ORIGINAL ARTICLE

An Update of Hepatic Biomarkers in Hepatocellular Carcinoma among HCV Patients in Egypt

BOTHAINA A. MADKOUR[1], OMAR M. SABRY[1], MARWA S. WAHDAN[1], AYMAN H. ABDELAZIZ[2], ALI ABDEL RAHIM[2] , SHEREIF A. MORSY[2], ALAA A. GAD[3]
[1]*Hematology Department, Theodor Bilharz Research Institute, Giza, Egypt*
[2]*Hepato-gastroenterology Department, Theodor Bilharz Research Institute, Giza, Egypt.*
[3]*Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Cairo, Egypt*
Correspondence to Marwa S. Wahdan Email: Marwa.wahdan@yahoo.com

ABSTRACT

Background: Hepatocellular Carcinoma (HCC) is a distinguished type of liver cancer with multifactorial risks. Epidermal growth factor (EGF), Interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), CD163 and tumor necrosis factor (TNF-α) are believed to have roles in deregulation of the inflammatory response and cancer development
Aim: To study the serum levels of five essential cytokines and growth factors; EGF, IL-6, VEFG, CD163 and TNF-α in the HCV Egyptian patients and healthy controls as well as to validate the relationship between these inflammatory biomarkers and the presence of HCC in HCV patients. Subjects and methods:
Methods: In this study we calculated the serum levels of TNF-α, EGF and IL-6 using ELISA technique as well as assessing the serum levels of VEGF, and CD163 using Human Luminex® screening assay premixed multiplex kit in a cohort of 165 subjects who were divided into 3 groups
Results: Regarding ELISA technique, there was a significant difference regarding EGF and IL-6 between the control group and the two diseased groups ($p< 0.05$); in EGF, the mean serum level was highest in the HCC group (631.7 pg/ml) and lowest in the control group (185.7 pg/ml). IL-6 serum values revealed highest significant levels in the HCC group (20.7 pg/ml) and lowest in the control group (4.7 pg/ml) ($p<0.05$). Additionally, ELISA technique's results revealed significant high levels of serum TNF-α in both HCV as well as HCC group compared to the control group with a ($p<0.05$), with no significance between HCC and HCV groups respectively.
Conclusion: TNF-α, EGF, IL-6, VEGF and CD 163 were increased in this cohort of Egyptian patients with chronic HCV infection compared to controls but failed to establish reliable diagnostic performance for the development of HCC as sole markers in those patients. However, based on the presented results most of these molecules are probably involved in the pathogenesis of host inflammatory response to HCV infection.
Keywords: IL-6, CD163, EGF, VEGF, TNF-α, Luminex assay, ELISA, hepatitis C virus

INTRODUCTION

There is rock-solid relationship between inflammation and cancer development which was established based on previous literatures and scientific studies[1]. Any disturbance in the inflammatory response will eventually lead to tumorigenesis, since there is a complex crosstalk between pro-inflammatory as well as tumorigenic mediators embodied in cytokines, chemokines, oncogenes, transcription factors and various enzymes, moreover, there are certain risk factors that might be incriminated in triggering the tumorigenic pathway, including extrinsic (environmental factors) and/or intrinsic (hereditary factors)]. Further pertained studies are compulsory for deeper exploration of inflammation-associated cancers aiming to find a novel therapeutic strategy targeted against these inflammatory mediators[3].

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are the main culprits of hepatocellular carcinoma (HCC) in countries of the Eastern Mediterranean Region and the Middle East where different strategies are required to treat them accordingly. A systematic review by Alavian and Haghbin [4] showed that HCC associated with HCV is very common in North African countries and Egypt has HCV prevalence of 79.8% in HCC patients[4]. The Egyptian government recently started a comprehensive nationwide screening and paid for treatment program with local generic forms of Direct-acting antiviral agents (DAAs) for all patients infected with HCV in order to eradicate the disease[5].

Hepatitis C virus "one of the known risks of liver cancer" is an RNA virus that lacks reverse transcriptase. Thus, its genome cannot be combined with a human, but on the other hand, it contains many proteins with specific transformative properties, thus viral hepatitis attacks the immune system with endothelial cells, which leads to immune dysfunction as well as abnormal angiogenesis and eventually neoplasm formation[6,7].

Hepatocellular carcinoma is considered one of the ten most common solid cancers globally and is the second cause of malignancy-related mortality after lung cancer, unfortunately, almost all current data point toward an increase in HCC incidence in many countries and the most effective way to combat this fatal malignancy and to reduce mortality is preventing its occurrence from the beginning[8]. HCC is a vivid example of inflammation-related cancer and represents a prototype of the link between tumor microenvironment (TME) and tumor progression [9]. In addition, presence or absence of HCV plays a critical role in HCC development since its presence may affect the mutation profile of the corresponding cancer genome[10,11].

Aside from genetic alteration, it is proposed to criminalize several markers of inflammation in the creation and maintenance of TME and some of these

to mention its implication in the pathogenesis of various auto-immune disease[34] .

Hepatocellular carcinoma (HCC) progression is discussed as being exclusively dependent on the extent of hepatitis, as the balance between pro-inflammatory and anti-inflammatory cytokines is the key feature to control disease progression; this means that a change in this balance can lead to disease progression[35].

