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Research Article

Up-regulation of hepatic expression of liver cancer stem cells (CD44) promotes evolution and progression of HCV-associated hepatocellular carcinoma

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Abstract

Background and aims: Molecular pathogenesis of hepatocellular carcinoma (HCC) is not yet clear despite the global widespread of this disease. Currently the significant association between cancer stem cells and various solid tumors acquired a great attention. Therefore, the aim of this study was to investigate the expression of hepatic cancer stem cells (CD44) in HCC patients and to correlate the results with the disease outcome. **Subjects and Methods:** A total of 20 patients with HCV-associated HCC were included in this study. They were compared to 20 patients with HCV-related cirrhosis without HCC and 20 healthy controls. The hepatic expression of CD44 protein was assessed by immunohistochemistry (IHC). **Results:** Compared with cirrhotic patients without HCC and healthy controls, cirrhotic patients with HCC had significantly higher hepatic expression of CD44. The hepatic expression of CD 44 was correlated positively with blood levels of alpha-fetoprotein (AFP) ($r = 0.29$; $p = 0.04$). Significant associations were found between the higher hepatic expression of CD44 and the increased frequency of patients with vascular invasion (93.7% vs 6.25%; $p = 0.001$). **Conclusion:** Increased hepatic expression of CD44 may have a role in growth and aggressiveness, of HCV-associated HCC, irrespective of the liver functional status.

Key words: Hepatitis C virus; Hepatocellular carcinoma; Liver cancer stem cells; Cluster of differentiation 44.

Introduction

Hepatocellular carcinoma (HCC) ranks fourth in terms of cancer-related mortality and is the sixth most common type of cancer, making it a global health concern[1]. Regardless of the underlying cause, 80–90% of patients with a history of liver cirrhosis experience its development [2]. Because of its broad prevalence in our locality, chronic hepatitis C virus (HCV) infection is one of the many etiological reasons of liver cirrhosis and the most common risk factor for developing HCC in Egypt [3–4].

HCC is quite variable; from a clinical perspective, over 80% of patients are identified with an advanced stage, necessitating palliative therapy. Other factors that contribute to this heterogeneity include a high 5-year recurrence rate[6], a variety of etiological risk factors [5]. Currently, the imaging methods utilized to diagnose and stage HCC are not very accurate

[7]. Furthermore, different histological subtypes may conflict with a precise diagnosis [8]. Regarding the molecular pathways, hepatocarcinogenesis, tumor growth, and metastasis are accelerated by a number of genetic and epigenetic changes that interact with the tumor microenvironment (TME) [9].

Evidence has been accumulating to support the hypothesis that solid tumors contain a small subpopulation of cancer stem cells (CSCs), which exhibit self-renewing capacities and are responsible for tumor maintenance and metastasis and possibly for resistance towards chemotherapy and radiation therapy[10]. One of the most important markers of CSC is cluster of differentiation-44(CD44) which is most frequently reported in HCC[10]. CD44 is a multi-structural and multifunctional transmembrane glycoprotein, which was initially identified as a receptor for hyaluronan that participates in both physiological and pathological processes[11]. The molecular structure of CD44 functions as a receptor for hyaluronic acid and other extra cellular matrix components, enabling CSCs to sense environmental changes and mediate signaling transduction to regulate CSC stemness properties[12]. Consequently, CD44 binding regulates CSC survival, self-renewal, maintenance, and chemoresistance[13], which at least in part explains why CD44 is critical for disseminated cancer cells to adapt to new environments and why CD44 is required for metastatic colonization[14].

Therefore, the aim of this study was to investigate the hepatic expression of CD44 protein, in Egyptian patients with HCV-associated HCC, and to correlate the results with the clinicopathological characteristic of the tumor and disease outcome.

