypertension, solitary lesion without major vascular invasion, and adequate future liver remnant (FLR) with at least 20% without liver cirrhosis or at least 30–40% with Child-Pugh class A, in addition to adequate vascular and biliary inflow/outflow.

Three patients underwent non-anatomical wide local resection, Two patients underwent anatomical segmental resection, 2 patients were submitted to left hepatectomy, and 18 patients were inoperable due to medical comorbidity, multiplicity of lesions, or metastasis which made patients unfit for surgery.

Inoperable patients were treated either with curative intent through TACE (15 patients), and RFA (5 patients) given to those with solitary mass not more 5 cm or 2–3 lesions each not more than 3 cm, or palliative intent through systemic therapy by sorafinib (5 patients) given to those with Child-Pugh A or B ECOG PS ≤ 2. Although HCC is minimally responsive to systemic chemotherapy, capecitabine and GEMOX regimens were given to some patients (five patients). Supportive treatment was only offered to this difficult population in an effort to provide care for those with Child class C cirrhosis and contraindications for transplantation (three patients); various hormonal and biologic agents have been tried with minimal success, including tamoxifen, antiandrogens (e.g., cyproterone and ketoconazole), and octreotide, and our treatments focused mainly on pain control, ascites, edema, and portosystemic encephalopathy management (Fig. 2).

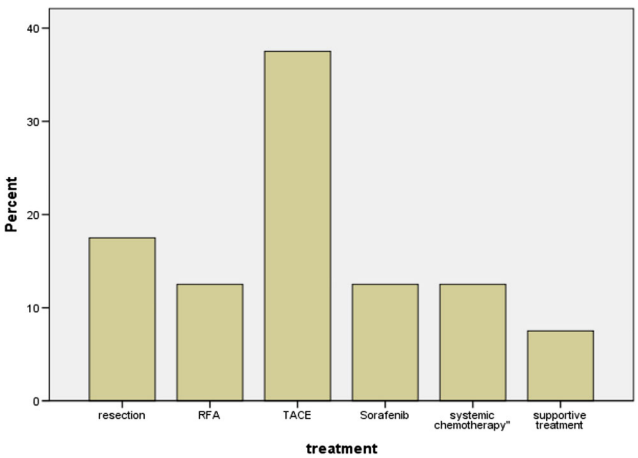

Fig. 2 Different types of treatment for HCC patients

Table 1 Laboratory characteristics of HCC patients and controls

Variable	Patients (40)	Controls (38)	*p* value
White blood cell count(10^9/L)	6.79± 0.3	7.66 ± 0.31	0.05
Hemoglobin (gm/dl)	9.40 ± 0.35	12.23 ± 0.196	*< 0.001*
Platelet count(10^9/L)	86.70 ± 5.17	253.46 ± 10.03	*< 0.001*
Total bilirubin (mg/dL)	2.90 ± 0.40	0.92 ± 0.067	*0.007*
Direct bilirubin (mg/dL)	1.83 ± 0.35	0.39 ± 0.036	*< 0.001*
Albumin (gm/L)	3.38 ± 0.14	4.47 ± 0.11	*< 0.001*
ALT (U/L)	122.03 ± 13.71	16.39 ± 0.48	*< 0.001*
AST (U/L)	105.66 ± 9.04	16.95 ± 0.46	*< 0.001*
AFP(ng/ml)	3398.8 ± 826.49	–	–

Data represented as means ± SEM. Independent *t* test, $p < 0.05$ is significant

ALT alanine transaminase, *AST* aspartate transaminase, *AFP* alpha-fetoprotein

Follow up

Traditionally, therapeutic response has been assessed by serial imaging of tumor burden according to either RECIST, WHO, or EASL criteria that based mainly on repeated tumor size measurement. However, in the setting of liver-directed therapies, and targeted therapies simple anatomical size changes are less informative, so reduction of tumor vascularity and tumor necrosis may not be associated with size shrinkage; these factors were assessed by contrasted dynamic CT or MRI that were done 1 month after surgical resection and ablative therapies, then every 2–3 months. Also, assessment of response to TKI (sorafenib) was done every 2 months but assessment of toxicity was done monthly that necessitated repeated CBC and blood chemistries; assessment continued to determine the overall survival of these patients. The range of follow-up period was 2–33 months with a mean of 10.487 months.

