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Relation Of Toll Like Receptor 2 And 4 To HCC Parameters.

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ABSTRACT

The crucial role of Toll-like receptor 2 and Toll-like receptor4 signaling have attracted much attention in the development of hepatocellular carcinoma (HCC) via inflammation-fibrosis-HCC axis and consider signaling pathways as potential therapeutic targets for HCC to open new avenues for development of novel strategies by cancer immunotherapy**.** We recruited 40 patients HCC on top of CHC and compared them with 33 patients chronic hepatitis c and 15 healthy matched controls; to compare serum TLR2 levels, serum TLR4 levels. The HCC group showed significantly higher levels of serum TLR2 and TLR4 levels compared with chronic hepatitis c (CHC) patients and controls. Level of TLR2 correlates positively with aging BMI, ALT, α Feto protein and the number, size of nodules and vascular invasion. Level of TLR4 correlates positively with ALT, AST, α Feto protein and the number, size of nodules and vascular invasion. Higher Level of TLR2 TLR4 are closely associated with the development of HCV related HCC patients Additionally, great value that correlated between toll like receptors 2 and 4 , act as part of innate immunity dysregulation in HCV related HCC patients with immunologic evasion that coexist in HCC.

Keywords: Hepatitis C virus. Hepatocellular carcinoma. Toll like receptors 2. Toll like receptors 4.

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INTRODUCTION

Hepatitis C virus is a hepatotropic virus approximately 3% of the world population is infected with HCV (1), annually there are about 3-4 million new cases of infection according to World Health Organization (2) that considered a major public health issue (3), causes chronic hepatitis that frequently progress to cirrhosis and gives rise to the high incidence of HCC (4) via multiple steps involves aberrant signaling pathways (5) are known to be frequently altered during HCC pathogenesis.

Furthermore, HCC is the second leading cause of cancer-related deaths globally (6) after lung cancer (7), acts as accounting for 9.1% of all cancer deaths worldwide, despite efforts for prevention ,screening and development of new technologies for HCC diagnosis and treatment (8). In Egypt, It acts the second leading cause of cancer deaths in men and the sixth most common cancers in women (5).

An effective innate immunity response relies on the toll-like receptors (TLR) found in several different liver cells which, through different ligands and many signaling pathways can elicit, not only a pro-inflammatory but also an oncogenic or anti-oncogenic response (9)

Toll-like receptors (TLRs) are pattern recognition receptors which may bridge the gap between inflammation and HCC due to the anatomical and functional relationship between gut and liver that is continuously exposed to gut-derived bacterial products, such as lipopolysaccharide (LPS) and bacterial DNA which stimulate TLRs. Activation of TLRs have shown to be involved in the progression of the inflammation-fibrosis-HCC axis that are triggered by damaged cells (10).

Aim of the work

Our aim is to attempt to identify the role of TLR2 and TLR4 in chronic hepatitis C (CHC) and HCC and its relation to disease parameters suggesting that TLR 2 and TLR4 represent therapeutic targets for HCC in advanced liver disease to open new avenues for development of novel strategies via TLR-mediated molecules for cancer immunotherapy.

PATIENTS AND METHODS

Study design

This prospective case-controlled ,hospital based study .

Study setting

The study was conducted on the three groups of the attendants of Internal Medicine Department,EL-Minia University Hospital, Egypt from December 2016 to September 2017 after the study protocol has been approved by Minia University ethics committee and conducted in accordance with the Helsinki Declaration with the international ethical guidelines.This study was included 3 groups :group I (included 40 patients with HCC on top of CHC, group II (included 33 patients with CHC and group III (Case Controls) included 15 apparently healthy volunteers confirmed by lab investigations.

Inclusion criteria

- Eligible patients were previously untreated adults 18 years of age to 65 years old who had CHC based on the presence of anti-HCV and detectable serum HCV RNA for 6 months or more ,absence of alcohol or drug abuse and absence of co-infection with HBV.
- HCC on top of chronic HCV diagnostic criteria were stratified according the Barcelona-Clinic Liver Cancer classification.