Subjects and Methods

Eligible subjects

Between May 2019 and February 2021, the Internal Medicine and Pathology Departments at Minia University Hospital, Egypt, collaborated with the Pathology Department at Minia Oncology Center, Egypt, to conduct the current retrospective, cross-sectional, comparative study. To achieve 99% power, 20 patients with HCV-related HCC were recruited in this study. It was calculated at the 0.05 level of significance using G Power 3.19.2 software. The study examined formalin-fixed paraffin-embedded liver tissues from 20 patients with HCC and HCV-associated liver cirrhosis. This group of patients was compared to two other groups: HCV-related liver cirrhosis patients who did not have HCC and healthy controls. Information about patients with cirrhosis and HCC was taken from their medical records in the archives of the Minia Oncology Center. Patients were only considered eligible if they had sufficient liver tissue and comprehensive clinicopathological data. Diabetes mellitus that was diagnosed according to the criteria of American Diabetic Association [15], endocrine disorders, and chronic liver illnesses other than chronic HCV infection were among the exclusion criteria., also end-organ failure, organ transplantation, hepatic resection, prior locoregional treatment for HCC, extrahepatic and hematological malignancies, autoimmune diseases, as well as steroid and immune-suppressive medications.

Hepatocellular carcinoma patients (group I): This group comprised 20 patients with HCV-related cirrhosis (15(75%) males, and 10(25%) females). They were recruited from attendants of Minia Oncology Center for liver biopsy. The diagnosis of HCC was based on the characteristic imaging criteria as defined by Bruix and Sherman [16] and histological evaluation [17].

Liver cirrhosis patients (group II)

This group comprised 20 patients with HCV-related cirrhosis (15(75%) males, and 5(25%) females). They were consecutively enrolled from those referred by outpatient clinics. The diagnosis of liver cirrhosis was built on the standard clinical criteria, in addition to the histopathological examination [18]. Presence of anti-HCV and detection of serum HCV-RNA for 6 months or more, were characteristic features of chronic HCV infection.

Healthy controls (group III):

A total of 20 healthy subjects were prospectively collected from subjects who underwent abdominal surgery in the Department of General Surgery at Minia University Hospital. They were 15(75%) males and 5(25%) females. All were clinically free and showed nothing abnormal in laboratory analyses.

Informed consent:
This study protocol was approved by the Institutional Ethics Committee of the Faculty of Medicine, Minia University and by Institutional Review Board of Minia Oncology Center, Egypt. Informed written consents were obtained from all subjects.

Clinical and laboratory assessment
Examining the medical files yielded demographic, clinical, and laboratory results, such as the peripheral hemogram, liver and kidney function tests, fasting and postprandial serum glucose levels, plasma levels of alpha fetoprotein (AFP), virological assays, and imaging studies. The Child-Pugh [19] and the Model for End-Stage Liver Disease (MELD) [20] scoring systems were used to assess the liver's functional state. The clinicopathological aspects were evaluated using the staging systems proposed by Okuda [22] and Tumor-Node-Metastasis (TNM) [21]. The healthy volunteers were asked to fill out a questionnaire on their age, sex, history of alcohol and tobacco use, and current medical conditions, such as diabetes mellitus. They used the commercially available kits in accordance with the manufacturer's instructions to give venous blood samples for the purpose of evaluating the previously specified laboratory tests.

Immunohistochemistry (IHC)
IHC was performed on 4-µm tissue sections taken from 10% buffered formalin-fixed, paraffin-embedded tissue blocks.Sections were deparaffinized in xylene bath and rehydrated in graded alcohol. Hydrogen peroxide was used to block endogenous peroxidase activity. Antigen retrieval was carried out utilizing citrate buffer concentrate (pH 6). Rabbit CD44 monoclonal antibody (1/100 dilution**, Abcam, ab51037**, **USA**), were used overnight as primary antibodies. Visualization was performed by Avidin-Biotin detection system (DAKO).