Patients were categorized according to the response to treatment as follows: complete response (CR); all target lesions gone, partial response (PR); ≥ 30% decrease from baseline, progressive disease (PD); ≥ 20% increase from smallest sum of longest diameter recorded since treatment started (best response), stable disease (SD); neither PD nor PR.

Statistics

The study was designed mainly to test the null hypothesis that toll-like receptors had no impact on treatment response and overall survival. Also, they were not correlated with different clinical and laboratory characteristics in HCC patients.

Descriptive data in the form of mean, median, range, standard deviation, and percentages were used; Mann-Whitney *U* test and independent *t* test were used to find a relation between two groups of quantitative variables, chi square test between ≥ 2 groups of categorical variables, and one-way ANOVA test for the relation between categorical and quantitative variables, and all of these relations were considered significant at *P* value < 0.05. Pearson correlation was used to determine the relations and magnitude of these relations between TLRs and different treatment outcomes. Kaplan-Meier was used for determination of the median overall survival and Log-rank test for determination of the impact of different prognostic factors on survival. Overall survival (OS) was defined as the interval from diagnosis to the date of death from any cause or last follow up. And all our results were calculated using SPSS ver. 21.

Results

The baseline laboratory characteristics of HCC patients and the controls (Table 1)

Both the hemoglobin concentration and platelet count were lower in HCC patients compared with controls. On the other hand, no significant difference was observed in WBC count between HCC patients and the controls.

The liver functions were significantly affected in HCC patients. The total and direct bilirubin, ALT, and AST were significantly increased in patients than in the controls. Albumin level was significantly lower in patients than in the controls.

Monocytes and their expression of TLR2 and TLR4 in HCC patients and the controls (Table 2)

Blood monocytes in HCC patients were significantly higher than in controls, with *p* value < 0.001. Also, the expression of both TLR2 and TLR4 on monocytes was significantly increased in HCC patients compared to the controls (*p* value < 0.001).

Table 2 Monocytes subsets and their TLR2 and TLR4 in hepatocellular carcinoma patients and the controls

Variable	Patients (40)	Controls (38)	*p* value
Monocytes (%)	11.34 ± 0.68	7.94 ± 0.39	*< 0.001*
TLR2 (%)	89.04 ± 7.91	45.39 ± 2.69	*< 0.001*
TLR4 (%)	103.29 ± 8.15	50.43 ± 2.26	*< 0.001*

Data represented as means ± SEM. Independent *t* test, $p < 0.05$ is significant

TLR toll-like receptor

Laboratory characteristics of patient groups according to hepatic focal lesions (Table 3)

According to hepatic focal lesions, our patients were divided into five groups.

- Group 1 included 9 patients with single focal lesion less than 5 cm
- Group 2 included 12 patients with single focal lesion more than 5 cm
- Group 3 included 5 patients with two focal lesions less than 5 cm
- Group 4 included 6 patients with two focal lesions more than 5 cm
- Group 5 included 8 patients with multiple focal lesions

We found that some laboratory data were significantly increased in patients with the increase in the number of hepatic focal lesions.

Patients in groups 3, 4, and 5 showed significantly higher levels of ALT, AST, and AFP than did patients in groups 1 and 2, with p value < 0.001 for ALT and AFP and p value = 0.02 for AST.

Also, the expression of TLR 2 on monocytes was significantly increased in patients of groups 3, 4, and 5 than those in groups 1 and 2, with p value = 0.02. The expression of TLR 4 on monocytes was significantly increased in patients of groups 4 and 5 than in patients in groups 1, 2, and 3, with p value < 0.001 implicating that TLR 4 was associated with higher HCC stages.