Exclusion criteria adopted in this study were patients younger than 18 years of age, Obese patient with body mass index (BMI) more than 25, patients with other causes of liver disease, co infection with hepatitis B , drug induced liver disease, autoimmune hepatitis, sclerosing cholingitis , hemochromatosis and hepatic decompensation (hepatic encephalopathy, ascites, varcial bleeding) for HCV patients. , Patients with heart

disease**,** Patients with chronic lung disease, Patients with serum creatinine >1.5 mg/dl, Patients with autoimmune diseases, chronic inflammatory diseases, patients with T2DM diagnosed according to the American Diabetes Association classification criteria , thyroid disorders , Patients with other malignants prior cancer therapy and history of previous HCC resection, patients with alcohol intake, patients who have received antiviral treatment and those were taking lipid lowering agents.

Clinical and laboratory assessment

The following data were collected from all patients: smoking habits of medical importance ,medical history ,life style characteristics ,age, gender, body weight ,height, BMI, calculated as weight divided by the square of the height, pulse ,blood pressure . Data from Child- Pugh class, staging was determined according the Barcelona-Clinic Liver Cancer classification in HCC on top of CHC patients that is awidely used classification method which takes into account variables related to tumor stage, liver function, physical status and cancer-related symptoms (with performance status scale 0–4 where 0 equal normal physical performance and 4 is bed ridden) to generate a treatment algorithm (11).In brief, at stage0 patients are optimal candidates for resection. At stage A patients are candidates for radical therapies (resection, liver transplantation or percutaneous treatments). At stage B patients may benefit from chemoembolization. At stage C patients may receive new agents as part of RCT and lastly at stage D patients had end-stage disease and can receive only symptomatic treatment (12).This classification was suggested to be best suited for treatment guidance, especially for patients who could benefit from curative therapies (13)

Sampling protocol and processing.

Venous blood was drawn after a 12 h overnight fasting by sterile venipuncture then these samples were divided into two tubes: 0.5 ml on EDTA containing tube: used to assess complete blood count (CBC) which determined by automated cell counter SYSMEX KX-2iN,Japan) , Prothrmbin time, concentration and international normalized ratio (INR) were done using Baiomerieux,Vitek.Inc.595, USA and the remaining blood was put on plain tube and left in room temperature to be clotted then centrifugated at 3000 rpm for 15 minutes .The expressed serum was used for the assay of :

Routine biochemical analysis that involved blood glucose, Liver chemical tests, renal functions, serum Alfa feto protein(AFP), viral markers using(Cobas E 411.Japan) , HCV-RNA PCR using Real time PCR) and the remaining serum was kept frozen at -80 ℃ for assessment of:serum TLR2 and serum TLR4 by ELISA (Wuhan Eiaab Science, China).

Statistical study

Data were coded ,entered and analyzed by the statistical for the social science (SPSS for windows version 20).

Symmetrically distributed continuous variables were summarized as a mean ± standard deviation (SD). The median and interquartile ranges were used for skewed continuous variables.

One way ANNOVA test was used for parametric quantitative data between 3 groups, independent sample (*t)* test for parametric quantitative data between 2 groups

Categorical variables were presented as frequency and percentage. Comparisons between groups were made by using the Mann–Whitney U test or the Student t test for continuous variables and the Chi-square (χ^2) or Fisher exact probability test for categorical data (Armitage and Berry,1987). The two-tailed, paired Student's t-test was used to test for significance of differences between TLR2 and TLR4 in HCV and HCC patients.

The pearson correlation coefficients were used to study the correlation between different parametric variables, Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables.

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Logistic regression analysis was used to model the association between TLR2, TLR4 and different covariates to determine the factors associated with HCC. The magnitude of these associations is reported as the odds ratio (OR with 95% CI). P values < 0.05 were considered significant. Linear regression analysis was used to reveal the predictors of TLR2 and TLR4 levels in HCC related to CHC, Receiver operating characteristics (ROC) analysis was used to identify the Sensitivity and specificity, TLR2 < 0.05 and TLR4< 0.05 were considered statistically significant.