Interpretation of CD44 immunoreactivity. The specimens were evaluated twice in different times by two experienced pathologists, blinded for the clinico-pathological data of the study subjects. The final staining scores of CD44 were calculated by multiplying the intensity score by the percentage score. The intensity of score was regarded as: absent: 0; weak: 1, moderate: 2, and strong: 3, whereas, the percentage score was graded as follows: none: 0, 1: 1–10%, 2:11–33%, 3:34–66%, and 4: 67–100%. For statistical analysis, a final staining score <4 was considered as low expression and a score >4 as a high expression.

Statistical analysis:
The analysis of the data was carried out using the IBM SPSS for windows (version 25).Normality of the data was tested using the One -sample Kolmogorov Smirnov test.Data were expressed as mean ±standard deviation (SD) and minimum and maximum of range for parametric quantitative data and by median interquartile range (IQR) for non-parametric quantitative data, in addition to both number and percentage for qualitative data.

Analyses were done between the three groups for parametric quantitative data using one way analysis of variance (ANOVA) test followed by Bonferroni post-hoc test between each two groups, analyses were done between the three groups for non-parametric quantitative data using Kruskal Wallis test followed by Mann Whitney test between each two groups, while the Chi-square and Fisher exact tests were used to compare categorical variables, when appropriate.

Analyses were done between the two groups for parametric quantitative data using Student's t-test, and for non-parametric quantitative data using Mann Whitney test.
Correlation between variables were done using Pearson's correlation for continuous variables and using Spearman's correlation for ordinal variable.
P-value ≤0.05 was considered statistically significant.

Results
The present study included 20 cirrhotic patients with HCC (**group 1**), of whom 15 (75%) were male. The mean age at initial diagnosis was 66 ± 8.1 years. All patients were positive for anti-HCV and PCR for HCV-RNA. Compared with 20 cirrhotic patients without HCC (**group II**), comprised 15 men and 5 women. Their ages ranged from 38 to 83 year old with mean ± SD of 61.9±11.9 years and 20 healthy controls **(group III)**. They comprised 15 men and 5 women. Their ages ranged from 50 to 75 year old with mean ± SD of 63.7±11.1 years.

The demographics of the 3 study groups were comparable as regards age ,sex and smoking exposure. Concerning BMI, there was no statistical significant difference between HCC and cirrhotic groups ,however both groups displaced statistically significant lower values when compared with healthy controls (25.8±3.7 kg/m^2 vs 29.2±2.6 kg/m^2, p<0.001 and 24.3±2.5 kg/m^2 vs 29.2±2.6 kg/m^2, p<0.001, respectively) (**Table 1).**

Table (1): Baseline demographics of the study groups

		Group I HCC	Group II Cirrhotic patients	Group III Control	p value			
		N=20	N=20	N=20	Among 3 groups	I vs II	I vs III	II vs III
Age (years)[1]	***Range*** ***Mean ± SD***	(50-83) 66.9±7.8	(38-83) 61.9±11.9	(50-75) 63.7±11.1	0.389	0.357	0.715	0.821
Sex (male/female) [n (%)][2]	***Male*** ***Female***	15(75%) 5(25%)	15(75%) 5(25%)	15(75%) 5(25%)	1	1	1	1
BMI (kg/m2)[1]	***Range*** ***Mean ± SD***	(20-34.6) 25.8±3.7	(20-28) 24.3±2.5	(26.8-32.5) 29.2±2.6	***<0.001****	0.104	***<0.001****	***<0.001****
Smoking [n (%)][2]	***No*** ***Yes***	12(60%) 8(40%)	14(70%) 6(30%)	15(75%) 5(25%)	0.583	0.507	0.311	0.723

HCC: hepatocellular carcinoma; N: number; BMI: Body mass index; (kg/m^2): Kilogram/meter^2; SD: standard deviation; ANOVA: analysis of variance.

1= Results are expressed as mean ± SD and compared by One way ANOVA test followed by Bonferroni post hoc test.