There were no significant differences in hemoglobin concentration, and WBC and platelets counts between the 5 groups. Also, there were no significant differences in levels of bilirubin, albumin, and blood monocytes between the groups.

Correlation between monocyte subsets and their TLR2 and TLR4 in hepatocellular carcinoma patients and some investigated parameters (Table 4)

There were strong positive correlations between levels of TLR 2 and TLR 4 on monocytes and ALT, AST, and AFP. While, there was negative correlation between albumin level and expression of TLR 4 on monocytes.

However, there were no significant correlations between the levels of monocytes, TLR2 and TLR4 with hemoglobin concentration, WBC count, platelet count, and bilirubin level in HCC patients (data not shown).

Relation between the mean percentages of TLRs 2 and 4 and treatment response (Table 5)

The mean percentages of TLR 2 and 4 were higher among HCC patients with poor response to treatment (170.5 ± 8

Table 3 Laboratory characteristics of patients groups according to hepatic focal lesions

Variable	Group 1 (9)	Group 2 (12)	Group 3 (5)	Group 4 (6)	Group 5 (8)	*p* value
Hemoglobin (gm/dl)	8.92 ± 0.82	9.58 ± 0.58	10.10 ± 1.22	9.074 ± 1.72	9.28 ± 0.60	0.9
Platelet count(10^9/L)	74.69 ± 8.899	95.49 ± 10.96	88.20 ± 11.56	74.77 ± 13.64	87.27 ± 8.50	0.6
Total bilirubin (mg/dL)	2.61 ± 1.22	1.84 ± 0.74	2.20 ± 0.61	2.99 ± 0.94	1.51 ± 0.31	0.9
Direct bilirubin (mg/dL)	2.30 ± 1.075	1.63 ± 0.65	1.93 ± 0.54	2.63 ± 0.83	1.33 ± 0.28	0.9
Albumin (gm/L)	3.55 ± 0.28	3.59 ± 0.28	3.25 ± 0.24	2.67 ± 0.28	3.18 ± 0.32	0.5
ALT (U/L)	105.20 ± 29.53	71.17 ± 9.61	166.94 ± 39.13	256.84 ± 10.60	157.70 ± 33.69	<0.001
AST (U/L)	99.87 ± 20.81	73.47 ± 10.81	134.86 ± 17.59	168.93 ± 9.83	130.57 ± 22.29	0.02
AFP (ng/ml)	39.14 ± 20.05	267.71 ± 26.56	1717.7 ± 578.00	7038.9 ± 1961.28	12,735.0 ± 800.64	<0.001
Monocytes (%)	13.13 ± 1.12	11.07 ± 0.85	12.24 ± 2.42	12.73 ± 2.20	8.73 ± 2.0974	0.3
TLR 2 (%)	74.98 ± 16.86	65.61 ± 9.72	106.25 ± 29.06	140.47 ± 4.51	118.75 ± 15.76	0.02
TLR 4 (%)	87.97 ± 17.99	82.51 ± 9.72	69.03 ± 7.20	129.74 ± 21.03	170.98 ± 8.02	<0.001

Group 1: 9 patients with single focal lesion less than 5 cm. Group 2: 12 patients with single focal lesion more than 5 cm. Group 3: 5 patients with two focal lesions less than 5 cm. Group 4: 6 patients with two focal lesion more than 5 cm. Group 5: 8 patients with multiple focal lesions. Data represented as means ± SEM. One-way ANOVA, $p < 0.05$ is significant

ALT alanine transaminase, *AST* aspartate transaminase, *AFP* alpha-fetoprotein, *TLR* toll-like receptor

Table 4 Correlations between monocytes and their TLR2 and TLR4 in hepatocellular carcinoma patients with some of the investigated parameters