RESULTS

All obtained results of different groups were summarized in tables 1-6 and figures 1-2.

Table (1): Laboratory data for all studied groups.

Data	HCC N=40	HCV N=33	Control N=15	P1 (IvsII)	P2 (IvsIII)	P3 (IIvsIII)
ALT(IU/l)	9-175 43.5±24.1	10-118 24.6±21.1	8-26 15.6±5.1	0.002**	0.001**	0.001**
AST(IU/l)	11.0- 594 130.7±21.9	8.1-77 47.2±29.5	10-34 17.5±6.7	0.0001***	0. 0001***	0.001**
Total bilirubin(mg/dl)	1.1-16.4 4.9±2.2	0.5-1.3 0. 5±0.4	0.7-0.9 0.2±0.1	0. 001**	0. 001**	0. 6
Direct bilirubin(mg/dl)	1-12.7 3.4±2.3	0.2-1 0.3±0.23	0.1-0.2 0.2±0.14	0.004**	0.001**	0.8
Albumin(g/dl)	1.8-4.1 3.2±0.9	3.6-4.9 4.4±0.5	4.2-5 4.6±0.6	0.001**	0.001**	0. 5
INR	1. 8-2.8 1.8±0.6	1-1.2 0.9±0.5	0.9-1.1 0.9±0.1	0.002**	0.001**	0. 9
Hb (g/dl)	6.5-12.9 9.9±1.9	11-13.1 11.4±1.6	12.2-14 12.9±1.01	0. 02*	0.01*	0.04*
Platelets (103/l)	59-218 104.2±74.1	165-380 265.9±51.6	185-360 275.9±31.6	0.001**	0.001**	0. 8
AFP(ng/ml)	210-71000 6351.1±170 78.4	75-98 85.9±6.6	2-7.2 2.9±0.6	0.003*	0.001**	0. 02*

Table (2): Comparison between the three groups as regard TLR2 and TLR4 .

Data	HCC N=40	HCV N=33	Control N=15	P1 (IvsII)	P2 (IvsIII)	P3 (IIvsIII)
Toll like receptor 2 (ng/ml)	0.9-36 18.6±8.5	0.2-31 14.1±5.8	0.5-22.1 8.6±4.5	0.02*	0.001**	0.001**
Toll like receptor 4 (ng/ml)	0.2-76 23.3±18.1	0.10- 46 10.8±15.7	0.4-49 4.1±8.7	0.04*	0.001**	0. 001**

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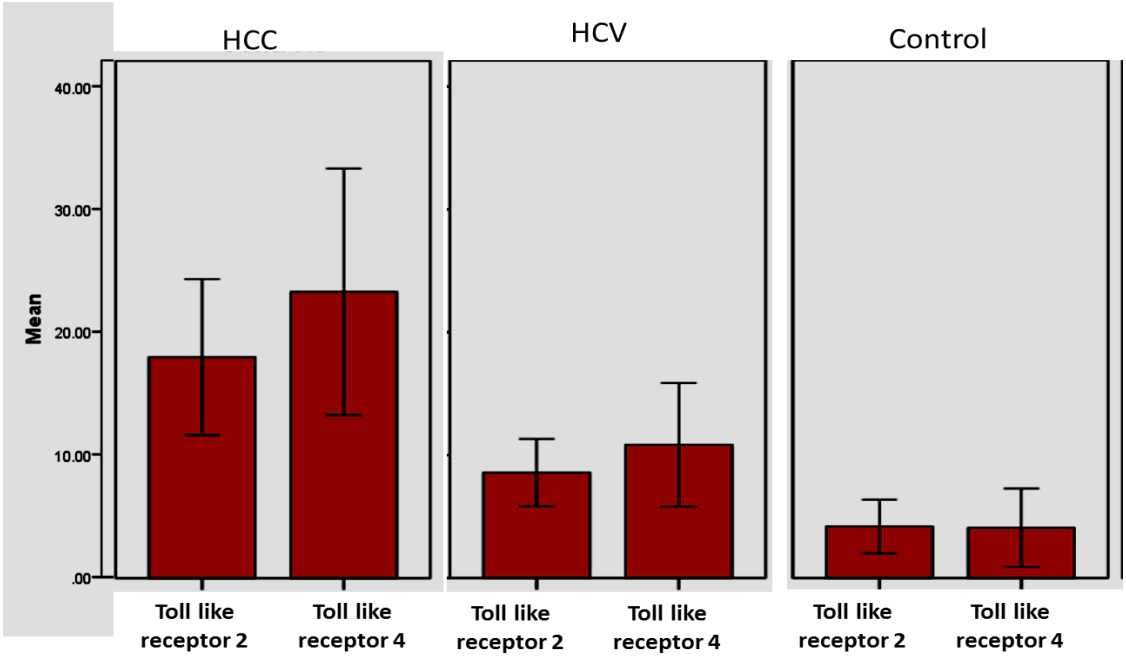