2= Results are expressed as frequency (percentage) and compared by Chi square test and Fisher's exact

****: Significant level at p value ≤ 0.0***

Table (2) shows that there was no statistically significant difference in any of the studied parameters between cirrhotic patients with HCC and those without. However , the serum levels of total bilirubin, ALT, AST, PT, INR and AFP were significantly higher in HCC patients than in healthy controls [1.2(0.9–1.5)mg/dl vs.0.8(0.7–0.9) mg/dl, p = 0.001; 42.5(34.3–75.8)IU/L vs. 26(18–38)IU/L, p<0.001;59(37.3–86.5) IU/L vs. 31(25–32)IU/L, p<0.001; 14.1±2.9sec. vs. 12.3±1.1sec., p = 0.04; 1.4±0.5 vs. 1.1±0.1, p = 0.041, and 56.5(22.5–390.3)ng/ml vs.5(3–8)ng/ml, p<0.001, respectively]. Whilst, they displayed statistically significant lower values of platelet count, and serum albumin versus healthy control group [185(130.5-238.8) (1×10^3/µl) vs. 235(190.8-248) (1×10^3/µl), p = 0.013, and 3.3±0.8 gm/dl vs. 4.2±0.5 gm/dl, p = 0.029, respectively].

In HCC group of patients there was classified according to TNM and Okuda stages where there was 5(25%) of them were TNM I+II and 15(75%) were TNM III+IV, while 11(55%) were classified Okuda stage 1 and 9(45%) were Okuda stage 2&3.

Table (2): Baseline characteristics in the study groups

		Group I HCC	Group II Cirrhotic patients	Group III Control	P value			
					Among 3 groups	I vs II	I vs III	II vs III
		N=20	N=20	N=20				
Hemoglobin (gm/dl) [1]	*Range* *Mean ± SD*	(7.8-15.9) 12.5±1.8	(7.8-15.2) 13±1.6	(11.7-15.5) 13.1±1.5	*0.496*	*0.624*	*0.512*	*0.982*
WBCs (1×10^3/µl)[2]	*Median* *IQR*	7.5 (5.3-9.8)	7.5 (4.9-9.2)	4.9 (4.6-12)	*0.417*	*0.797*	*0.192*	*0.356*
PLT (1×10^3/µl) [2]	*Median* *IQR*	185 (130.5-238.8)	183 (158.3-214)	235 (190.8-248)	***0.005****	*1*	***0.013****	***0.001****
Total bilirubin (mg/dl) [2]	*Median* *IQR*	1.2 (0.9-1.5)	1 (0.8-1.2)	0.8 (0.7-0.9)	***<0.001****	*0.338*	***0.001****	***0.001****
ALT (IU/L) [2]	*Median* *IQR*	42.5 (34.3-75.8)	42.9 (36-65.9)	26 (18-38)	***0.001****	*0.892*	***0.001****	***0.001****
AST (IU/ L) [2]	*Median* *IQR*	59 (37.3-86.5)	57.9 (44.6-81.6)	31 (25-32)	***<0.001****	*0.924*	***<0.001****	***<0.001****
Albumin (gm/dl) [1]	*Range* *Mean±SD*	(2.3-4.6) 3.3±0.8	(2.3-4.9) 3.9±0.8	(3.6-4.3) 4.2±0.5	***0.035****	*0.207*	***0.029****	*0.634*
PT (seconds) [1]	*Range* *Mean±SD*	(11.3-24) 14.1±2.9	(11.3-24) 13.7±3.1	(11-14) 12.3±1.1	***0.046****	*0.737*	***0.042****	*0.200*
INR[1]	*Range* *Mean±SD*	(1-2.4) 1.4±0.5	(1-2.4) 1.3±0.4	(1-1.3) 1.1±0.1	***0.046****	*0.794*	***0.041****	*0.224*
Urea (mg/dl) [2]	*Median* *IQR*	37 (27-44.5)	30.5 (27-37.8)	29 (26-49.3)	*0.529*	*0.244*	*0.438*	*0.724*
Creatinine (mg/dl) [2]	*Median* *IQR*	0.9 (0.6-1.1)	0.9 (0.7-1)	0.7 (0.6-0.8)	*0.076*	*0.935*	*0.084*	0.1480
AFP (ng/dl) [2]	*Median* *IQR*	56.5 (22.5-390.3)	20 (5.1-400)	5 (3-8)	***<0.001****	*0.078*	***<0.001****	***<0.001****
TNM stage [n. (%)] **I+II** **III+IV**		5 (25%) 15(75%)	–	–	–	–	–	–
Okuda stage [n. (%)] **1** **2+3**		11(55) 9(45%)	–	–	–	–	–	–