The current study aimed at investigating the serum levels of 5 essential biomarkers namely TNF-α, EGF and IL-6 using ELISA technique as well as VEFG, and CD163 using Luminex assay in the HCV infected Egyptian patients and healthy controls as well as to validate the relationship between these inflammatory biomarkers and the presence of HCC in patients with HCV infection.

Patients and methods:

Study population and demographic information: The study was executed at Theodor Bilharz Research institute (TBRI) where 165 subjects were involved and categorized into 3 groups; group (A) comprised 55 patients with established diagnosis of chronic HCV infection, group (B) comprised 60 patients diagnosed with HCC on top of chronic HCV infection and classified according to Barcelona clinic liver cancer (BCLC) system and Triphasic CT scan for HCC staging. Finally, group (C) who constituted 50 healthy individuals to serve as the control group. Exclusion criteria included those with HBV comorbidity, schistosomiasis, alcohol consumption as well as patients receiving antiviral therapy. A written informed consent was attained from the participants. All the procedures used were approved by TBRI ethics committee according to Helsinki Declaration.

Group A included 27(49.1%) males besides 28(50.9%) females with their age ranged from 34 to 67 years (mean±SD=44.3±13.9). while in group B comprised 42(70%) males as opposed to 18(30%) females with age ranged from 48 to 60 years (Mean± SD= 46.8±15.9) were enrolled. Finally, group C comprised 36 (72.0%) males and 14 (28.0%) females with their age ranged from 32 to 57 years (Mean ± SD= 46.7±13.3).

All patients under the presented study were subjected to the following: Complete history taking, pelvi-abdominal ultrasonography and Spleen & Liver stiffness measurement (SSM) using Echoscence 502 FibroScan® equipment with XL probe to overcome presence of ascites. Routine laboratory investigations including complete blood count (CBC) using the automated hematology analyzer DxH500 Beckman Coulter, USA. Blood smears were spread and stained for determination of differential leucocytic count, coagulation profile: Prothrombin time (PT), international normalized ratio (INR) and partial thromboplastin time (PTT) were performed using Stago Compact, Diagnostica Stago, Spain. Liver function tests: serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin were performed using AU480 chemistry analyzer, Beckman Coulter, USA. Serologicaltests including HCV IgGAb and HBs Ag were performed using ADVIA Centaur CP, Siemens, Germany. In order to confirm active viremia in HCV Ab reactive cases, quantitative RT-PCR using Cobas® HCV assay was performedon Cobas ® 4800 System, Roche, Switzerland.Alpha fetoprotein (AFP) was markers include interleukin-6 (IL-6), vascular endothelial growth factor (VEFG), epidermal growth factor (EGF), CD163 and tumor necrosis factor (TNF-α). The first marker is IL-6, a pro-inflammatory cytokine known to be an integral marker of a cancer-related cytokine that results in stimulation of the immune system as well as cancer-induced immunosuppression that ultimately preserves the survival of cancer cells[12]. Second, VEGF is one of the main mediators of angiogenesis which plays a critical role in carcinogenesis by promoting the growth of cancer cells[13]. VEGF activates multiple signaling pathways that promote the growth of endothelial cells, as well as their migration, differentiation and increased vascular permeability[14,15], serum VEGF levels may influence vital hepatic functions in addition to angiogenesis and tumor progression and thus can be used as a good predictor of HCC progression and prognosis[16].

Several studies have established the presence of a plausible cross talk between VEGF and EGF via EGFR signaling pathway[17,18,19]. EGFR is a tyrosine kinase trans-membrane receptor expressed on the epithelial cells' surface. It controls the development of carcinogenesis, comprising cancer cell survival, cell cycle progression besides angiogenesis[20]. EGF ligand binds to EGFR and this activates signal transduction and causes up-regulation of transcription factors which lead to proliferation and differentiation of epidermal together with epithelial tissue[21]. Moreover, initiation of EGFR signaling pathway causes up-regulation of VEGF ligand in addition to its receptor VEGFR2 on endothelial cells, leading to increased vascular permeability and stimulating angiogenesis[22,23].

When it comes to the TME, macrophages possess a significant role in triggering tumorogenesis and metastasis in several tumors since an abundant infiltrate of macrophage is always found in the specimen of solid tumors [3, 24]. Macrophages are intriguingly studied in recent years and CD163 is one of its important markers expressed on almost all circulating macrophages predominantly M2 macrophages possessing anti-inflammatory function[25,26]. CD163 is a transmembrane protein which is a member of cysteine-rich family receptors[27]. It was reported that CD163 +ve macrophages are associated with poor prognosis in various tumors, such as melanoma, renal cell carcinoma and bladder cancer[28,29,30]. In addition, serum level of soluble form of CD163 (sCD163) was elevated after macrophage activation and its over expression could be rendered a potential marker of a progressive inflammatory response[31,32]. Hence, CD163 serum level in HCV patients who developed HCC could be of value.

Last but not the least is TNF-α, it is a member of a superfamily of cytokines comprising nineteen ligands which is secreted from the macrophage and other immune activated cells to induce cell necrosis and apoptosis (necroptosis)[33], additionally, it is known to be a key mediator of both acute and chronic systematic inflammatory reactions via inducing both its own secretion as well as induction of other inflammatory cytokines and chemokines, hence it possesses a chief role in cancer-associated chronic inflammation and tumorogenesis not

was performed, the Mann-Whitney test was used for unusual variables and the 2 test or Fisher's exact test was used to determine the distribution of categorical variables between groups.