Figure (1): Shows high TLR2 and TLR4 in HCV related HCC group than other groups.

Table 3: Significant correlation with TLR2, aging, BMI, ALT, and α Feto protein (p > 0.02* 0.01*, 0.05*, 0.03* respectively) .

Parameter	Correlation (r)*	P value
Age	0.14	0.01*
BMI	-0. 1	0.05*
ALT	0.37	0.03*
α Feto protein	0.1 1	0. 05*

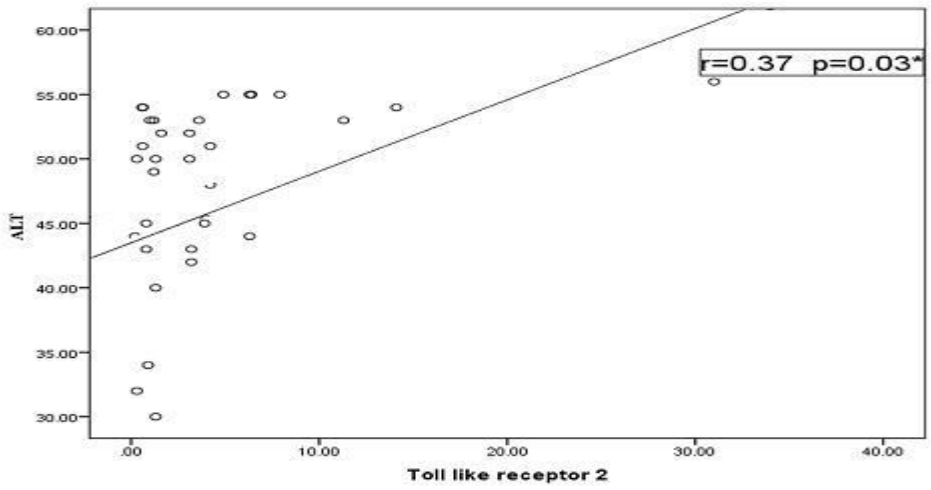

Figure 2:Shows positive correlation between TLR2 and ALT among HCV related HCC.

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Table 4: Significant positive correlation between TLR2 and tumor characters in patients with HCV related HCC.

Number of nodules Single Multiple	 1-36 0.9-32	 8.5±9.9 15.4±7.3	0. 05*
Size of nodules <5cm ≥5cm	 1.6-19 0.9-32	 7.8±4.8 19.3±10.1	0. 02*
Vascular invasion **Present** **Absent**	 2.1-36 3.8-22	 21. 1±6.1 10±2.2	0. 01**

Table 5: Significant positive correlation with TLR4 and BMI, ALT, and α Feto protein .

Parameter	Correlation (r)*	P value
BMI	-0. 1	0.03*
ALT	0. 8	0.01*
α Feto protein	0. 3	0.03*

Table 6: Significant positive correlation with TLR4 and tumor characters in patients with HCV related HCC.