HCC: hepatocellular carcinoma; N:number; SD:standard deviation; ANOVA: analysis of variance; IQR: interquartile range; WBCs: white blood cells; PLT: platelets; ALT:alanine transaminase; AST: aspartate transaminase; PT:prothrombin time; INR: international normalized ratio; AFP: alpha fetoprotein. TNM: Tumor, Nodes and Metastasis.

1= Results are expressed as mean ± SD and compared by One way ANOVA test followed by Bonferroni post hoc test .

2=Results are expressed as median(IQR) and compared by Kruskal Wallis test followed by Mann Whitney test between each two groups

****: Significant level at p value ≤ 0.05***

Table 3 shows that there was no statistical significant difference between cirrhotic patients with HCC and those without as regard the different hepatic functional scoring systems (Child-Pugh score, class and MELD score).

Table (3): Comparison of scores of hepatic functional status in cirrhotic patients with hepatocellular carcinoma versus those without.

		Group I HCC	Group II Cirrhotic patients	P value
		N=20	N=20	
Child-Pugh score	*Range* *Mean ± SD*	(5-8) 6.3±1.3	(5-8) 5.6±0.9	*0.053*
Child-Pugh - class	*Class A+ B* *Class C*	14(70%) 6(30%)	16(80%) 4(20%)	*0.465*
MELD score	*Median* *IQR*	10 (7.3-13)	7 (6-11)	*0.154*

HCC: hepatocellular carcinoma; N: number; IQR: interquartile range; ANOVA: analysis of variance; MELD: Model for End-stage Liver Disease,
1. Data are expressed as mean ± SD and compared using Student's t-test
2. Data are expressed as frequency(percentage) and compared using Chi-square and Fisher's exact test when appropriate.
3. Data are expressed as median (IQR) and compared using Mann Whitney test

The hepatic expression of CD 44 was found to be significantly higher in cirrhotic patients with HCC compared to those without HCC (3.8(1.3-6) vs. 1.4(1–3.8), p=0.042), and between HCC and control group (3.8(1.3-6) vs. 1(1–2), p=0.004).(**Table 4)**

Table (4): Comparison of hepatic expression of CD 44 proteins in the study groups.

Hepatic expression		Group I HCC	Group II Cirrhotic patients	Group III Control	p value			
		N=20	N=20	N=20	Among 3 groups	I vs II	I vs III	II vs III
CD 44 score	*Median* *IQR*	3.8 (1.3-6)	1.4 (1-3.8)	1 (1-2)	***0.009****	***0.042****	***0.004****	0.313

HCC: hepatocellular carcinoma; N=number; IQR: interquartile range.
Data are expressed as median (IQR)and compared using Kruskal Wallis test followed by Mann Whitney test .
****: Significant level at p value ≤ 0.05.***

It was also found that the hepatic expression of CD 44 was correlated positively with smoking exposure (r = 0.33; p = 0.011), and AFP (r = 0.29; p = 0.04). **(Table 5).**

Table (5): Correlations of hepatic expression CD 44 immunoreactivity with different clinicobiochemical parameters.