The diagnostic performance of TNF-α, EGF, VEGF, IL-6 and CD163 was evaluated by receptor operating characteristic (ROC) curves. The area under the ROC (AUROC) was used as an index to compare the accuracy of the tests. The threshold for the diagnosis of the study group was taken from the common maximum sensitivity and specificity point. The sensitivity and specificity of the relevant cutting processes were also shown. Spearman's rank correlation coefficient (r) was performed to show the correlation between the different variables in this study. The odds ratio (OR), 95% confidence interval (95% CI) (single variable), and multivariate analysis models were used to test the preferential association between each group studied on the incidence of HCC after adjustment for all other significant potential influences.

RESULTS

The presented study was carried out in the Hepato-Gastroenterology department at Theodor Bilharz Research institute (TBRI) where 165 patients were recorded and divided into three groups; 55 patients were diagnosed with chronic HCV infection; 60 patients were diagnosed with HCC on top of HCV and 50 healthy age and sex-matched individuals who served as the control group. Demographic, laboratory and clinical data are presented in (Table 1).

The levels of EGF, IL-6, and VEGF in all diseased groups were significantly increased by the control group and HCV (p-value <0.001). In the event that the serum TNF-α level increased significantly in the HCV and HCC groups, significantly when compared to the control group (p-value <0.002), while it was not significant in the HCC group when compared to the HCV group. Regarding serum specific to CD163 in the HCV and HCC groups was significantly increased in the control group (p-value <0.001), while mortality significantly increased in the HCC group compared to the HCV group (Table 2).

In order to differentiate the HCC group from the HCV group, receiver operation (ROC) curves were generated to show the diagnostic performance of TNF-α, EGF, IL-6, VEGF and CD163 in the studied groups. It was found that serum EGF at a cut-off value >360, with a sensitivity of 50% and a specificity of 96.7% with an area under the curve (AUC) was 0.7 (p-value=0.003) and an accuracy of 73.3%, serum IL-6 at a cutoff value of >14.5, with a sensitivity of 93.3%. A specificity of 50% with AUC was 0.68 (p-value=0.006), an accuracy of 71.7%, and serum CD163 at a cutoff value of >391.0, with a sensitivity of 86.7% and a specificity of 50.0% with AUC was 0.72 (p-value = 0.001) and an accuracy of 68.3% but serum TNF-α. And VEGF was not significant and could not distinguish between HCC and HCV patients (Table 3) and (Fig.1).

measured using Quantikinehuman AFP immunoassay ELISA (Cat.No. DAFP00), R&D systems, USA. AFP was used as the screening tool for HCC and all positive patients underwent subsequently multi-slice computer tomography (MSCT) to confirm the presence of HCC.

Sample Collection and Biomarker Measurement Sampling Procedure: Blood samples were collected under aseptic conditions by clean venipuncture using disposable vacuum blood collection system. A total volume of five ml blood was withdrawn from each subject and collected using the vacutainer as follows: one ml in an EDTA tube for performing CBC and differential count then the plasma was separated and stored in -80 ultra low freezer Nuair, USA for quantitative RT-PCR.

Quantification of serum level of TNF-α, EGF and IL6:

ELISA technique was used for quantitative determination of Human TNF-α (Cat.No.DTA00C), Human EGF (Cat.No.DEG00) and Human IL-6 (Cat. No. D6050) all from Quantikine® Immunoassay, R&D Systems, USA. The procedures given by the manufacturer were strictly followed.

Quantification of Biomarkers using Human Luminex Assay: Serum samples required a 2-fold dilution (sample 75 µL of sample + 75 µL of Calibrator Diluent RD6-52) were mixed thoroughly. Human Luminex® Screening Assays premixed multiplex kit (Catalogue number. LXSAH-02/ Lot number 120705, R&D Systems, Inc., USA), was used to carry out simultaneous analysis of multiple biomarkers. Luminex uses applied bead-based technology in which a fluorescence polystyrene beads were used; each bead set was coated with antibody specific to a particular analyte. Within the analyzer, laser excites the internal dyes that tag each microsphere particle together with capturing any reporter dye during the assay. In this way, this technology allows multiplexing of up to 100 unique quantitative assays within a single sample.

Biomarkers serum levels were analyzed for the following analytes: VEGF and CD163 using Human Premixed Multi-Analyte Kit according to manufacturers' instruction (R&D Systems, USA), Luminex 100 ™ readout System was used. Ultimately, the Levels of each analyte were automatically calculated from standard curves using xPONENT ® software.

Standard concentrations were applied to the certificate of analysis and 3-fold dilutions were calculated for the remaining levels. Duplicate reads were averaged for each standard and sample, and the mean blank mean fluorescence intensity (MFI) was subtracted. A standard curve was generated for each analysis by minimizing data with xPONENT® software capable of generating a logistic curve compatibility (5-PL) of five variables. The samples were diluted, so the readable concentration was multiplied from the standard curve by the dilution factor 2.

