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Hepatitis C Virus as risk factor for development of hepatocellular carcinoma in Egypt: I- HCV promotes HCC progression by increasing cancer stem marker (CD133 and CD44) expression

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ABSTRACT

Hepatitis C virus (HCV) represents a major public health problem, affecting 3% of the world's population. In majority of infected patients, HCV can effectively evade innate immunity resulting in chronic hepatitis, which can progress to cirrhosis and hepatocellular carcinoma (HCC). Similar to most solid tumors, HCCs are believed to contain poorly differentiated cancer stem cell-like cells (CSCs) that initiate tumorigenesis and confer resistance to chemotherapy. The present work attempted to study the mRNA expression of cancer stem cell markers (CD133 and CD44) in patients with chronic hepatitis C virus (reflecting the role of HCV) and their correlation with progression toward cirrhosis and HCC. Peripheral blood mononuclear cell (PBMC) prepared from chronic HCV patients (either with or without complications) were probed for mRNA expression of CD133 and CD44 by RT-PCR and compared to that of non-HCV cirrhotic patients as well as healthy control subjects. Our results showed that mRNA expression of CD133 was significantly elevated in all HCV patients either with or without complications but not in those with non-HCV cirrhosis, with maximal expression in patients without complications (HCV patients only). On the other hand, maximal CD44 mRNA expression was recorded in HCC patients. Taken together, these results suggests that, chronic HCV infection appear to predispose cells towards the path of acquiring cancer stem cell traits by inducing CD133 and CD44 expression and it prove the hypothesis that the viral interference with signaling network of normal stem cells leads to their transformation into CSCs.

Keywords: Hepatitis C Virus, hepatocellular carcinoma, Egypt, I- HCV, HCC, CD133, CD44

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INTRODUCTION

Hepatitis C virus (HCV) infection remains a serious burden to public health affecting 2-3% of the world population ⁽¹⁾. HCV is a positive-strand RNA virus with an enveloped virion belonging to the family *Flaviviridae* ⁽²⁾. The viral genome (9,600 nucleotides) encodes a single polyprotein that is processed co-translationally into three structural proteins (core, envelope E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) ⁽³⁾.

It is believed that control of HCV infection is determined by the interplay between virus and the host immune system ⁽⁴⁾. In the majority of HCV infected patients (70-80%), HCV can effectively evade immune challenge ⁽⁵⁾ resulting in delayed HCV specific immune response in addition to alteration in maturation and functional differentiation of immune cell leading to persistent viral infection ⁽⁶⁻⁸⁾. Banerjee et al., (2010) ⁽⁹⁾ reported that, HCV proteins interact with host-cell factors that are involved in cell cycle regulation, transcriptional regulation, cell proliferation and apoptosis which contribute to the viral oncogenic processes. Strong positive correlation between HCV-induced chronic liver disease and the development of hepatocellular carcinoma (HCC) is widely accepted, the molecular mechanisms underlying hepatocarcinogenesis is not fully elucidated ⁽¹⁰⁾.

Hematopoietic stem cells are the only cells that possess the ability of both multi-potency and self-renewal ⁽¹¹⁾. Warr et al., (2011)⁽¹²⁾ proved that, cancer stem cells may arise from an intrinsic mutation leading to disturbance of signaling network implicated in regulating self-renewal of normal stem cells as well as proliferation or differentiation of progenitor cells. Therefore, blood derived CSCs represent an interesting source of biological information to understand the malignancy-associated dissemination, drug resistance and treatment-induced cell death ⁽¹³⁾. They found that co-expression of CD44 antigen in addition to

CD133 in HCC cells represented more precise stem cell properties of CSCs, including self-renewal, differentiation, aggressive proliferation and hematogenous metastasis ^(14,15).

Mak et al., (2012) ⁽¹⁶⁾ demonstrated that CD133 is located well-suited for a cell protrusion to participate the signaling pathway regulating the epithelial mesenchymal transition. Also, CD133 expression associated with suppression of cancer cell differentiation.

Mima et al., (2012) ⁽¹⁷⁾ found that CD44 plays an important role in inducing epithelial mesenchymal transition and in maintaining the mesenchymal phenotype in HCC. Also its elevation is correlated with increased metastasis, recurrence, resistance to chemo- or radiation therapy, and decreased survival ⁽¹⁸⁾.