	N	CD 44 score	
		r	***P value***
Age (years) (P)	60	0.154	0.239
BMI (kg/m2) (P)	60	-0.233	0.073
Smoking (S)	60	**0.33**	**0.011***
Hemoglobin (gm/dl)(P)	60	0.216	0.097
WBCs(x10³)/ml(P)	60	0.191	0.143
PLT(x10³)/ml(P)	60	-0.168	0.200
Total bilirubin (mg/dl)³ (P)	60	-0.107	0.415
ALT (IU/L) (P)	60	0.117	0.374
AST (IU/L) (P)	60	0.168	0.199
Albumin (gm/dl) (P)	60	-0.052	0.691
PT (seconds) (P)	60	0.006	0.962
INR (P)	60	0.086	0.515
Urea(mg/dl) (P)	60	0.093	0.480
Creatinine (mg/dl) (P)	60	0.094	0.476
AFP (ng/ml)P (P)	60	**0.29**	**0.04***
CHILD Class (S)	40	-0.136	0.401
Child-pugh score (P)	40	-0.079	0.629
MELD score (P)	40	-0.181	0.263

N:number of patients; CD 44: cluster of differentiation 44; BMI: body mass index; WBCs: white blood cells; ALT: alanine transaminase; AST: aspartate transaminase; AFP: alpha fetoprotein; PT: prothrombin time; INR: international normalized ratio; MELD: Model for End-Stage Liver Disease.

(P) Pearson's correlation; (S) Spearman's correlation

Grading of correlation coefficient:0-0.29=mild;0.30-0.49=moderate; 0.50-1=marked

****: Significant level at p value ≤ 0.05***

Table 6 revealed that, higher hepatic expression of CD 44 exhibited significantly higher frequency of HCC patients with vascular invasion (87.5% vs. 12.5%; p = 0.001).

Table (6): Relationship between hepatic expression of CD 44 proteins and clinicopathological features of the tumor in hepatocellular carcinoma patients.

Variable	Hepatic expression of CD44			
	n.	no/low n. (%)	High n. (%)	P-value
Age (year)				
≤ 60	10	6 (60)	4 (40)	1
> 60	10	7 (70)	3 (30)	
Gender				
Male	17	15(88.2	2(11.8)	1
Female	3	2(66.7)	1(33.3)	
Child-Pugh class				
A	14	9(64.3)	5(35.7)	0.341
B+C	6	4(66.7)	2(33.3)	
MELD Score				
≤ 12	10	5 (50)	5 (50)	1
> 12	10	5 (50)	5 (50)	
AFP (ng/ml)				
≤ 100	10	5 (50)	5 (50)	1
> 100	10	5 (50)	5 (50)	
Tumor number				
Single	12	8 (66.7)	4(33.3)	0.650
Multiple	8	3 (37.5)	5(62.5)	
Tumor size (cm)				
≤ 5	10	5 (50)	5 (50)	1
> 5	10	5 (50)	5 (50)	
Vascular invasion				
No	4	1 (25)	3 (75)	**0.001***
Yes	16	2(12.5)	14(87.5)	
Lymphatic permeation				
No	18	8 (44.4)		
Yes	2	1 (50)	10(55.6) (50)	0.832
TNM [n.(%)]				
I+II	12	6 (50)	6 (50)	1
III+IV	8	4 (50)	4 (50)	
Okuda [n.(%)]				
1	11	6 (54.5)	5 (45.5)	0.931
2+3	9	4 (44.4)	5 (55.5)	

n: number of patients; CD 44: cluster of differentiation 44; MELD: Model for End-Stage Liver Disease; AFP: alpha fetoprotein ; TNM: Tumor, Lymph nodes and Metastasis.
Data are expressed as proportions and percentages, and compared using Chi-square statistic or Fisher's exact test
: Significant level at p value ≤ 0.05

Figure 1 (a-c), shows the hepatic expression of CD 44 proteins in the 3 studied groups where it was highly expressed in HCC group**.**

