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Original Article 1

Circulating cancer associated fibroblast and cancer stem cell markers as diagnostic and prognostic tools of hepatitis C induced hepatocellular carcinoma

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Background
Several studies have been carried out on the crosstalk between cancer-associated fibroblasts (CAFs) and liver cancer stem cells (LCSCs) and their roles in tumorigenesis and metastasis in various malignancies, including hepatocellular carcinoma (HCC). Identification of cancer stem cells (CSCs) and CAFs in these studies has typically been carried out based on their markers' expression in hepatic tumor tissues.
Objective
To detect CAFs and CSCs markers in peripheral blood, which can be used as non-invasive diagnostic and prognostic tools for HCV induced fibrosis and carcinogenesis in Egyptian patients.
Materials and methods
A case-control study was conducted on 200 subjects. Four groups were included in the study: A) healthy control group, B) chronic hepatitis C (CHC) non-cirrhotic group, C) CHC-cirrhotic group and, D) CHC-HCC group. Peripheral blood detection of LCSC markers (CD133&CD44) was done by Flow-cytometer analysis, and ELISA was used to detect CAFs markers such as Collagen Type XI Alpha I Chain (COL11A1) and α-smooth muscle actin (α-SMA).
Results and conclusion
Significant difference in the level of CD133, CD44 and COL11A1 in CHC patients compared to the control group (<0.001). The level of previous markers increased with the progression of the disease. However, α-SMA level decreased in both the non-cirrhotic and HCC groups. Sensitivity of CD133 was 77.78% with specificity 88.24% followed by COL11A1 with higher sensitivity of 83.33%, but a slightly lower specificity of 73.33%. So, CD133 emerged as the most promising diagnostic marker for HCC, followed by COL11A1. Regular detection of CSCs and CAFs in circulation may aid in the diagnosis and prognosis of liver cirrhosis and HCC.

Keywords Hepatocellular carcinoma, hepatitis C infection, cancer stem cells, cancer associated fibroblasts

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Introduction

Despite Egypt's successful transition from having high infection rate of hepatitis C virus (HCV) in the world to one of the lowest as prevalence of infection reduced from 10% to 0.38% [1] and despite applying national preventive and treatment programs which eradicate HCV infection, elimination of the risk of Hepatocellular carcinoma (HCC) development was still not complete [2,3]. Therefore, all patients with advanced fibrosis must be under continued surveillance programs [4]. Hepatocellular carcinoma is the seventh common malignancy worldwide [5]. Eighteen percent (18%) of HCC patients have only 5 year survival rate. It has a poor prognosis due to the high rate of recurrence post-surgery and liver metastasis [6].

The main role of HCV was to establish a microenvironment that led to increase carcinogenic cascade. It was reported that HCV proteins have a direct effect on the initiation and progression of HCC [7]. This may be explained by the induction of epithelial-mesenchymal transition (EMT) state and the generation of cancer stem cells (CSCs) in

liver cells [8]. Chronic liver injury by HCV, inflammation, fibrosis and cirrhosiswhich preceded tumor formation, is considered a premalignant microenvironment [9]. Once malignancy occurred, the premalignant microenvironment was replaced by the tumor microenvironment (TME) to sustain the progression of the tumor [10]. Several cells are involved in HCC as immune stromal cells associated with the tumor including B and T cells, tumor-associated macrophages (TAMs), CSCs, cancer-associated fibroblasts (CAFs), neutrophils, and endothelial cells [11]. The bidirectional crosstalk between HCC cells and TME cells reinforced proliferation, migration, metastasis, chemo-resistance, and induction of tolerance against tumor cells [12,13]. Persistent injury of liver cells leads to the activation of hepatic satellite cells (HSCs). Once EMT occurrs, the source of CAFs will mainly be from activated HSCs. In 2019, Huang et al. found that a specific type of CAFs could increase the proliferation and progression of CSCs. increasing the stemness of cancer. In turn, CSCs can maintain CAFs in an activated state through the releasie of specific cytokines as a positive feedback loop through the CAF-CSC crosstalk [14]. In 2016, Attieh and Vignjevic reported that CAFs can lead and guide malignant cells, creating a specific path for them [15].