Statistical analysis: The mean±standard error (SE) of the mean was used to express all of the findings. The data have been analysed using SPSS version 11. The significance among the groups was also compared using Duncan's post-hoc test. When P<0.05, a difference was deemed significant.To compare the means of naturally distributed variables between groups, a Student's test

Table 1: Demographic, laboratory and clinical data among the studied groups:

Demographic and clinical data	Control (n=50)	HCV (n=55)	HCC (n=60)
Gender M/F N (%)	36(72.0%)/ 14(28.0%)	27(49.0%)/ 28(51.0%)	42(70.0%)/ 18(30.0%)
TLC ($X10^3$/µl)	7.1 (5.8- 8.4)	4.2 (3.5- 10.3)[a]	3.9 (3.2- 9.9) [a]
HB (g/l)	12.6±1.1	10.5±1.5[a]	9.8±1.9 [a]
Platelet ($X10^6$/µl)	281.8±95.7	100.2±37.3 [aa]	85.8±35.4 [aa, b]
PT (sec)	12.5±1.0	15.0±4.1 [a]	17.7±4.5 [aa]
PC (%)	87.5±11.7	75.7±22.0 [a]	66.7±22.0 [a]
INR	1.1±0.1	1.4±0.5 [a]	1.6±0.6 [a]
PTT (sec)	32.4±5.2	38.5±4.2 [a]	40.7±6.4 [aa]
ALT (U/l)	26.0 (17.3- 33.5)	38.5 (23.3- 55.8) [a]	47.5 (33.0- 72.8) [a]
AST (U/l)	33.5 (24.0- 40.8)	56.5 (38.5- 78.8) [a]	61.0 (42.0- 85.3) [a]
Albumin (mg/dl)	4.2±0.7	3.2±0.5 [a]	2.9±0.9[aa]
T.Bilirubin (mg/dl)	0.7 (0.4- 1.1)	1.8 (09-2.3) [a]	2.7 (0.9- 4.5)[aa, b]
D.Bilirubin (mg/dl)	0.1 (0.1- 0.3)	0.9 (0.5- 1.4) [a]	1.8 (0.5- 2.3)[aa, b]
Creatinine (mg/dl)	0.9 (0.7- 1.0)	0.8 (0.7- 1.3)	0.9 (0.8- 1.2)
Urea (mg/dl)	30.0 (27.3- 31.8)	44.4 (28.0- 83.0) [a]	62.5 (42.3- 93.0) [a, b]
Glucose (mg/dl)	90.0 (85.0- 95.0)	127.5 (106.8- 191.3)[a]	122.5 (107.0- 159.8) [a]
AFP (0-11 ng/ml)	3.0(2.8 - 3.8)	10.8 (9.4 - 11.6)[a]	325.0 (187.5 - 587.5)[aa, bb]
SplenomegalyY/N N (%)	0 (0.0%)/ 50 (100.0%)	50 (91.0%)/ 5 (9.0%) [a]	60 (100.0%)/ 0 (0.0%) [a]
Ascites Mild/ moderate N (%)	0 (0.0%)/ 0 (0.0%)	20 (36.7%)/ 9 (16.6%) [a]	28 (46.7%)/ 18 (30.0%) [a]
Child-Pugh score	-	7.2±1.8	10.5±1.8 [c]
MELD	-	10.2 (7.3- 21.7)	14.7 (10.5- 30.1) [c]
Fibroscan (KPa=Kilo Pascal)	4.7±1.18	14.3±2.9 [aa]	28.5±2.8 [aa, bb]
HCC staging according to Barcelona classification (BCLC)			A 16.7% / B 26.6% / C 33.4% / D 23.3%

Gender, Splenomegaly, Ascites and BCLC are presented as number and %, the data were analyzed by *X2* test, while HB, PLT, PT, PC, INR, PTT, Albumin a Child-Pugh score and Fibroscan are represented as Mean ± SD, the data were analyzed by t. test, and TLC, ALT, AST, T.Bili, D.Bili, Creat., Urea, Glucose MELD score and AFP are represented as Median [interquartile range] (25%-75%) the data were analyzed by Mann-Whitney U test. Fibroscan units kilopascals (kPa); F0/F1= 2.5 - 7 kPa, F2=7-9.5 kPa, F3=9.5-12.5 kPa, F4>12.5 kPa
[a]*P*: Regarding to the comparison of HCV group vs control, [b]*P*: Regarding to the comparison of HCC group vs control, [c]*P*: Regarding to the comparison of HCC group vs HCV group. [1 Initial]*P* value ≤ 0.05 significant while [2 Initials]*P* value ≤ 0.01 highly significant

Table 2: Serum Biomarker levels among the studied groups:

Biomarkers	Control	HCV	HCC	P. value [a]p	[b]p	[c]p
TNF-α pg/ml	5.2(0.8-9.8)	13.2(8.4-20.8)	11.9(8.5-25.9)	0.002**	0.002**	0.8
EGF pg/ml	185.7 ± 39.08	321.9 ± 47.5	631.7 ± 67.8	0.001**	0.001**	0.001**
IL-6 pg/ml	4.74 ± 1.3	11.94 ± 3.1	20.72 ± 5.1	0.001**	0.001**	0.001**
VEGF pg/ml	58.4(26.6-117.5)	164.9(133.8-259.7)	286.3(141.1-514.1)	0.001**	0.001**	0.001**
CD163 pg/ml	37.2 (23.3- 43.9)	383.3 (163.8- 859.3)	157.4 (108.5- 329.8)	0.001**	0.001**	0.003**