Parameters		Monocytes	TLR 4	TLR 2
ALT (U/L)	r	− 0.2	*0.7*	*0.7*
	p value	0.3	*< 0.001*	*0.002*
AST (U/L)	r	− 0.2	*0.7*	*0.7*
	p- value	0.3	*< 0.001*	*0.002*
AFP (ng/ml)	r	− 0.3	*0.7*	*0.8*
	p value	0.2	*< 0.001*	*< 0.001*
OS	r	+ 0.142	*− 0.7*	*− 0.4*
	p value	0.383	*< 0.001*	*0.002*
Albumin (gm/L)	r	0.1	− 0.4	− 0.3
	p value	0.7	0.05	0.2

Pearson correlation; r Pearson's correlation coefficient, significant *p* value <0.05

ALT alanine transaminase, *AST* aspartate transaminase, *AFP* alpha-feto-protein, *TLR* toll-like receptor, *OS* overall survival

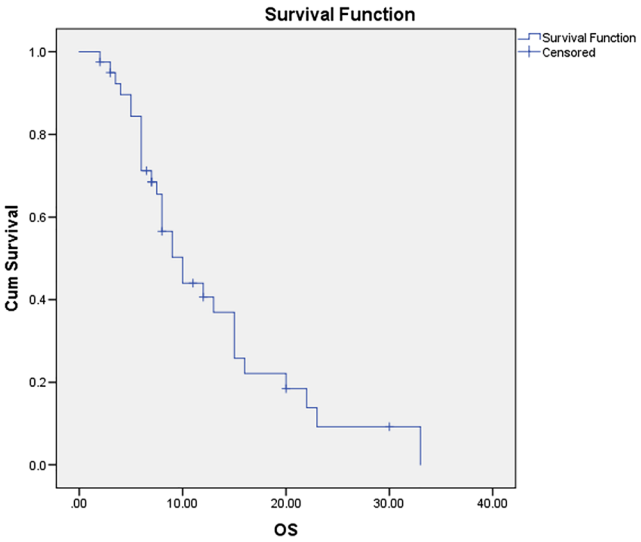

Fig. 3 The median OS of all HCC patients was 10 months (95% CI 7.4–12.6)

and 118.5 ± 18 in patients with PD and 114.1 ± 10 and 90 ± 10 in patients with SD, respectively) than among HCC patients with good response to treatment (43.1 ± 3and 71 ± 19 in patients with CR and 87 ± 11and 79 ± 19 in patients with PR, respectively). The difference was significant for TLR 2 ($p < 0.0001$) but not for TLR 4 ($p = 0.2$).

Analysis of the overall survival among HCC patients

The mean overall survival (OS) of our HCC patients was 12.7 ± 1.5 months and the median was 10 months (95% CI 7.4–12.6) (Fig. 3).

TLR2 and TLR4 have shown moderate to strong negative correlations with the mean OS ($r = -0.7$, $p < 0.001$ and $r = -0.4$, $p = 0.002$, respectively) (Table 4 and Fig. 4). Furthermore, linear regression analysis of factors affecting OS has shown that TLR4 was the most influential factor on OS followed by TLR2 (Table 6).

Table 5 Relation between the mean percentages of TLRs 2 and 4 and treatment response

	CR $N = 9$	PR N = 9	SD $N = 14$	PD $N = 8$	*p* value
TLR2 (%)	43.1 ± 3	87 ± 11	114.1 ± 10	170.5 ± 8	< 0.0001
TLR4 (%)	71 ± 19	79 ± 19	90 ± 10	118.5 ± 18	0.2

Data expressed as mean percentages; one-way ANOVA test for significance

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease

Discussion

Liver functions, indicating patient overall status, are useful prognostic factors of poor outcome in advanced cancer patients. Consistent with previous studies [32–37], the liver functions of our patients were significantly affected. The total and direct bilirubin, ALT, and AST were significantly increased in HCC patients than in the controls. Groups of patients with more advanced hepatic focal lesions (group 3, 4, and 5) showed significant increase in the levels of ALT and AST than did the other groups. Moreover, serum albumin levels of our HCC patients were significantly decreased compared to the controls.