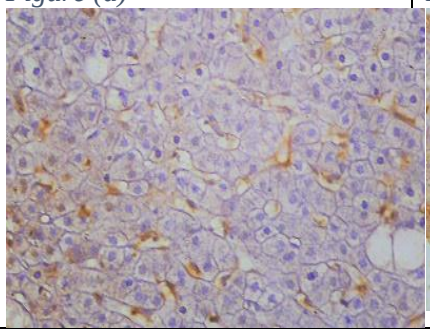
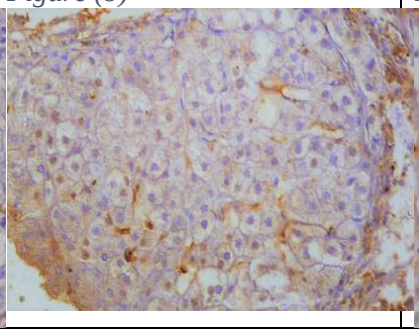
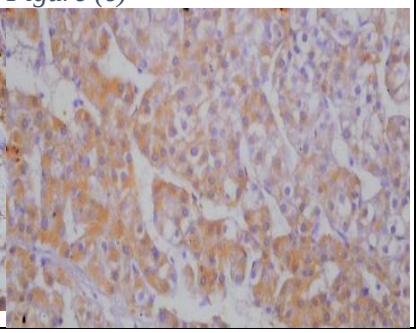
	Control	Cirrhosis	HCC
CD44	*Figure (a)*	*Figure (b)*	*Figure (c)*
			

Figure 1: (a-c): Hepatic expression of CD 44 protein in the study groups Magnification 200 X scale bare 100um.

Figure 2 (a-d) shows examples of hepatic expression of CD44 protein in a variety of stromal cells of tumor microenvironment (TME) in HCC and cirrhotic patients

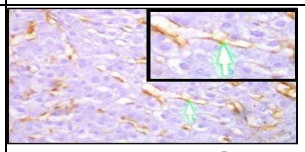
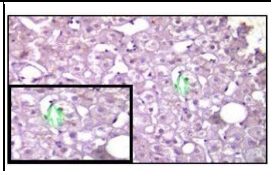
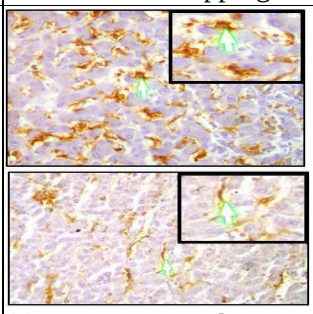
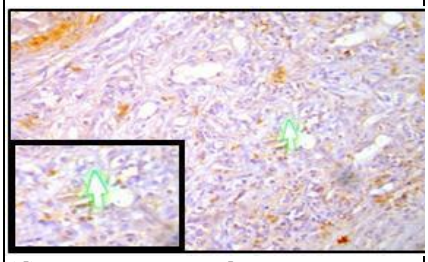
Immunohistochemical marker and detection site	Hepatocellular carcinoma	Liver cirrhosis
CD44 expression in sinusoidal cells	 (a): positive cytoplasmic immunoexpression at sinusoidal wrapping cells	 (b): negative cytoplasmic immunoexpression
CD44 expression in stromal stem cells	 (c): positive cytoplasmic immunoexpression	 (d): negative cytoplasmic immunoexpression

Figure 2 (a-d): Examples of hepatic expression of CD44 protein in a variety of stromal cells of tumor microenvironment in hepatocellular carcinoma and cirrhotic patients. Images are presented at 20X magnification power with 200X zoom in boxes.

Discussion

HCC has a high death rate and is still an incurable disease despite a number of treatment options, such as radiotherapy, chemotherapy, local ablation, and surgical excision[23]. Consequently, it is critical to investigate the molecular pathways behind HCC in order to find elements that may aid in the creation of treatments that would increase patient survival.