TNF-α, VEGF and CD163 are represented as median with (Inter Quartile Range 25 and 75 Percentiles, %) the data were analyzed by Mann-Whitney U test, while EGF and IL-6 are represented as Mean ± SD the data were analyzed by t. test.
[a]*p*: Regarding to the comparison of HCV group vs control,
[b]*p*: Regarding to the comparison of HCC group vs control,
[c]*p*: Regarding to the comparison of HCC group vs HCV group.
[1 Initial]*p* value ≤ 0.05 significant while [2 Initials]*P* value ≤ 0.01 highly significant

Table 3: Comparison between the HCV and the HCC groups:

HCV & HCC	Markers cutoff	Sn.	Sp.	PPV	NPV	Diagnostic accuracy	AUC	p. value
TNF-α	25.3	26.7%	86.7%	66.7%	54.2%	56.7%	51.1%	0.8
EGF	360.0	50.0%	96.7%	93.8%	65.9%	73.3%	70.0%	0.003**
IL-6	14.5	93.3%	50.0%	65.1%	88.2%	71.7%	68.4%	0.006**
VEGF	145.0	100.0%	30.0%	58.8%	100.0%	65.0%	62.1%	0.09
CD163	391.0	86.7%	50.0%	63.4%	78.9%	68.3%	72.7%	0.001**

Sn: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: negative predictive value, AUC Area under curve and *C.I*: 95% Confidence Interval.
* *p* value <0.05 is significant, ** *p* value <0.01 is highly significant.

VEGF and CD163 in comparison between HCV with HCC group.

This study included a correlation study between different parameters; the results revealed that there was a significant positive correlation between the serum levels of both AST, bilirubin with TNF-α, IL-6, EGF, and CD163. Moreover, EGF, IL-6 and CD163 showed a positive significant correlation with ALT. Also, Child Pugh and MELD scores showed positive correlation with TNF-α, IL-6, EGF and CD163

Ultimately, serum biomarker levels regarding TNF-α, EGF, IL-6 and CD 163 were all significantly positively correlated with each other, while VEGF was only significantly correlated with EGF. Detailed data are shown in (Table 4).

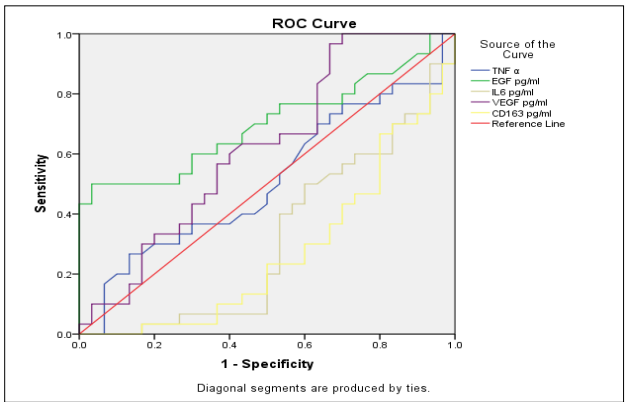

Figure (1): Receiver operating characteristic (ROC) curve showing the diagnostic performance of TNFα, EGF, IL6,

Table 4: Correlation study between the studied groups:

	TNF-α		EGF pg/ml		IL-6 pg/ml		VEGF pg/ml		CD163 pg/ml	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
ALT	0.132	0.247	0.321**	0.001	0.285*	0.01	0.076	0.432	0.320**	0.004
AST	0.280*	0.013	0.389**	0.001	0.510**	0.001	0.045	0.831	0.484**	0.001
Albumin	-0.388**	0.001	-0.495**	0.001	-0.705**	0.001	-0.075	0.526	-0.625**	0.001
T.Bilirubin	0.356**	0.001	0.514**	0.001	0.658**	0.001	0.071	0.462	0.677**	0.001
D.Bilirubin	0.307**	0.006	0.507**	0.001	0.665**	0.001	0.084	0.172	0.671**	0.001
Child Pugh score	0.400**	0.001	-0.508**	0.001	0.728**	0.001	0.022	0.847	0.760**	0.001
MELD score	0.439**	0.001	-0.590**	0.001	0.628**	0.001	-0.002	0.983	0.661**	0.001
TNF-α			0.301*	0.01	0.532**	0.001	0.138	0.342	0.438**	0.001
EGF pg/ml					0.463**	0.001	0.516**	0.001	0.294*	0.01
IL-6 pg/ml							0.219	0.051	0.837**	0.001
VEGF pg/ml									0.082	0.468

* *p* value <0.05 is significant, ** *p* value <0.01 is highly significant.

study by **liu [40]** validated the potential role of EGF in promoting motility of HCC cells through fibronectin.

Being a prominent pro-inflammatory cytokine; IL-6 serum levels revealed significant difference between the 3 studied groups, being highest in the HCC group and lowest in the control group. The current results were in concordance with several studies which demonstrated that elevated serum levels of IL-6 were associated with increased risk of HCC in chronic HCV patients. Moreover, a study by Lippitiz[11] showed increased serum levels of IL-6 in HCC patients in comparison to the control group.