However, the role of HCV infections in promoting the expression of potential cancer stem cell markers and its role to initiate tumor growth is poorly understood. So, the present study was design to find out the relation that link between HCV as one of causative agent for persistent inflammation and the expression of CSC marker (CD133 and CD44) that mirror CSC state to understand the factor that has an important role in cancer initiation.

SUBJECTS AND METHODS

Subjects and clinical parameters:

The present study was conducted on a total of 58 subjects. Of them, 48 were patients recruited from those admitting and attending the in- and out-patient clinics of the Department of Internal Medicine, Medical Research Institute, Alexandria University. They included 4 main groups: The first group chronic HCV patients without manifestations of liver cirrhosis (12 patients), the 2nd was chronic HCV patients with radiological and serological evidence of liver cirrhosis (12 patients) and another group of 12 non-HCV patients with radiological and serological evidence of liver cirrhosis. The last group was chronic HCV patients with HCC

confirmed by radiological and laboratory assessment (12 patients).

In addition, 10 healthy age- and sex-matched individuals were involved in the study as healthy controls. The control group had absent serological markers of HBV, HCV, Bilharziasis and autoimmune hepatitis. In addition, they had normal liver transaminase and with normal liver homoeostasis assessed by unremarkable radiographic findings on ultrasound study. The HCC group was diagnosed by abdominal ultrasonography, triphasic CT abdomen, serum alfa fetoprotein and confirmed histopathologically; Cirrhotic group was diagnosed by abdominal ultrasonography while patients with chronic HCV were diagnosed by clinical examination, abdominal ultrasound, laboratory investigations and liver biopsy. All cases were newly diagnosed cases that had not received prior chemotherapy. All HCV studied cases were HCV-positive and HBV negative as confirmed by polymerase chain reaction (PCR) and serologic tests.

Written informed consent was obtained from all participants prior to enrollment in the study, which conformed to the ethical guidelines of the 2004 Declaration of Helsinki. Ethical approval for the study was obtained from the Local Ethical Committee of the Medical Research Institute, Alexandria University.

Methods:

RNA Extraction

Blood samples from peripheral veins (15 ml) were obtained from each group. The mononucleated cell fraction was isolated by centrifugation on a Ficoll-Hypaque gradient at 400g for 30 minutes⁽¹⁹⁾. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany) and generate cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to manufacturer's instructions.

Quantitative reverse transcription PCR analysis (q-RT-PCR)

Real-time quantitative (q-RT-PCR) analysis was performed with the SYBR Green PCR Master Mix (Applied Biosystems, USA). Primers for CD-133 (#QT00075586), CD-44 (#QT00998333) (Applied Biosystem, AB) and for GAPDH as house keeping gene were used in the analysis.

Real-Time PCR was performed in total volume of 25 µl containing 12.5 µl SYBR Green PCR Master Mix, (Applied Biosystems, USA), 2 µl of each reverse transcribed DNA (cDNA), 2.5 µl of the relevant primer (Applied Biosystems, USA) and 8 µl of distilled water. Cycling conditions were 95°C for 15 minutes, followed by 40 cycles at 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. Real-time PCR assays were carried out in duplicate for each sample and mean values were used for the calculation of the mRNA levels.

Data interpretation: Relative quantification (RQ) was calculated from the following equation:

$$\Delta C(t)_{\text{sample}} = C(t)_{\text{target}} - C(t)_{\text{house keeping gene}}$$

$$\Delta C(t)_{\text{calibrator}} = C(t)_{\text{target}} - C(t)_{\text{house keeping gene}}$$

The $\Delta\Delta C$ is then determined using the following formula:

$$\Delta\Delta C(t) = \Delta C(t)_{\text{sample}} - \Delta C(t)_{\text{calibrator}}$$

Quantitative expression of the target gene normalized to the house keeping gene and relative to the calibrator (RQ) = $2^{-\Delta\Delta C(t)}$.

RESULTS

CD133 mRNA expression

The fold increase in CD133 mRNA expression in control subjects ranged from 0.31-1.7 with mean±SD of 1.35±1.29; while in patients with chronic HCV, it ranged from 1.45-14.82 with mean±SD of 9±5.6. In HCV patients with liver cirrhosis, CD133 ranged from 0.76-10.3 with mean±SD of 3.6±3.3. In non HCV patients with liver cirrhosis, CD133 ranged from 0.11-5.8 with mean±SD of 1.3±1.67. Finally, in HCV patients

with HCC, it ranged from 0.417-10.8 with mean±SD of 4.4±3.1 (table 1).