Cancer stem cells were found to be responsible for chemotherapy resistance and cancer recurrence [16,17]. Identifying CSCs subpopulations inside a tumor provides a unique idea concerning diagnosis, prevention and treatment of tumor [18]. Several markers were associated with liver CSCs such as CD44, CD133, and CD90, and their expression has been linked to poor prognosis [19,20].

In 2005, Katayama defined CD44 as a receptor for hyaluronic acid, glycoprotein class I trans-membrane [21]. It was associated with cell homing, interactions, and new blood angiogenesis. It was expressed in many mammalian cells as neutrophils and monocytes [22, 23]. CD44 was negative or lowly expressed in normal liver tissue with variable levels of expression in viral hepatitis, HCC, peri-HCC, HB (hepatoblastoma) [24]. CD44+CSC in HCC was usually accompanied by other CSC markers such as CD90 and CD133 [25,26]. Inverse correlation was reported between the level of CD44 expression and survival time [27].

Prominin-1 (CD133) was defined as a hematopoietic stem cell marker [28, 29]. Normally, it was not expressed in hepatocytes [30, 31]. However, it was expressed in many tumors and liver-related diseases [32-37]. Hepatitis C virus enhanced CD133 expression as reported by Ma, [38]. Both CD44 and CD133 were considered prognostic markers as they were associated with a higher rate of recurrence, carcinogenetic potential and lower overall survival [39, 40]. An inverse association between CD 133 expression and overall survival rate was found by Song et al., in contrast to CD44, CD133 was associated with tumor grade, stage and alpha feto-protein (AFP) serum level [41].

Also, multiple markers have been associated with CAFs identification as fibroblast activation protein (FAP), fibroblast specific protein 1 (FSP1 or S100A4), α-smooth muscle actin (α-SMA), platelet-derived growth factor (PDGF), Collagen Type XI Alpha I Chain (COLL11A1) fibronectin, integrin α-11 and podoplanin [42].

Alpha smooth muscle actin was expressed on the vascular smooth muscle cells. It was involved in the process of fibrogenesis [43]. Previously, it was associated with early stages of liver damage and treatment efficacy monitoring [44]. Collagen Type XI Alpha I Chain was associated with the development of bones and the assembly of collagen fiber. It was increased in many cancers, and its high level was reported with recurrence, chemoresistance, and poor outcome. In solid tumors, it was overexpressed on CAFs beside malignant cells, highlighted as a specific marker for CAFs [45].

The goal of surveillance is to detect subclinical lesions that can be potentially curative [46]. Prevention of recurrence can be done by serial screening follow-up in order to detect any malignant cells before cancer cells become evident or suspected. Hepatocellular carcinoma has a long subclinical proliferative period, enabling curative therapies to be usually effective [47].

Our study aimed to study the expression of markers of both CSCs (CD133 & CD 44), and CAFs (α-SMA & COLL11A1), in the peripheral blood as non-invasive diagnostic tools with prognostic value in chronic hepatitis C patients in Egypt to open new avenues for overcoming HCC progression.

Materials and methods

Subjects and samples collection

The present case-control study was conducted on 200 subjects recruited in the outpatient clinic and inpatient wards at Hepato-Gastroenterology Department. They were divided into four equal groups: 50 non-cirrhotic patients, 50 cirrhotic patients, 50 HCC patients, and 50 healthy controls. All hepatic patients were post-CHC infection as confirmed by positive anti-HCV antibodies testing using 3rd generation enzyme-linked immuno-sorbent assay (ELISA), viral load was detected by HCV quantitative real-time RNA PCR. Cirrhotic patients were confirmed by abdominal ultrasound and fibro-scan. Triphasic computerized tomography (CT) scan and α-FP (α-FP $\geq$ 100 μg/ml) were used to diagnose HCC patients. All HCV patients co-infected with other viruses such as

hepatitis B virus (HBV) or human immunodeficiency virus (HIV), any patient with a history of cancers other than HCC, patients with previous liver transplantation, immunosuppression or any autoimmune diseases were excluded from the study. Laboratory work was conducted at Theodor Bilharz Research Institute (TBRI). Routine chemical and hematological lab tests were done to all groups included: complete blood picture (CBC) (Quintus five parts differential, Sweden), serum total and direct bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), serum albumin (Cobas 8000 auto-analysis, Japan), serum potassium (K), sodium (Na), serum urea and serum creatinine (Beckman AU480 Analyzer, USA), Prothrombin time and international normalization ratio (INR) (Stago STA compact max, France), Anti-HBV surface antigen (anti-HBsAg), HCV antibody (HCV Ab), & HIV Ab (Abbott Alinity Analyzer, USA).