Regarding to VEGF, there was a significant difference in the levels of VEGF between the 3 studied groups being highest in the HCC group and lowest in the control group; these results were concomitant with other studies made by Kaseb[15] & Debes[41] which revealed that higher serum VEGF level was associated with advancement of HCC disease together with poor overall survival and this suggests that lower levels may confer a favorable prognosis in the studied groups, however; these results were not consistent with Li[42] who didn't validate the role of high serum levels of VEGF in HCC development.

Finally, in regards to CD163, highest significant levels were detected in HCV group compared to HCC group and controls; these results were concomitant with Kong[24] and Minami[43] who reported that the serum level of sCD163 was only related to active hepatitis-related

DISCUSSION

Many coetaneous studies have been executed to explore the implications of hepatic inflammatory biomarkers in hepatocarcinogenesis-related pathways. These studies have aimed to establish novel therapeutic approaches that suppress their deleterious immune response. Recently, deeper insights into the pathogenesis of HCC have revealed the critical role of different inflammatory pathways in liver carcinogenesis[36,37]. The current study aimed to investigate and evaluate the serum levels of TNF-α, EGF, IL-6, VEFG, and CD163 in Egyptian patients with chronic HCV infection and healthy controls as well as to validate the relationship between these inflammatory biomarkers and the presence of HCC ontop of chronic HCV infection.

Results obtained in the presented study showed significant difference in the serum TNF-α levels between HCV as well as HCC group compared to the control group with no significant difference between HCV and HCC groups. This finding is congruous with Wang[19] who suggested the implication of high serum levels of TNF-α in the inflammatory pathway and HCC development.

As regards to EGF, there was a significant difference between the 3 groups. Again, these results were consistent with the studies performed by Tanabe[38] & Abu Dayee[39] which showed that high circulating EGF levels are associated with increased risk of HCC development in patients with liver cirrhosis, moreover the

patients with chronic HCV infection as well as various inter-relations between these markers. TNF-α, EGF, IL-6, VEGF and CD163 were proved to be potentially diagnostic and/or prognostic biomarkers for assessment of the extent of liver inflammation and eventually liver cirrhosis. The investigation of these biomarkers' combination might deliver more accurate information and cost-effectiveness for the early approach to HCC diagnosis and/or prognosis. Nonetheless, the diagnostic and predictive abilities of these biomarkers are still bounded by the heterogeneous environmental as well as genetic natures of HCCs; hence further studies in this direction "comprising larger sample size" are recommended to establish these biomarkers as novel diagnostic tools in assessing the stage of liver cellular damage and thus early diagnosis, prevention of progressive damage and ultimately prevention of HCC development. In addition, in the context of personalized medicine this would help in identifying individualized interventions between these markers.

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Conflict of Interest: none

Ethical approval: This project was approved by the TBRI ethical committee as well as TBRI institutional review board, FWA: 00010609. All procedures consistent with the ethical standards of the Institutional Ethical Research Committee and the 1964 Declaration of Helsinki and its subsequent amendments or similar ethical standards were followed in the study, particularly that included human participants.

Informed Consent: Written consent was obtained from all participants participating in this study.

REFERENCES

1. Balkwill F, Charles A, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer cell, 2005; 7(3): 211-217.
2. Chai P, Siveen S, Shanmugam K. Analysis of the intricate relationship between chronic inflammation and cancer. Biochem J, 2015; 468(1): 1-15.
3. Marelli G, Sica A, Vannucci L. Inflammation as target in cancer therapy. CurrOpinPharmacol, 2017; 35, 57-65.
4. Alavian, M and Haghbin H. Relative Importance of Hepatitis B and C Viruses in Hepatocellular Carcinoma in EMRO Countries and the Middle East: A Systematic Review. Hepat Mon, 2016; 16(3).
5. Waked I, Esmat G, Elsharkawy A. Screening and Treatment Program to Eliminate Hepatitis C in Egypt. N Engl J Med 2020; 382:1166-1174.
6. Ali N, Allam H, May R. Hepatitis C virus-induced cancer stem cell-like signatures in cell culture and murine tumor xenografts. J Virol 2011; 85: 12292-12303.
7. Mazzanti R, Arena U and Tassi R. Hepatocellular carcinoma: Where are we? WJEM, 2016; 6(1): p.21.
8. Evangelista S, Margherita M, Antonio F. Epidemiological and etiological variations in hepatocellular carcinoma. Infection, 2020; 48(1):7-17.
9. Mantovani A. The inflammation–cancer connection. FEBS J, 2018; 285(4): 638-640.
10. Nishida N and Kudo M. (2013). Recent advancements in comprehensive genetic analyses for human hepatocellular carcinoma. Oncology, 2013; 84(1): 93-97.
11. Madkour B, Gad A, Hamdy S. Interleukin-6-572 promoter gene polymorphism and its association with chronic hepatitis C-induced hepatocellular carcinoma: an Egyptian study. Comp Clin Path, 2018; 27:161-165.

factors and the degree of cirrhosis but not to tumor invasion, rendering its significance in HCC very limited, this was contradictory with Kazankov[44] who suggested that sCD163 could be of prognostic value in case of HCC progression.