The overexpression of AFP in human HCC may be useful not only in the diagnosis but also in the prognosis and follow-up, beside screening and monitoring of the treatment responses in HCC [38–40]. However, serum AFP values are influenced by non-neoplastic factors, such as the presence of viral hepatitis or cirrhosis [41]. Our patients showed high levels of AFP with significant increase in groups with more advanced hepatic focal lesions (group 3, 4, and 5) than the in other groups.

The breakdown of the gut barrier and translocation of intestinal bacteria and bacterial pathogen-associated molecular patterns (PAMPs) may trigger chronic inflammation which is the major contributor to hepatocellular carcinomas [7, 22, 42–44]. TLR4 promote liver's inflammatory response which may result in hepatocarcinogenesis [45] and hepatic fibrosis [46, 47] through binding to microbial lipopolysaccharide (LPS-TLR4) [29, 42, 48]. Intestinal microbiota and TLR4 were not required only for HCC initiation, but also for HCC progression, mediating increased proliferation, production of proinflammatory cytokines (TNF-a, IL-6), expression of the hepatomitogen epiregulin, and prevention of apoptosis. Gut

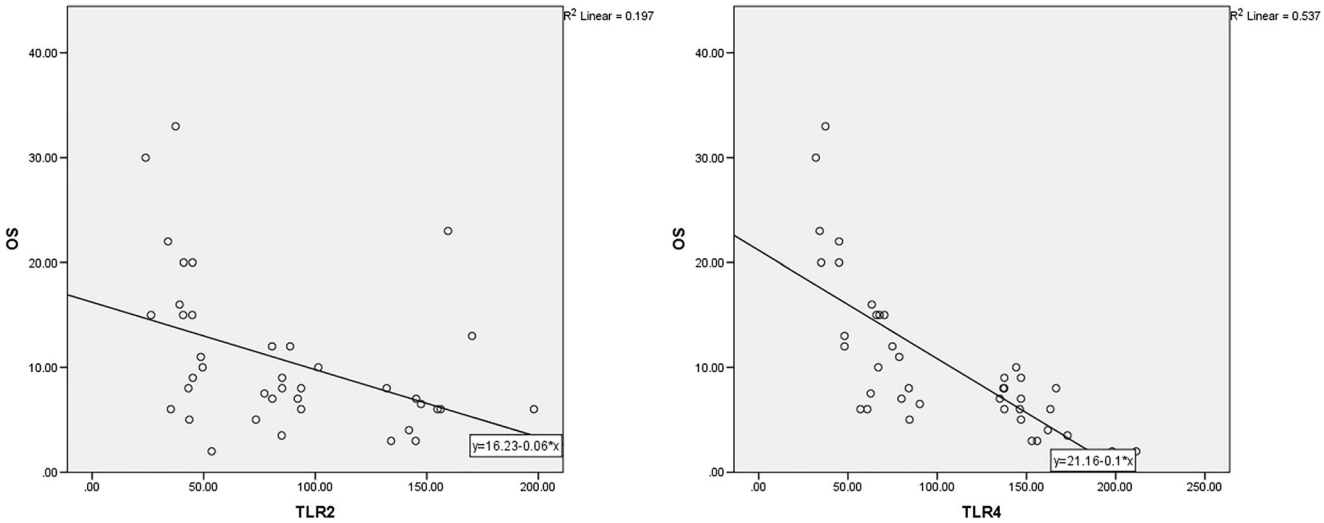

Fig. 4 Correlations between the expression of TLR2 and 4 on monocytes with the mean overall survival duration among HCC patients

sterilization, or TLR4 inactivation, significantly reduced the development of HCC [30]. Mice deficient in TLR4 and myeloid differentiation factor (MyD88), but not TLR2, have marked decreases in the incidence, size, and number of chemical-induced liver cancer, indicating a strong contribution of TLR signaling to hepatocarcinogenesis [11, 49]. Therefore, it seems clear that TLRs play a role in the inflammation-associated liver cancer development. This is consistent with our results; the expression of TLR 4 on monocytes was significantly higher in patients than in controls, in patients with more progressive HCC than in those with lower progression and also in patients with poor response to treatment than in those with good response.