Ethical approval

The study was approved by Theodor Bilharz Research Institute's Human Research Ethics Committee and the ethical committee of the faculty of medicine, Ain Shams University. All participants provided written informed consent.

Analysis of cancer stem cells by flow-cytometer

Peripheral blood mononuclear cell layers were separated using the Ficoll-hypaque method [48]. Then, analysis for liver circulating cancer stem cells (LCSC) markers such as anti-human cluster of differentiation 133 (CD133, Prominin-1) and anti-human/mouse cluster of differentiation 44 (CD44) monoclonal antibody conjugated with phycoerythrin (PE) (eBioscience), analysis was done by flow-cytometer (Beckman Coulter Epics XL-MCL, USA).

Detection of cancer associated fibroblasts markers

CAFs markers (α-SMA & COLL11A1) were detected in the serum by commercially available ELISA kits (Chongqing Biospes Co., Ltd, China).

Statistical methods

The current results were conducted by the 26th version of the statistical Package for the social science (SPSS) (IBM Corp., Chicago, IL, USA) and Excel (Microsoft Office 2010). Descriptive quantitative statistics were presented as mean ± SEM (standard error of the mean) for all quantitative variables. Descriptive qualitative statistics as percentages and numbers for all qualitative variables. Chi-square test (χ2- test) to compare among different groups for all qualitative variables. Analysis of variance (ANOVA) test for comparisons between means of different groups. Post Hoc test (Bonferroni) to study significance between individual groups. True- and false-positive fractions of all assessed markers at different cutoff points were conducted by receiver operating characteristic (ROC) curve. Sensitivities, specificities and areas under the ROC curve (AUC) were computed. Significant level (p-value) was expressed as follows $p>0.05$ was considered non-significant, $p<0.05$ was considered significant. $p<0.01$ was considered highly significant.

Results

Subjects and investigations

A total of 200 subjects were included in this case-control study, 112 males (56%) and 88 females (44%) with an age above 18 years. They were divided into four groups: 50 chronic hepatitis C (CHC)-non-cirrhotic patients, 50 CHC-cirrhotic patients, 50 patients with HCC on top of CHC and 50 healthy controls. The demographic, biochemical, and radiological profiles are presented in Tables 1 and 2.

Flow-cytometric analysis for cancer stem cells (CSCs) detection

A significant increase in CD 133 and CD 44 was observed in non-cirrhotic and cirrhotic groups compared to healthy controls ($p<0.001$) and in HCC group compared to other groups ($p<0.001$). However, their percentages did not show any significant differences between non-cirrhotic and cirrhotic groups ($p>0.05$). (Table 3).

Detection of cancer-associated fibroblasts (CAFs) markers

Alpha-smooth muscle actin was significantly increased in the non-cirrhotic group compared to the healthy controls ($p<0.05$). However, it showed a significant decrease in cirrhotic and HCC groups compared to the healthy controls and non-cirrhotic group ($p<0.01$). There were no significant differences observed between the cirrhotic and HCC groups ($p>0.05$) (Table 4). COLL11A1 also showed a significant increase in non-cirrhotic, cirrhotic, and HCC groups compared to the healthy controls ($p<0.001$). However, it did not show any significant differences between the cirrhotic group and the non-cirrhotic group, as well as between the HCC group and both non-cirrhotic and cirrhotic groups ($p>0.05$) (Table 4).