Interestingly there was a significant positive correlation between VEGF and EGF, which suggests the ongoing crosstalk between both markers that results in angiogenesis and tumorigenesis[23] not only in hepatic disorders but also seen in gastric carcinoma, breast cancer and lung cancer, hence several studies were concerned with developing therapeutic strategies that target VEGF/STAT3 and (PI3K/Akt and NF-κB/ MMP-9/VEGF pathways[45,46].

Furthermore, there was a significant positive correlation between elevated liver functions including AST and bilirubin with high levels of IL-6, TNF-α, and CD163. This could be explained by their inter-mingling role in initiating hepatic inflammatory response via NF-KB signaling pathway activation[47]. These findings magnified the role of these pro-inflammatory cytokines in the disruption of liver functions leading to inflammation, fibrosis and cirrhosis; especially IL-6 and CD 163 which are proved to be crucial biomarkers of Kupffer cells' activation in diverse liver diseases.

Ultimately, there was a significant positive correlation between IL-6, TNF-α, and CD 163 with EGF which is supported by quite recent studies including Shostak and Chariot[21] and Liu[39] which enforced the close relationship between EGF and tumorigenesis as well as metastasis.

To our knowledge, this is the first study to investigate the potential diagnostic values of all the five biomarkers' serum levels in HCV, HCC and controls. In this study, ROC analysis showed diagnostic sensitivity and specificity values regarding TNF-α, EGF, IL-6, and CD163 aiding in the distinction between the 3 studied groups. Moreover, the studied markers have shown valuable diagnostic relevance in prediction of disease emergence but failed to demonstrate an acceptable diagnostic performance to establish HCC development on top of HCV. For example, regarding IL-6, Schmidt-Arras[48] showed that serum levels of IL-6 was high in HCC patients compared to the controls and this showed a moderate diagnostic accuracy in predicting the future HCC development. Furthermore, Xu[49] revealed that IL-6 in combination with IL-17 and IL-8 possesses significant diagnostic accuracy thus will be very helpful in HCC diagnosis. Interestingly VEGF with a NPV of 100% could be a very useful marker in ruling out the presence of HCC in HCV cirrhotic patients.

Ultimately, among many markers tested so far, only AFP has been adopted for diagnostic purposes and endorsed by relevant international guidelines. Some other biomarkers including PIVKAII, GP3, CSTB and SCCA1 have been tested but were proved to be diagnostically inferior compared to AFP[50].

CONCLUSION

Five important cytokines and growth factors had shown associations with the development of HCC in Egyptian