Statistical analysis of these results revealed that mRNA gene expression of CD133 was significantly elevated in all HCV infected patients (either with or without hepatic complications) but not in those with non-HCV cirrhosis as compared to normal controls (p=0.0005, 0.025, 0.01 and 0.647 respectively). Interestingly, the mRNA expression levels in

chronic HCV patients without complications was highly significant as compared to those with complications (cirrhosis and HCC); with p=0.005 and 0.025 respectively. In addition, CD133 mRNA expression was significantly higher in cirrhotic HCV patients when compared to their non-HCV cirrhotic partners (p=0.025); however, no significant difference was recorded upon comparing HCV patients with cirrhosis and HCC (p=0.19) (table 1).

Table (1): Statistical analysis of the results of relative quantification (RQ) of CD133 among HCV infected patient groups either without complications (group II), with liver cirrhosis (group III) or HCC (group V) as compared to non-HCV cirrhotics and healthy subjects.

Groups CD133 (fold increase)	Healthy subjects (group I)	HCV patients without complication s (group II)	HCV patients with liver cirrhosis (group III)	non-HCV cirrhotic patients (group IV)	HCV patients with HCC (group V)
Range	0.31-1.7	1.45-14.82	0.76-10.3	0.11-5.8	0.417-10.8
Mean	1.35	9	3.6	1.3	4.4
SD	1.29	5.6	3.3	1.6	3.1
P ₁		0.0005*	0.025*	0.647	0.01*
P ₂			0.005*	0.0005*	0.025*
P ₃				0.025*	0.19

*Statistical significant at p ≤ 0.05; P1= p value between studied groups and control subjects;

P2= p value between HCV patients and other studied groups; P3= p value between HCV patients with liver cirrhosis and other studied groups

CD44 mRNA expressions

CD44 mRNA expression in control subjects ranged from 0.13-3.03 with mean±SD of 1.26±0.81; while in patients with chronic HCV, it ranged from 2.46-5.3 with mean±SD of 3.95±1.1. In HCV patients with liver cirrhosis, CD44 ranged from 0.4-5.5 with mean±SD of 2.1±1.7 while in non-HCV cirrhotic patients, CD44 ranged from 0.1-4.2 with mean±SD of 1.1±1.4. Finally, in HCV patients with HCC, CD44 mRNA expression ranged from 3.1-10.8 with mean±SD of 6.8±2.7 (table 2).

Statistical analysis of these results revealed that mRNA gene expression of CD44

was significantly elevated in chronic HCV patients and in HCC patients as compared to normal subjects (p=0.0001). On the other hand no significant variation in CD44 expression was recorded in cirrhotic patients either HCV or non-HCV as compared to normal subject (p=0.1, 0.86 respectively). In addition, HCV patients with HCC still had significantly elevated CD44 mRNA expression when compared to HCV patients without complications (p=0.005) and HCV cirrhotics (p=0.0001). Also, CD44 was found to be significantly elevated in HCV patients without complications when compared to those with liver cirrhosis (p=0.005).

Furthermore, no significant difference was observed in CD44 mRNA expression between cirrhotic patients either due to HCV or non-HCV infection (table 2).

Table (2): Statistical analysis of the results of relative quantification (RQ) of CD44 among HCV infected patient groups either without complications (group II), with liver cirrhosis (group III) or HCC (group V) as compared to non-HCV cirrhotics and healthy subjects.

Groups CD44 (fold increase)	Healthy subjects (group I)	HCV patients without complications (group II)	HCV patients with liver cirrhosis (group III)	non-HCV cirrhotic patients (group IV)	HCV patients with HCC (group V)
Range	0.13-3.03	2.46-5.3	0.4-5.5	0.1-4.2	3.1-10.8
Mean	1.26	3.95	2.1	1.1	6.8
SD	0.81	1.1	1.7	1.4	2.7
P ₁		0.0001*	0.1	0.86	0.0001*
P ₂			0.005*	0.0001*	0.005*
P ₃				0.1	0.0001*

*Statistical significant at $p \leq 0.05$; P₁= p value between studied groups and control subjects; P₂= p value between HCV patients and other studied groups; P₃= p value between HCV patients with liver cirrhosis and other studied groups

Correlation studies

Pearson`s correlation was performed between all parametrs among all subjects under study. The results revealed the following:

1. Significant positive correlation between CD133 and CD44 (figure 1).
2. Significant positive correlation between CD44 and SGPT (figure 2).
3. Significant negative correlation between CD44 and albumin and prothrombin activity (figure 3).