Data	Toll like receptor 4		
	Range	mean±SD	P
Number of nodules Single Multiple	 0.2-76 0.5-46	11.1±9.3 10.5±6.1	0. 03*
Size of nodules <5cm ≥5cm	 1.5-28 4.5-46	8.1± 4.9 13.8±6.6	0.01*
Vascular invasion **Present** **Absent**	3.7 -17.2 1-9.4	13.1± 1.9 5. 8±0.6	0.01*

DISCUSSION

This cross-sectional prospective, case-control study,we assessed the association between TLR 2 and TLR4 and HCC related HCV in a hospital based study .To our knowledge, this is the first single study to report positive association between serum TLR 2, TLR4 and the presence of HCC. Our data demonstrate higher levels of TLR2 and TLR4 in HCV related HCC group than CHC patients and healthy matched controls.

In this study ,As regards: TLR2 : We found strong association between the higher level of TLR2 and the development of HCC in agreement with Zhu et al., (14) who reported that the role of TLR2 in chronic hepatitis C and HCC as predictor factors in the HCC microenvironment, chemokine receptors, play a critical role in tumorgenesis and metastasis. Furthermore, in accordance of Kiziltas, (1) who explained that by activation not only TLR2 but also TLR4 by HCV core protein and NS3 promotes hepatic inflammation and injury through the production of IFN-β , Moreover, (Broering et al., (15) report that LPS, the HCV core protein and IFN-γ have been suggested to amplify inflammatory monocyte/macrophage activation via formation of MyD88-IRAK complexes, increased NF-κB activation and increased production of TNF-α, leading to the loss of TLR tolerance to induce persistent inflammation during chronic HCV infection.

Our studies showed a highly significant increase in serum TLR 2 in both patients groups compared to controls as a predictor of hepatitis C virus-associated HCC.(p<0. 02 ,0.001**<0.001) respectively in agreement

with Kataki et al.,(16) who found a significant upregulation of TLR2 expression in HCC that is explained by Maeda ,(17) that report the TLR2 expression in hepatocytes is upregulated by LPS, TNF, and others, suggesting that hepatocytes become more sensitive in the inflammatory condition.

Unfortunatly, this disagreement with French et al., (18) who demonstrates that TLR2 is downregulated in hepatocytes, Kupffer cells and peripheral blood monocytes in hepatitis patients as hepatitis B antigen positive , on the other hand, TLR2 expression and TNF-α are up-regulated probably mediated by precore proteins.

Moreover, our results demonstrates that TLR2 correlates with increased tumor size ,number and vascular invasion with no previous studies.

Lopes et al.,(9) report that TLR2 still presents an ambiguous role, possibly depending on liver's stage in the inflammation-cirrhosis-carcinoma axis to exert its protumorigenic or anti tumorigenic capacity .

Furthermore,in our study : as regard TLR 4 : we found that TLR 4 levels with a highly significant increase in serum TLR 4 in both patients groups compared to controls as a predictor of HCC ($p<0.04$,0.001**<0.001) respectively .These results are in agreement with many previous studies in agreement with (Jiang et al .,(19) who show that revealed an association between TLR4 expression, tumor aggressiveness and a poor prognosis for patients with HCC although the mechanisms by which TLR4 promotes cancer progression are still unknown, Furthermore, Dong et al., (20) stated that TLR 4-mediated signaling has been shown to be important to cell survival, invasion and metastasis in a variety of cancers that is explained by the involvement of the Akt and mitogen-activated protein kinase (MAPK) pathways in LPS-enhanced TLR4 ,TLR4/STAT3 activation along with increased cell proliferation.

Additionally, Hsiao et al.,(21) demonstrates that TLR 4 has role in chemoresistance in HepG2 cells in agreement with Tsukamoto et al.,(22) who demonstrate that TLR4 increases tumor-initiating activity and chemoresistance.

Moreover, our results demonstrates that TLR4 correlates with increased tumor size ,number and vascular invasion in agreement with experimental studies postulated a role for TLR4 levels that were reduced in MyD88-deficient mice, Gu et al., (23) report that the classical TLR4/MyD88 signaling pathway is also responsible for progression and invasion of HCC by activation of the NF-κB signaling pathway .