patients with chronic viral hepatitis B and C. Hepatol. 2014; 60: 521–30.
32. Solé C, Pose E, Solà E. Macrophage activation markers predict prognosis and decompensation in patients with cirrhosis—linking gut permeability, inflammation and cirrhosis progression. Aliment Pharm Ther, 2018; 47 (6): 851-853.
33. Chu M. Tumor necrosis factor. Cancer Lett, 2013; 328(2): 222-225.
34. Maziveyi M, Subramaniam V, Dash S. Altered Tumor Necrosis Factor-Alpha Signaling Pathway Between High and Low Metastatic Carcinoma Clones Derived from Single Tumor. J Compr Cancer Res, 2017; 1(1): 100005.
35. Sghaier I, Mouelhi L, Rabia A, Genetic variants in IL-6 and IL-10 genes and susceptibility to hepatocellular carcinoma in HCV infected patients. Cytokine, 2017; 31 (89): 62-7.
36. Taniguchi K and Karin M. NF-κB, inflammation, immunity and cancer: coming of age. Nat Rev Immunol, 2018.
37. Yasi P, Huarong C, Jun Y. Biomarkers in Hepatocellular Carcinoma: Current Status and Future Perspectives. Biomedicines, 2020; 8(12):576.
38. Tanabe K, Lemoine A, Finkelstein M, Epidermal growth factor gene functional polymorphism and the risk of hepatocellular carcinoma in patients with cirrhosis. JAMA 2008; 299:53-60.
39. Abu Dayyeh K, Yang M, Fuchs C. A functional polymorphism in the epidermal growth factor gene is associated with risk for hepatocellular carcinoma. Gastroenterology, 2011; 141:141-149.
40. Liu Z, Chen D, Ning F. EGF is highly expressed in hepatocellular carcinoma (HCC) and promotes motility of HCC cells via fibronectin. J cell biochem, 2018; 119(5):4170- 4183.
41. Debes D, van Tilborg M, Groothuismink M. Levels of cytokines in serum associate with development of hepatocellular carcinoma in patients with HCV infection treated with direct-acting antivirals. Gastroenterology, 2018; 154(3): 515-517.
42. Li Z, Xue Q, Chen Y. Predictive values of serum VEGF and CRP levels combined with contrast enhanced MRI in hepatocellular carcinoma patients after TACE. Am J Cancer Res, 2016; 6(10): 2375.
43. Minami K, Hiwatashi K, Ueno S. Prognostic significance of CD68, CD163 and Folate receptor β positive macrophages in hepatocellular carcinoma. ExpTher Med, 2018; 15(5), 4465-4476.
44. Kazankov K, Tordjman J, Møller J. Macrophage activation marker soluble CD163 and non-alcoholic fatty liver disease in morbidly obese patients undergoing bariatric surgery. J GastroenterolHepatol, 2015; 30(8), 1293-1300.
45. Tomasi L and Ramani K. Sumoylation and phosphorylation cross-talk in hepatocellular carcinoma. TranslGastroenterolHepatol, 2018; 3(4): 20.
46. Xiao H, Tong R, Ding C. γH2AX promotes hepatocellular carcinoma angiogenesis via EGFR/HIF-1α/VEGF pathways under hypoxic condition. Oncotarget, 2015; 6(4): 2180.
47. Feng Y, Zu L, Zhang L. MicroRNA-26b inhibits the tumor growth of human liver cancer through the PI3K/Akt and NF-κB/MMP-9/VEGF pathways. Oncol rep, 2018; 39(5): 2288-2296.
48. Chen S, Zheng P, Wang W. Abberent expression of NOR1 protein in tumor associated macrophages contributes to the development of DEN-induced hepatocellular carcinoma. J cell physiol, 2018; 233(6): 5002-5013.
49. Schmidt-Arras D and Rose-John S. IL-6 pathway in the liver: From physiopathology to therapy. J Hepatol, 2016; 64(6): 1403-1415.
50. Xu F, Yi J, Wang F. Involvement of soluble B7-H3 in combination with the serum inflammatory cytokines interleukin-17, -8 and-6 in the diagnosis of hepatocellular carcinoma. Oncology Lett, 2017; 14(6): 8138-8143.
12. Lippitz E and Harris A. Cytokine patterns in cancer patients: A review of the correlation between interleukin 6 and prognosis. Oncoimmunology, 2016; 3; 5(5): e1093722.
13. Wu X, Xin Z, Zhang W. Polymorphisms in the VEGFA promoter are associated with susceptibility to hepatocellular carcinoma by altering promoter activity. IJC, 2013; 133(5): 1085-1093.
14. Dan G, Simona O, Dana C. Potential Circulating Biomarkers of Recurrence after Hepatic Resection or Liver Transplantation in Hepatocellular Carcinoma Patients. Cancers (Basel), 2020; 12(5): 1275.
15. Mashreghi M, Azarpara H, Bazaz M. Angiogenesis biomarkers and their targeting ligands as potential targets for tumor angiogenesis. J cell physiol, 2018; 233(4): 2949-2965.
16. Kaseb O, Morris S, Hassan M. Clinical and prognostic implications of plasma insulin-like growth factor-1 and vascular endothelial growth factor in patients with hepatocellular carcinoma. J ClinOncol, 2011; 29 (29): 3892.
17. Zhou Q, Cheung B, Jada R. EGFR Intron 1 polymorphism in Asian Populations and its correlation with EGFR gene expression and amplification in breast tumor tissues. Cancer BiolTher, 2006; 5: 1445-1449.
18. Hansen F, Sorensen B, Spindler L. Microvessel density and the association with single nucleotide polymorphisms of the vascular endothelial growth factor receptor 2 in patients with colorectal cancer. Virchows Arch, 2010; 456: 251-260.
19. Wang H, Liu J, Hu X. Prognostic and therapeutic values of tumor necrosis factor-alpha in hepatocellular carcinoma. IMJECR, 2016; 22: 3694.
20. Natthaporn S, Anchalee T, Ravat P. HCC-derived EGFR mutants are functioning, EGF-dependent, and erlotinib-resistant Cell & Bioscience, 2020; 10: 41.
21. Shostak K and Chariot A. EGFR and NF-κB: partners in cancer. Trends mol med, 2015; 21(6): 385-393.
22. Ai J, Li J, Lin M. R-Smad signaling-mediated VEGF expression coordinately regulates endothelial cell differentiation of rat mesenchymal stem cells. Stem Cells Dev, 2015; 24(11): 1320-1331.
23. Wang F, Wang L, Li Y. PAC-1 and its derivative WF-210 Inhibit Angiogenesis by inhibiting VEGF/VEGFR pathway. Eur. J pharmacol, 2018; 821: 29-38.
24. Kong Q, Zhu D, Xu X. The clinical significance of the CD163+ and CD68+ macrophages in patients with hepatocellular carcinoma. PLoS One, 2013; 8(3): e59771.
25. Ayon B , Rani A, Madison F. Soluble Receptors Affecting Stroke Outcomes: Potential Biomarkers and Therapeutic Tools. Int J MolSci, 2021; 22: 1108
26. Vano Y, Giraldo A, Fridman H. The Human Tumor Microenvironment. In Oncoimmunology, 2018; 5-21. Springer, Cham.
27. Hogger P, Dreier J, Droste A., Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid inducible member of the scavenger receptor cysteine-rich family (CD163). J Immunol, 1998; 161: 1883–1890.
28. Jensen O, Schmidt H, Moller J, Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J ClinOncol, 2007; 27: 3330–3337.
29. Komohara Y, Hasita H, Ohnishi K, Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. Cancer Sci, 2011; 102: 1424–1431.
30. Aljabery F, Olsson H, Gimm O. M2-macrophage infiltration and macrophage traits of tumor cells in urinary bladder cancer. UrolOncol-SeminOri, 2018; 36 (4):159-e19.
31. Kazankov K, Barrera F, Moller J. Soluble CD163, a macrophage activation marker, is independently associated with fibrosis in