In HCV-related HCC patients, HCV infection causes the development of different inflammatory and fibrotic mediators such as proinflammatory cytokines, cell death signals,

and reactive oxygen species[24], as well as, hepatic stellate cells activation[25].

Hepatocarcinogenesis is initiated and promoted by the cirrhotic microenvironment created by all of these processes, which is known as "field cancerization"[26]. Growing data in recent times has characterized the function of CSCs [27] and EMTs [28] in the development of HCC.

In this study; we evaluated the CD44 molecule; which is non-kinase cell surface transmembrane glycoprotein that is overexpressed in CSCs and frequently undergoes alternative splicing to support cancer progression[29]. It is also known that CD44 serves as the primary cell surface receptor for hyaluronate, the main component of the extracellular matrix .It belongs to the family of cell adhesion molecules, which are crucial for intercellular adhesion and communication as well as between cells and the extracellular matrix[30].

We found that HCC patients exhibited significantly higher hepatic expression of CD44 than those of cirrhosis and control groups. Our findings were consistent with those of Rozeik et al.,[31], who sought to assess hepatic expression of CD44 in Egyptian patients with HCV-induced chronic liver diseases and hepatocellular carcinomas. They discovered that the mean expression of CD44 values increased significantly with disease progression, rising from 33.7% in chronic non-cirrhotic hepatitis to 58.65% in the cirrhotic group and up to 78.35% in the HCC group. It was also consistent with the findings of Mustika et al.,[32], who examined the expression of CD44 in Indonesian patients with advanced liver disease and found that there were substantial differences in CD44 expression among the HCC, chronic hepatitis, and liver cirrhosis groups. In contrast, Zhao et al.,[33] observed that the presence of HCC had no effect on CD44 expression patterns. These conflicting results may be attributed to the different patients' characteristics and methodologies.

Furthermore; we found that the hepatic expression of CD-44 was correlated positively with the serum level of AFP and that the higher hepatic expression of CD44 was associated with the significantly higher frequency of patients with vascular invasion. These findings were in agreement with those of other researchers (Luo&Tan[10]; Zhang et al.,[34]; Xie et al.,[35]), who found that CD44 was overexpressed in HCC and that HCC patients with high expression level of CD-44 exhibited more aggressive malignant features than those with low expression, revealing the significant role of CD44 in cancer cell local invasion, intravasation, migration, and the establishment of metastatic lesions. CSCs can undergo EMT, invade, circulate in the bloodstream, and spread to distant regions, resulting in metastatic tumors.CSCs appear to enter a more quiescent state (G0), with lower proliferative activity.In this stage, cells defy chemotherapy and persist, resulting in recurrence. [36].Furtheremore, CSCs exhibit increased DNA repair and reduced apoptosis[37]. The TME protects CSCs by delivering anti-apoptotic, stemness-maintaining factors, and matrix components[38].

Another noteworthy finding of this study is the increased hepatic expression of CD44 in a number of HCC microenvironmental cells, including liver sinusoidal endothelial cells (LSECs) and stromal inflammatory cells in HCC patients. LSECs contribute to the development of chronic liver damage and hence tumourigenesis by permitting the persistence of chronic viral infections, worsening of fibrosis, acquisition of angiogenesis, and EMT.[39]. Hepatocyte stellate cells, fibroblasts, endothelial cells, adipocytes, and immune cells; which include CD8+ T cells, regulatory T cells, dendritic cells, and macrophage sare the primary stromal inflammatory cells in the HCC microenvironment. These cells interact with HCC to create an environment that is conducive to the growth of tumors[40].

In conclusion, by using IHC method, the hepatic expression of CD44 was found to be significantly upregulated in HCC patients. Moreover, HCC patients with high hepatic expression of this molecule exhibited more tumor aggressiveness, irrespective of the functional status of liver. However, the ultimate utility of these results in practice warranted further validation by other large prospective, multi-center studies.

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