In our study ,When evaluated patients with HCV related HCC by using receiver-operator characteristic (ROC) curves of serum TLR2 and TLR4, we found AUC of TLR2 and TLR4 (0.72±0.06, 0.77±0.05 respectively) with high sensitivity specificity+ve Predictive value and -ve Predictive value so TLR2 and TLR4 have crucial role in pathogensis of HCV related HCC so these results raise the possibility that by targeting TLR2 and TLR4 with high affinity pharmacological stimulants may be able to control HCV infection by induction of IFN-α and direct activation of antiviral mechanisms in hepatocytes. Additionally, they provide insight about the potential use of them as a new set of molecular markers for prognosis and outcomes of chronic HCV infection and HCC and we hope to early stage recognition of HCC make these patients eligible for potentially curative therapies, as therapeutic targets for HCC by inhibiting TLRs with antagonists has the potential to be a novel therapeutic technique for HCCor TLR agonists as immune tolerance, this was explained by Brackett et al., (24) who report that TLRs activation as an anticancer innate immune response is highly desirable because of its inherent ability to generate an adaptive antitumor T-cell response.

CONCLUSIONS

Our findings indicated that a higher TLR2 and TLR4 are closely associated with the development of HCV related HCC patients. Additionally, great value that correlated between TLR2 and TLR4 , act as part of innate immunity dysregulation in HCV related HCC patients with immunologic evasion that coexist in HCC. It was found that the serum TLR2 and TLR4 correlated positively with the number, size of nodules and vascular invasion. Since TLR2 and TLR4 are modifiable risk factors and therapeutic targets for HCC.

Recommendation

The link between increased serum TLR2 and TLR4, chronic hepatitis and HCC support that have stronger effects on the susceptibility of HCC, play an important roles in hepatomitogen expression and promotion liver tumorgenesis suggest that a better understanding of these signaling pathways in the liver, giving the distinction it deserves to intrigue the biopharmaceutical field and provide a new therapeutic target for HCC. In further studies, we recommend for large number of cases and measuring level of TLR2 and TLR4 in HCV related HCC patients, giving it the distinction it deserves to intrigue the biopharmaceutical field.

Ethical approval

The study protocol was approved by the ethics committee of faculty of medicine, Minia University. Informed consent was obtained from each patient before participation in the study.

REFERENCES

[1] Kiziltas, E. E., Kiziltas, A., Rhodes, K., Emanetoglu, N. W., Blumentritt, M., & Gardner, D. J. (2016). Electrically conductive nano graphite-filled bacterial cellulose composites. Carbohydrate polymers, 136, 1144-1151.

[2] 2-World Health Organization. (WHO). (2015). Guidelines on the management of Hepatitis C.

[3] Morozov VA , Lagaye S. Hepatitis C virus: Morphogenesis, infection and therapy 3-2018

[4] Zhang Y, Zhang YI, Ge L, Lin Y, Kwok HF.The Roles of Protein Tyrosine Phosphatases in Hepatocellular Carcinoma. Cancers (Basel). 2018 Mar 20;10(3).

[5] Ziada, D. H., El Sadany, S., Soliman, H., Abd-Elsalam, S., Salama, M., Hawash, N., ... & Elsabagh, H. M. (2016).Prevalence of hepatocellular carcinoma in chronic hepatitis C patients in Mid Delta, Egypt: A .single center study. Journal of the Egyptian National Cancer Institute, 28(4), 257-262.

[6] El Khodiry, A., Afify, M., & El Tayebi, H. M. (2018). Behind the curtain of non-coding RNAs; long non-coding RNAs regulating hepatocarcinogenesis. World journal of gastroenterology, 24(5), 549.

[7] Mohamoud YA, Mumtaz GR, Riome S, et al. The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis. BMC Infect Dis 2014;13:288.