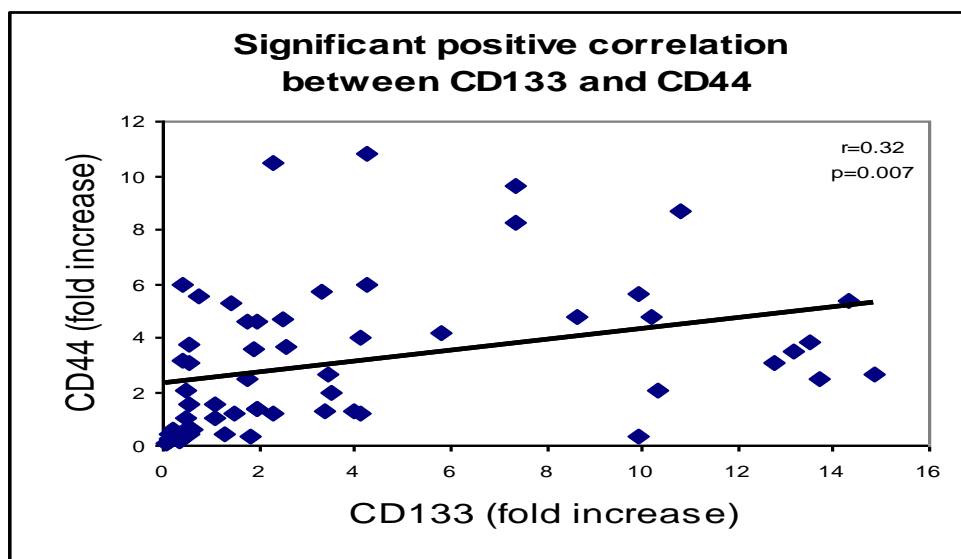


Figure 1: Significant positive correlation between CD133 and CD44

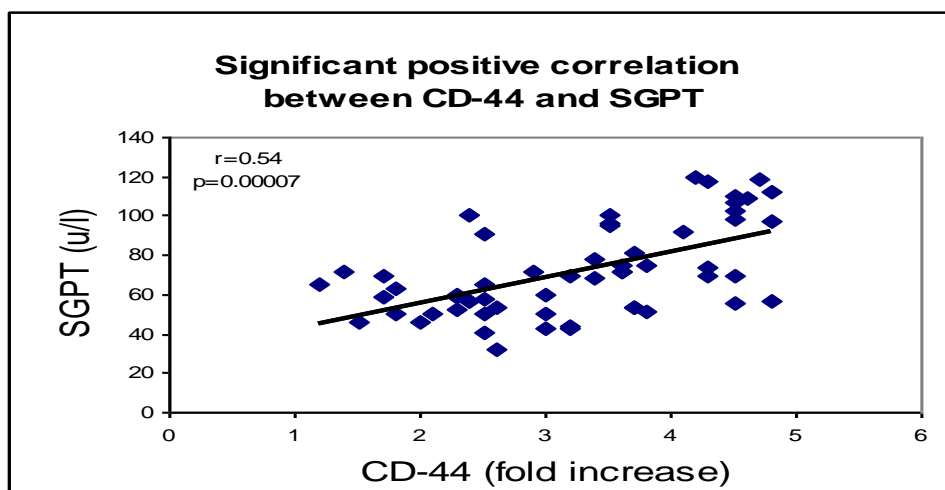


Figure 2: Significant correlations between CD-44 and SGPT

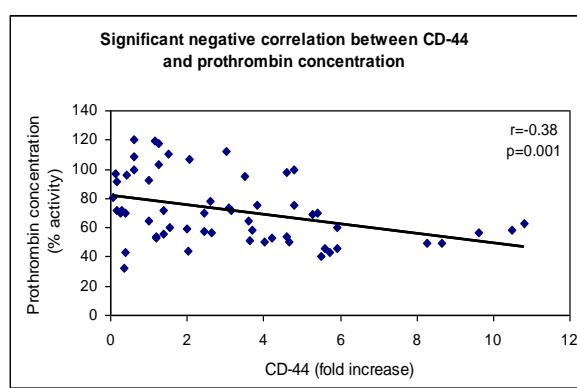
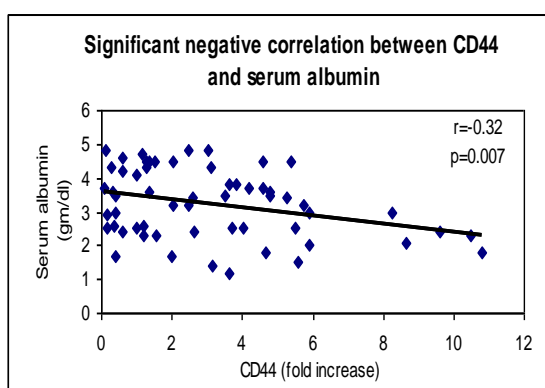


Figure 3: Significant correlations between CD-44 and other study parameters

DISCUSSION

The hematological and liver function data of the present study revealed that platelet counts were reduced in all patient groups relative to healthy individuals. These results were accompanied with parallel decrease in serum albumin level and prothrombin activity with elevated serum total bilirubin and serum glutamic pyruvic transaminase (SGPT).

The results of CD133 and CD44 mRNA expression of the present study revealed that maximal expression was recorded in chronic HCV patients with no complications. Those with hepatic complications had relatively lower expression rate with no significant difference between patients with cirrhosis and HCC was noticed. Meanwhile, minimal expression was registered in non-HCV cirrhotic patients. Concerning CD44 mRNA transcripts, maximal

expression was noticed in PBMCs of HCC patients that was even significantly higher than their HCV cirrhotic partners and, interestingly, in chronic HCV patients than cirrhotic and controls. Again, minimal expression was observed in non-HCV cirrhotic subjects.

Our results of CD133 were in accordance with Bahnassy et al., (2014) (20) who found that CD133, determined by flow cytometry, was significantly higher in blood of chronic HCV patients compared to those with HCC where both were higher as compared to healthy controls. The authors explained this finding on the basis that CD133+ cells represent a subset of "normal" stem cells that are released from the bone marrow into circulation during early inflammatory stage of HCV-associated liver disease in order to repair hepatic damage, compensate for the cell loss and prevent or