Diagnostic performance of cancer stem cells (CSCs) and cancer-associated fibroblasts (CAFs) markers

Concerning HCC, the receiver operating curve (ROC) showed that, CD133 was the most significant predictor with the largest area under the curve (AUC) of 0.962, along with a sensitivity of 77.78% and specificity of 88.24%, followed by

COLL11A1 with AUC of 0.785, higher sensitivity of 83.33%, but a slightly lower specificity of 73.33%. CD44 and CD133/CD44 exhibited lower AUC values (0.647 and 0.345, respectively), sensitivities (63.16% and 64.71%, respectively), and specificities (71.43% and 73.58%, respectively). Alpha-smooth muscle actin (α-SMA) had the lowest AUC value of 0.229, with a sensitivity of 61.54% and specificity of 66.67%. On the other side, AFP, at a value of >19 ng/ml, had an AUC of 0.754, sensitivity of 66.67%, and specificity of 81.82% (Table 5, Figure 1).

Discussion

Regular detection of CSCs and CAFs in circulation may aid in the diagnosis and prognosis of cirrhotic and HCC patients. Since the presence of LCSCs and CAFs themselves, irrespective of their number, in the peripheral blood was diagnostic for the presence of ongoing tumor growth and metastatic lesions, finding an accurate simple non-invasive tool to diagnose and screen high risk hepatic patients is mandatory.

In 2021, Espejo-Cruz et al. reported that circulating CSC was derived during EMT or directly from the primary tumor [49]. Liver cancer stem cells have the ability to circulate within the body. Metastatic cancer stem cells (CTCs / LCSCs) EMT, could invade lymphatic/ blood stroma by intra-vasation, then secondary tumor growth could be initiated by extravasation [50, 51].

Therefore, detection of CTCs and CAFs markers in circulation can be used as diagnostic markers for ongoing metastasis and/or relapse. Up to our knowledge, no previous studies were conducted to detect circulating cancer stem cells (CCSCs) and CAFs in the peripheral blood of HCC patients, by using combination of CSCs and CAFs markers, as all previous researches were conducted on liver biopsies.

Our study showed a gradual increase in the level of CD44 and CD133 with significant differences between the different studied groups. There was a significant increase in non-cirrhotic and cirrhotic groups compared to the healthy control group ($p<0.001$) and in the HCC group compared to other studied groups ($p<0.001$). Our results agreed with previous researchwhich reported that CD 133 +cells were highly expressed in cirrhotic livers and HCC, while totally absent in normal liver biopsies as reported by previous studies [27, 30,52]. Indeed, in 2017, Rozeik et al. reported metastatic behavior of LCSC by changing the site of CD133 and CD44 expression in the liver. They were localized in fibrous septa and portal areas in non-cirrhotic and cirrhotic patients, while they were found within invaded vessels and peri-tumoral adjacent connective tissue in HCC biopsies enforced the migratory and metastatic invasion behavior of CSCs [53].

It was reported that CD133 was associated with a higher rate of lymphatic metastasis with great invasiveness in HCC [54, 55]. It was found that overexpression of CD133 was associated with poor prognosis and advanced tumor stage in HCC as reported by previous research [56-59]. The same was found concerning CD44, as Zhu et al. reported that CD44 was expressed predominantly on CD133+ population in HCC [25]. Although in our study, there were no significant differences in the level of CD133 and CD44 between cirrhotic and non-cirrhotic patients, there were significant differences between HCC and both cirrhotic and non-cirrhotic patients. This increase with disease progression indicates the predictive value of CSCs markers in HCC, enforcing their usage in diagnosis and follow up. Cancer stem cells CD133+/CD44+ were reported to be an important population of HCC cells resistant to Sorafenib as they could survive under this therapy [60, 61]. Our results mirrored those of liver biopsy results which revealed significant differences in the level of expression of the CSCs markers in tissue biopsies [53].

Concerning α-SMA, a significant increase was found in non-cirrhotic patients compared to controls followed by a progressive decrease in both cirrhotic and HCC groups compared to healthy controls and non-cirrhotic groups ($p<0.01$). There was controversy in previous research concerning the level of α-SMA.

Contrarily, Yamaoka et al. found that increased α-SMA positive cells were always associated with the progression of fibrosis in CHC and alcoholic liver disease. [62]. Also, Liu et al. demonstrated up-regulation of α-SMA and fibro-nectin coding genes leading to activation of HSC [63]. Hautekeete & Geerts found that elevated α-SMA levels could suppress T-cell response leading to tumor tolerance and progression of HCC [64].