[8] Lopez-Valdes,Crus MM. The Relationship of Aflatoxin B1 and Hepatocellular Carcinoma. Journal of Liver Research, Disorders & Therapy.Volume 3 Issue 6 – 2017.

[9] Lopes, J. A. G., Borges-Canha, M., & Pimentel-Nunes, P. (2016). Innate immunity and hepatocarcinoma: Can toll-like receptors open the door to oncogenesis?. World journal of hepatology, 8(3), 162.

[10] Song, I. J., Yang, Y. M., Inokuchi-Shimizu, S., Roh, Y. S., Yang, L., & Seki, E. (2018). The contribution of toll-like receptor signaling to the development of liver fibrosis and cancer in hepatocyte-specific TAK1-deleted mice. International journal of cancer, 142(1), 81-91.

[11] Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma.Lancet 2003;362:1907–17.

[12] Cillo U, Bassanello M, Vitale A, Grigoletto FA, Burra P,Fagiuoli S. The critical issue of hepatocellular carcinoma prognostic classification: which is the best tool available? J

[13] Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma:diagnosis and treatment. Gastroenterology 2002;122:1609–19.

[14] Zhu Y, Gao XM, Yang J, Xu D, Zhang Y, Lu M,Zhang Z, Sheng Y. C-C chemokine receptor type 1 mediates osteopontin-promoted metastasis in hepatocellular carcinoma. Cancer Sci. 2018 Mar; 109(3): 710–723.

[15] Broering R, Lu M, Schlaak JF. Role of Toll-like receptors in liver health and disease. Clin Sci (Lond) 2011;121:415–426.

[16] Kataki K1,2, Borthakur P1, Kumari N1, Deka M1, Kataki AC2, Medhi S1. Association of mRNA expression of toll-like receptor 2 and 3 with hepatitis B viral load in chronic hepatitis, cirrhosis, and hepatocellular carcinoma. J Med Virol. 2017 Jun;89(6):1008-1014.

[17] Maeda S . NF-κB, JNK, and TLR Signaling Pathways in Hepatocarcinogenesis. Gastroenterology Research and Practice .Volume 2010 (2010), Article ID 367694, 10 pages

[18] French SW1, Oliva J, French BA, Li J, Bardag-Gorce F. Alcohol, nutrition and liver cancer: role of Toll-like receptor signaling. World J Gastroenterol. 2010 Mar 21;16(11):1344-8.

[19] -Jiang ZC, Tang XM, Zhao YR, Zheng L. A functional variant at miR-34a binding site in toll-like receptor 4 gene alters susceptibility to hepatocellular carcinoma in a Chinese Han population. Tumour Biol. 2014 Dec;35(12):12345-52.

[20] Dong YQ, Lu CW, Zhang L, Yang J, Hameed W, Chen W. Toll-like receptor 4 signaling promotes invasion of hepatocellular carcinoma cells through MKK4/JNK pathway. Mol Immunol. 2015 Dec;68(2 Pt C):671-83

[21] Hsiao CC, Chen PH, Cheng CI, Tsai MS, Chang CY, Lu SC, Hsieh MC, Lin YC, Lee PH, Kao YH.

[22] Tsukamoto H1,2,3, Mishra L4, Machida K5,6. Alcohol, TLR4-TGF-β antagonism, and liver cancer. Hepatol Int. 2014 Sep;8 Suppl 2:408-12.

[23] Gu J, Sun R, Shen S, Yu Z. The influence of TLR4 agonist lipopolysaccharides on hepatocellular carcinoma cells and the feasibility of its application in treating liver cancer Onco Targets Ther. 2015; 8():2215-25.

[24] Brackett, Craig M.; Kojouharov, Bojidar; Veith, Jean; Greene, Kellee F.; Burdelya, Lyudmila G.; Gollnick, Sandra O.; Abrams, Scott I.; Gudkov, Andrei V. Toll-like receptor-5 agonist, entolimod, suppresses metastasis and induces immunity by stimulating an NK-dendritic-CD8+ T-cell axis Science.gov2016-01-01.