Besides its known role in chronic inflammation, TLR2 signaling also mediates immune evasion of tumor cells and tumor advancement [50]. Still, its role in HCC progression remains obscure. In line with previous reports [50, 51], our HCC patients have shown significantly increased expression of TLR 2 on monocytes compared with controls. Its expression was also higher in patients in groups 3, 4, and 5 than in patients in groups 1 and 2, in addition to patients with poor response to treatment than to patients with better treatment response.

Moreover, we detected significant direct correlations between levels of TLR 2 and TLR 4 on peripheral monocytes and ALT, AST, and AFP, in addition to significant inverse correlations between levels of TLR 2 and TLR 4 on monocytes and the overall survival of HCC patients. These results were consistent with those of Zhe et al. [51] who studied the expression levels of TLR2 and TLR4 on HCC cells. Bo Hao et al.'s [49] meta-analysis proved that high expression of TLR4 was significantly associated with poor OS (pooled HR = 2.05; 95% CI (1.49–2.49, $P < 0.001$), and our results came in agreement with the previous one implicating that TLR4 is a novel prognostic biomarker that could potentially help to improve treatment decision-making of many solid tumors including HCC.

Table 6 Linear regression analysis of some factors associated with overall survival

	Beta	*p* value
TLR4	− 0.7	< 0.0001
TLR2	− 0.4	0.03
AST	0.2	0.5
ALT	− 0.1	0.7
Albumin	− 0.09	0.6
AFP	0.1	0.4

Significant *p* value <0.05

ALT alanine transaminase, *AST* aspartate transaminase, *AFP* alpha-fetoprotein, *TLR* toll-like receptor

Study limitations

The expression of TLR2 and TLR4 was investigated in the peripheral blood only. The expression level should have been investigated in the liver tissue to give a real image for what is happening on the tumor microenvironment.

Conclusion

The results of the current study suggest that increased expression ofTLR2 and TLR4 on peripheral monocytes might reflect the development and progression of HCC and can be used to indicate poor prognosis. In addition, high expression of TLR2 correlated significantly with poor response to treatment, while high expression of both TLR2 and TLR4 were associated with poor survival. Our findings will help to design more studies on

the role of TLRs in HCC pathogenesis and prognosis which may provide new therapeutic targets for HCC.

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Compliance with ethical standards

The study was approved by the institutional review board and all patients provided written informed consent before enrollment.

Conflict of interest The authors declared that they have no conflict of interest.