eliminate fibrosis. They stated also that, when these cells fail to clear viral infection and/or repair the damage, they set the stage for the development of HCC on top of the chronically inflamed or cirrhotic liver due to their ability to proliferate unlimitedly. The same authors concluded that CD133 expression may predispose to a poor prognosis in HCC patients, since it was significantly correlated with tumor size and stage. They indicated that monitoring CD133 might be a good prognostic marker for the development of HCC in high risk patients ⁽²⁰⁾.

In a recent study conducted by Romano et al., (2015) ⁽²¹⁾, it was found that CD133 over-expression is associated with rupture of tumor capsule that prevent the spread of tumor cells. It seems that CD133 tends to be actively expressed in tumors showing potential for invasion and metastasis. Moreover, Ma et al., (2008) ⁽²²⁾ proved that CD133 was upregulated in association with the liver regeneration process. They found that isolated CD133⁺ HCC cells have higher proliferative and tumorigenic potential and express lower levels of normal mature hepatocyte markers. They also detected that the expression of CD133⁺ cells acquires the HCC resistance to conventional chemotherapy. However, they found that CD133 was expressed in only a minimal proportion of HCC cells, and not expressed at all in normal hepatocytes suggesting that the CD133⁺ cells are responsible of the origin of liver cancer. Moreover, Sasaki et al., (2010) ⁽²³⁾ and Chan et al., (2014) ⁽²⁴⁾ demonstrated that cytoplasmic expression of CD133 in HCC is associated with accelerated tumor invasion to the major branch of the portal vein and also with elevated serum α -fetoprotein levels and histologically high tumor grade. They showed also that, a significant association was observed between cytoplasmic expression of CD133 and the overall survival of patients with HCC attributing that to multifocal

carcinogenicity and hematogenous metastasis to the liver and other remote organs.