Although our results concerning α-SMA weren't in line with the majority of previous results. This variation may be attributed to differences in the type of studied samples, as previous research depended on the detection of the level of expression in liver biopsies differing from our method which depends on measuring the level of secreted protein which reflects the secretory function of cirrhotic livers. We explain the decreasing level of secreted α-SMA by decreasing secretory function of liver cells with the progression of the disease and the occurence of fibrosis which may be accompanied by high expression of the marker without an increase in

the secretory function. We are also in line with other explanations found by other researchers such as Lau et al., who investigated different stages of fibrosis in CHC by reporting staining patterns of α-SMA in 21 liver biopsies [65]. They observed that α-SMA–positive cells were observed in stage 0 fibrosis, suggesting early activation of HSC. However, they decreased with advanced fibrosis. Also, they reported inactive state return of HSCs once fibrosis was well established without ongoing hepatic inflammation. In 2017, Anggorowati et al. reported that α-SMA expression was higher in benign ovarian tumors compared to malignant tumors They explained the previous result by the differences in the maturity of blood vessels, as, blood vessels in malignant tumors (which results from angiogenesis) were less mature than those in benign tumors [66]. Also, Karata et al. reported that some markers were found to be down-regulated during invasion and dissemination during epithelial-mesenchymal transition [51]. As α -SMA, was reported as a marker for the EMT process, it could be affected according to the degree of blood vessel maturity. Levy et al. reported that there was no association between development of fibrosis and α-SMA-positive HSCs [67].

To our knowledge, COL11A1 has not investigated in HCC as in other tumors. COL11A1 was reported as a central component of the ECM in many cancers, which was predominantly produced by CAFs [68]. Normally, COL11A1 was expressed in mesenchymal stem cells and cartilaginous tissues, while its expression was almost undetectable in other normal tissues, including resident fibroblasts differing from other CAF markers. In 2015, Raglow & Thomas reported that poor prognosis and aggressive tumor phenotypes were associated with high levels of COL11A1 in several types, such as breast, ovarian, colorectal and pancreatic cancers [69]. COL11A1 over-expression has only been observed in desmoplastic areas of tumors composed mostly of different cancers not in inflammatory diseases, suggesting that COL11A1 could be a unique marker for CAFs [70]. In our study, a significant increase in the level of COLL11A1 was seen in hepatic patients compared to normal controls ($p<0.001$). In contrast to our results, many researchers found that COL11A1 was a sensitive biomarker that could discriminate between malignant cells and chronic inflammatory cells in the pancreas and predict cancer prognosis [71-73].

In our study, CD133 has specificities of 88.24% and sensitivities of 77.78% followed by COL11A1 which emerged as 2nd significant predictor of HCC with specificities of 73.33% and sensitivities of 83.33%. On the other hand, CD44, CD133/CD44 and α-SMA exhibited lower sensitivities, and specificities. In line with our findings, Makled et al. reported that CD133 demonstrated a high sensitivity of 97% and specificity of 80% in the detection of HCC, suggesting its effectiveness in detecting HCC with high sensitivity [74]. Additionally, Jun et al. also reported high specificity and sensitivity for CD133 (both 70%) in the detection of HCC [75].

In spite of being sensitive and specific (83.33%, 73.33%) by ROC curve, COL11A1 couldn't discriminate between cirrhotic patients and HCC patients by post -Hoc test, although there was a difference in the levels detected (51.28±6.74, 56.22±4.26). Salimian et al. reported that COL11A1 could potentially be used as a useful diagnostic marker in other malignancies such as breast cancer, colorectal cancer and gastric cancer [76]. Also, Sun et al. identified COL11A1 as a potential diagnostic marker for gastric cancer, with an AUC value of 0.934 (95% CI: 0.906–0.962), respectively [77]. Di et al. revealed high specificity and sensitivity of COL11A1 for the diagnosis of oral squamous cell carcinoma ($AUC = 0.781$, $p<0.05$) [78].