References

1. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology. 2012;142:1264–1273.e1.
2. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. Hepatology. 2003;37:429–42.
3. Chaturvedi VK, Singh A, Dubey SK, Hetta HF, John J, Singh MP. Molecular mechanistic insight of hepatitis B virus mediated hepatocellular carcinoma. Microb Pathog. 2019;128:184–94.
4. Zahran AM, Abdel-Meguid MM, Ashmawy AM, Rayan A, Elkady A, Elsherbiny NM, et al. Frequency and implications of natural killer and natural killer T cells in hepatocellular carcinoma. Egypt J Immunol. 2018;25:45.
5. Hetta HF. Impact of hepatitis B viral load and liver histopathology on the decision to treat chronic hepatitis B patients with persistent normal alanine transaminases. EC Microbiol. 2016;4:647.
6. Miura K, Ishioka M, Minami S, Horie Y, Ohshima S, Goto T, et al. Toll-like receptor 4 on macrophage promotes the development of steatohepatitis-related hepatocellular carcinoma in mice. J Biol Chem. 2016;291:11504–17.
7. Seki E, De Minicis S, Gwak G-Y, Kluwe J, Inokuchi S, Bursill CA, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest. 2009;119:1858.
8. Wiest R, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. Hepatology. 2005;41:422–33.
9. Moynagh PN. TLR signalling and activation of IRFs: revisiting old friends from the NF-κB pathway. Trends Immunol. 2005;26:469–76.
10. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4:499–511.
11. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. Hepatology. 2008;48:322–35.
12. Dolganiuc A, Garcia C, Kodys K, Szabo G. Distinct toll-like receptor expression in monocytes and T cells in chronic HCV infection. World J Gastroenterol: WJG. 2006;12:1198.
13. O'Neill LA, Bryant CE, Doyle SL. Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev. 2009;61:177–97.
14. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell. 1998;2:253–8.
15. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, et al. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002;168:4531–7.
16. Gelman AE, Zhang J, Choi Y, Turka LA. Toll-like receptor ligands directly promote activated CD4+ T cell survival. J Immunol. 2004;172:6065–73.
17. Eiró N, Altadill A, Juárez LM, Rodríguez M, González LO, Atienza S, et al. Toll-like receptors 3, 4 and 9 in hepatocellular carcinoma: relationship with clinicopathological characteristics and prognosis. Hepatol Res. 2014;44:769–78.
18. Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology. 2007;133:1869–1869.e14.
19. Swann JB, Vesely MD, Silva A, Sharkey J, Akira S, Schreiber RD, et al. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. Proc Natl Acad Sci. 2008;105:652–6.
20. Heidland A, Klassen A, Rutkowski P, Bahner U: The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? , 2006.
21. Hetta HF, Elkady A, Tohamy TA, Badary MS. Regulatory B cells: key players in hepatocellular carcinoma progression. Gastroenterol Hepatol Open Access. 2016;5:00136. https://doi.org/10.15406/ghoa.2016.05.00136.
22. Shata MT, Abdel-Hameed EA, Hetta HF, Sherman KE. Immune activation in HIV/HCV-infected patients is associated with low-level expression of liver expressed antimicrobial peptide-2 (LEAP-2). J Clin Pathol. 2013;66:967–75.
23. Hetta HF, Mekky MA, Khalil NK, Mohamed WA, El-Feky MA, Ahmed SH, et al. Extra-hepatic infection of hepatitis C virus in the colon tissue and its relationship with hepatitis C virus pathogenesis. J Med Microbiol. 2016;65:703–12.
24. Hetta HF, Mekky MA, Khalil NK, Mohamed WA, El-Feky MA, Ahmed SH, et al. Association of colonic regulatory T cells with hepatitis C virus pathogenesis and liver pathology. J Gastroenterol Hepatol. 2015;30:1543–51.
25. Mehta M, Hetta HF, Abdel-Hameed EA, Rouster SD, Hossain M, Mekky MA, et al. Association between IL28B rs12979860 single nucleotide polymorphism and the frequency of colonic Treg in chronically HCV-infected patients. Arch Virol. 2016;161:3161–9.
26. Helal F, Hetta MJM, Shata MTM. Gut immune response in the presence of hepatitis C virus infection. World J Immunol. 2014;4:52.
27. Hetta HF, Elkady A, Mekky MA, Abdelmalek MO, Sayed HI, Bazeed SE, et al. Interplay between gut microbiota and T lymphocytes in colorectal cancer. Colorec Cancer. 2017;3:12.
28. Kawai T, Akira S. TLR signaling. Cell Death Differ. 2006;13:816–25.
29. Mencin A, Kluwe J, Schwabe RF. Toll-like receptors as targets in chronic liver diseases. Gut. 2009;58:704–20.
30. Roh YS, Seki E. Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. J Gastroenterol Hepatol. 2013;28:38–42.
31. Lopes JAG, Borges-Canha M, Pimentel-Nunes P. Innate immunity and hepatocarcinoma: can toll-like receptors open the door to oncogenesis? World J Hepatol. 2016;8:162–82.
32. Chen CH, Su WW, Yang SS, Chang TT, Cheng KS, Lin HH, et al. Long-term trends and geographic variations in the survival of patients with hepatocellular carcinoma: analysis of 11 312 patients in Taiwan. J Gastroenterol Hepatol. 2006;21:1561–6.
33. Changchien C-S, Chen C-L, Yen Y-H, Wang J-H, Hu T-H, Lee C-M, et al. Analysis of 6381 hepatocellular carcinoma patients in southern Taiwan: prognostic features, treatment outcome, and survival. J Gastroenterol. 2008;43:159–70.
34. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, et al. A model to predict survival in patients with end-stage liver disease. Hepatology. 2001;33:464–70.