In a most recent study by Zhong et al., (2015) ⁽²⁵⁾ who proved that CD133 over expression was significantly correlated with conventional clinicopathological aspects of HCC, such as low tumor differentiation, advanced tumor stage, vascular invasion and vascular thrombosis.

These results support the hypothesis that CD133 possesses the capacity to play an important role as stem cell marker in HCC development. On the other hand, HCV infection itself increases CSC traits during chronic infection by accelerating CD133 expression. This goes in accordance with Ali et al., (2011) ⁽²⁶⁾ who demonstrated that the expression of an HCV subgenomic replicon in cultured cells results in the acquisition of CSC traits. These traits include enhanced expression of CD133, α -fetoprotein, cytokeratin-19 (CK19), and c-Myc. Conversely, curing of the replicon from these cells results in diminished expression of these factors. The authors further demonstrated that HCV-expressing cells in liver tissues from chronic HCV patients are predisposed to the acquisition of characteristics of CSCs (including CD133 over-expression) and hepatic progenitor cells.

As a further investigation to the relationship between HCV infection and the CD133-dependent pathway predisposing to hepatic malignancy, Park et al., (2009) ⁽²⁷⁾ proposed that the NS5A of HCV polyprotein increased the stability of β -catenin (central molecule of the signaling pathway predisposing to CD133 over expression) through protein interplay in hepatoma cell lines, and thus β -catenin was accumulated in NS5A stable cells and in the HCV replicon cells. Also, Liu et al., (2011) ⁽²⁸⁾ showed that HCV core protein enhances Wnt/ β -catenin signaling activity, hence playing an important role in HCV-associated carcinogenesis. These observations are indications for the links that contribute to the association between chronic HCV infection and

the development of HCC. Similarly, Ding et al., (2013) ⁽²⁹⁾ and Kanno et al., (2012) ⁽³⁰⁾ showed that mRNA and protein levels of HDAC6 (intimately associated with CD133 expression) were also up-regulated in HCV infected cells, HCC tissues and cell lines, and contributes to accelerated migration and invasion activity of HCC cells. These observations are all in favor of the relationship between chronic HCV infections and the development of virus-induced malignancy.

The results of the present study revealed also a 6.8 folds increase in CD44 mRNA expression in HCC patients than controls. This was accompanied with a parallel increase in chronic HCV patients without complications.

These results are in agreement with Lingala et al., (2010) ⁽³¹⁾ who found that the degree of CD44 protein staining (by anti-CD44 in immunohistochemical (IHC) assays) in liver biopsies from patients with chronic viral hepatitis was significantly higher than other stages of the disease (including cirrhosis and HCC). A possible explanation for this discrepancy may be due to differences between liver tissue (targeted in their study) and PBMCs (targeted in the present study). Another possible reason is that liver specimens were recruited from heterogenous population of patients with HBV and HCV either with or without advanced fibrosis or cirrhosis unlike the current study that was almost devoted to HCV patients where negativity for HBV was a major exclusion criteria. The authors found cell surface expression of CD133, CD44, CD90 and ALDH in peri-HCC, mildly inflamed or nearly normal liver tissue, not only in patients with advanced disease indicating that circulating, bone marrow-derived stem cells may be attracted to normal or inflamed livers to facilitate tissue repair and that regenerative populations of hepatocytes might be the source of positive staining for CSC markers in livers from viral inflammation or even from healthy individuals. In addition, Oliva., (2010) ⁽³²⁾

showed that HCC tissues had a more intense IHC staining for variable CSC markers (including CD44) than in cirrhotic liver tissues; a finding that goes in accordance with the present study.

On the other hand, recent findings by Hu et al., (2014) ⁽³³⁾ and Niu et al., (2015) ⁽³⁴⁾ indicated that high CD44 expression in patients with HCC is positively correlated with circulating α -fetoprotein level, tumor size, tumor differentiation, tumor stage, portal vein thrombosis, vascular invasion, tumor recurrence and metastasis. These findings suggest that CD44 expression might be useful as a predictive marker for HCC progression, metastasis and recurrence.