35. Marwa khalaf MAM, Kamel SI, AbdelRahman ME-T, Abdelmalek MO, Sayed HI, Hetta HF. Could we depend on HBV DNA level to predict significant liver fibrosis in chronic hepatitis B patients with persistently normal alanine aminotransferase PNALT. EC Gastroenterology and Digestive System. 2017;2:247.
36. McMillan DC. Systemic inflammation, nutritional status and survival in patients with cancer. Curr Opin Clin Nutr Metab Care. 2009;12:223–6.
37. Tsai H-J, Hsieh M-Y, Tsai Y-C, Liu Z-Y, Hsieh H-Y, Lee C-M, et al. Liver function tests may be useful tools for advanced cancer patient care: a preliminary single-center result. Kaohsiung J Med Sci. 2014;30:146–52.
38. Chan SL, Chan AT, Yeo W. Role of α-fetoprotein in hepatocellular carcinoma: prognostication, treatment monitoring or both? Future Oncol. 2009;5:889–99.
39. Chan SL, Mo FK, Johnson PJ, Hui EP, Ma BB, Ho WM, et al. New utility of an old marker: serial α-fetoprotein measurement in predicting radiologic response and survival of patients with hepatocellular carcinoma undergoing systemic chemotherapy. J Clin Oncol. 2009;27:446–52.
40. Murugavel KG, Mathews S, Jayanthi V, Shankar EM, Hari R, Surendran R, et al. Alpha-fetoprotein as a tumor marker in hepatocellular carcinoma: investigations in south Indian subjects with hepatotropic virus and aflatoxin etiologies. Int J Infect Dis. 2008;12:e71–6.
41. Chan SL, Mo F, Johnson PJ, Siu DY, Chan MH, Lau WY, et al. Performance of serum α-fetoprotein levels in the diagnosis of hepatocellular carcinoma in patients with a hepatic mass. HPB. 2014;16:366–72.
42. Yu LX, Yan HX, Liu Q, Yang W, Wu HP, Dong W, et al. Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. Hepatology. 2010;52:1322–33.
43. Cirera I, Bauer TM, Navasa M, Vila J, Grande L, Taurá P, et al. Bacterial translocation of enteric organisms in patients with cirrhosis. J Hepatol. 2001;34:32–7.
44. Almeida J, Galhenage S, Yu J, Kurtovic J, Riordan SM. Gut flora and bacterial translocation in chronic liver disease. World J Gastroenterol: WJG. 2006;12:1493–502.
45. Dapito DH, Mencin A, Gwak G-Y, Pradere J-P, Jang M-K, Mederacke I, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell. 2012;21:504–16.
46. Seki E, De Minicis S, Österreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-β signaling and hepatic fibrosis. Nat Med. 2007;13:1324–32.
47. Singh A, Koduru B, Carlisle C, Akhter H, Liu R-M, Schroder K, et al. NADPH oxidase 4 modulates hepatic responses to lipopolysaccharide mediated by toll-like receptor-4. Sci Rep. 2017;7:14346.
48. Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. Gastroenterology. 2006;130:1886–900.
49. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science. 2007;317:121–4.
50. Shi W, Su L, Li Q, Sun L, Lv J, Li J, et al. Suppression of toll-like receptor 2 expression inhibits the bioactivity of human hepatocellular carcinoma. Tumour Biol. 2014;35:9627–37.
51. Zhe Y, Li Y, Liu D, Su DM, Liu JG, Li HY. Extracellular HSP70-peptide complexes promote the proliferation of hepatocellular carcinoma cells via TLR2/4/JNK1/2MAPK pathway. Tumour Biol. 2016;37:13951–9.