Our study indicated also that CD44 mRNA is significantly expressed in PBMCs of patients with chronic HCV even without complications suggesting a role for HCV infection per se as important factor contributing to enhanced CD44 expression. This goes in agreement with Abe et al., (2012) ⁽³⁵⁾ who investigated that cell surface expression of CD44 detected by DNA microarray analysis was upregulated in Huh7 cell line harboring an HCV subgenomic RNA replicon of genotypes 1b and 2a. This finding is enforced by a more recent observation by Andre et al., (2015) ⁽³⁶⁾ who found increasing CD44 expression in cells harboring viral replicon and that blocking of HCV viral replication led to a reduction in CD44 expression.

Furthermore, Iqbal et al., (2014) ⁽³⁷⁾ investigated that HCV infection induces epithelial mesenchymal transition and cell migration via osteopontin/CD44-dependent pathway followed by β -catenin activation which can lead to tumor progression and epithelial mesenchymal transition of human hepatoma cells.

The current study show also that the unelevated CD133 and CD44 expression in cirrhotic patients might be due to the fact that chronic HCV infections can continuously trigger production of reactive oxygen species

(including hydrogen peroxide, hydroxyl radicals and superoxide radicals) by macrophages, neutrophils and cytotoxic T lymphocytes⁽³⁸⁾, which induces cell cycling, resulting in stem cell differentiation⁽³⁹⁾.

Our data revealed also mismatched pattern of CD133 and CD44 expression between study groups indicating that individual alterations of the CD133 and CD44 expression is not sufficient for HCC development, suggesting that there might be further changes in the liver microenvironment facilitating both HCC initiation and progression.

In the present study, correlation analysis revealed that CD44 was positively correlated with CD133, SGPT while showed negative correlation with albumin and prothrombin activity. The negative correlation between prothrombin activity and CD44 indicating that as long as the disease progresses, the expression of CD44 increases since prothrombin activity is one of the surrogate markers of profound liver affection. Also, there was a strong positive correlation between SGPT and CD44 indicating the loss of hepatocytes as a result of virus infection and persistent inflammation inducing cytokine production and inhibiting albumin synthesis (this was also manifested by negative correlation with CD44 that induces cell proliferation increasing metastasis potential). These data are confirmed by the observation of Khalifa et al., (2008)⁽⁴⁰⁾ who reported that the levels of CD44s were significantly higher in Non-Hodgkin's lymphoma patients than in controls correlating negatively with albumin and positively with clinical outcome of the disease. The authors attributed this finding to the release of high levels of inflammatory cytokines (TNF- α , in particular). They added that increased levels of CD44s are associated with high tumor burden and poor prognostic criteria and are potentially recommended as prognostic markers in Non-Hodgkin's lymphoma.

In addition, the present study showed positive correlation between CD44, CD133. In accordance with these findings, immunohistochemical study of human HCC specimens by Hou et al., (2012)⁽¹⁵⁾ revealed that the number of CD133⁺ CD44⁺ cells is increased and associated with portal vein invasion, indicating the strong association between CD44, CD133 in invasion, metastasis, recurrence and HCC progression.

These findings highlight the important role of deregulated signaling network induced by HCV infection in transformation of stem/progenitor cells and initiation of tumor. Our results and previous findings in this research area firmly indicate that CD133/CD44 positive HCC cells were critical for hematogenous metastasis; where CD133 is not only responsible for tumor initiation or progression, but also essential for self-renewal function. Additionally, CD44 seems to be important for invasion and extra hepatic metastasis. However, both molecules play an important role in inducing epithelial mesenchymal transition and maintaining the mesenchymal phenotype in HCC.

It seems likely that, chronic HCV infection appears to predispose circulating stem cells towards the pathway of acquiring cancer stem cell characteristics, eventually by inducing accelerated expression of CD133/CD44. The current data together with other related investigations might promote potential assumptions that tumors not only arise as a result of successful escape from immune surveillance, but also as a result of breakdown of signaling network in normal stem cells due to persistent HCV infection that leads to their transformation into CSCs. These events might lead to modification in niche surrounding stem cell, thus increasing the migration, invasion potential and facilitate tumor progression. However, the underlying mechanisms responsible for virus entry into stem cell are still remaining unclear. Whether HCV infection *per se* creates the physiological and

biochemical milieu predisposing to stem cell transition or the later should be invaded with viral particles to shuttle down to this destination remains another area of discrepancy and active research.

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