Previous discrepancies in the results could be due to differences in sample size and type of studied sample. As we previously mentioned serum sample analysis might not correlate with the level of expression. Another cause was the lack of research that investigated COL11A1 in HCC or even hepatic patients as all previous researches were conducted in other types of tumors. The major drawback of this study was that the levels of the studied markers were not correlated with HCC stage and response to treatment. Also, a larger sample size is needed to validate these markers. Regular detection of CSCs and CAFs in circulation may serve as diagnostic and prognostic markers in patients with liver cirrhosis and HCC. Further research and validation studies are necessary to confirm the utility of these markers in clinical settings correlating them with tissue samples, tumor staging and their ability to predict relapse.

Table 1 Demographic, clinical and radiological data among groups.

Groups / Variables	Healthy control group	Non-cirrhotic group	Cirrhotic group	Hepatocellular carcinoma group	*p-value*
Age	53.16±1.82	51.92±2.18	55.40±3.09	60.17±1.12	NS●
Gender (males/females)	11/14	12/13	10/15	23/2	NS♠
Smoking					
No	18 (72%)	17 (68%)	20 (80%)	14 (56%)	<0.05♠
Yes	7 (28%)	8 (32%)	5 (20%)	11 (44%)*	
Hypertension					
No	16 (64%)	15 (60%)	16 (64%)	17 (68%)	NS♠
Yes	9 (36%)	10 (40%)	9 (36%)	8 (32%)	
Diabetes					
No	14 (56%)	18 (72%)	9 (36%)	8 (32%)	<0.01♠
Yes	11 (44.0%)	7 (28.0%)	16 (64.0%)**	17 (68.0%)**	
Stages of fibrosis by abdominal sonar ultrasound					
F0	25 (100%)	14 (56%)	0	0	<0.001♦
F1	0	11(44%)	0	0	
F2	0	0	0	0	
F3	0	0	6 (24%)	0	
F4	0	0	19 (76%)	25 (100%)	

● Data for age are expressed as mean±SEM; Categorical data expressed as number (percentage); ♠ Chi-square test (χ2- test); ♦ ANOVA (Post Hoc test –Bonferroni); *P<0.05 significant increase than healthy control and cirrhotic groups; **P<0.01 significant increase than healthy control and non-cirrhotic groups; NS= statistical not significant differences between groups. F0: no fibrosis; F1: portal fibrosis with insignificant abnormal areas; F2: portal fibrosis with septa and abnormalities in wider areas; F3: numerous septa without cirrhosis and prominent abnormalities; and F4: cirrhosis

Table 2 Routine lab investigations among groups.

Groups / Variables	Healthy control group	Non-cirrhotic group	Cirrhotic group	Hepatocellular carcinoma group
Hemoglobin (g/dl)	13.07±0.27	13.80±0.29	9.68±0.44[a]	11.4±0.32[b]
RBS/glucose(mg/dl)	114.68±4.57	106.88±3.28	179.12±21.46[c]	163.23±13.49[c]
WBC (x103/µl)	8.72±0.35	6.76±0.37[d]	5.58±0.58[d]	8.38±0.77
Platelet count (x103/µl)	301.20±15.89	222.92±13.25	102.29±10.98[a]	126.31±14.31[a]
PC	96.96±0.69	94.24±1.64	62.84±3.46[a]	60.87±2.49[a]
PT	13.88±0.13	13.95±0.16	19.49±0.96[e]	19.58±0.75[e]
INR	1.04±0.009	1.04±0.012	1.47±0.078[e]	1.49±0.062[e]
Urea (mg/dl)	34.44±2.66	53.04±3.03	61.37±9.13[e]	97.96±10.48[e]
Creatinine (mg/dl)	0.75±0.05	0.97±0.06	0.98±0.08	1.51±0.1[f]
Na (meq/L)	140.36±0.99	139.52±0.67	135.12±1.20	133.62±1.13
K (meq/l)	4.26±0.09	4.11±0.08	4.12±0.09	4.35±0.11
AST (IU/L)	37.0±1.75	42.60±6.49	49.64±7.76	179.78±28.47[f]
ALT (IU/L)	44.65±1.84	47.04±6.85	27.04±2.87[a]	61.04±5.84[b]
Serum albumin (g/dl)	4.17±0.09	4.37±0.07	2.73±0.11[c]	2.81±0.11[c]
Total bilirubin (mg/dL)	0.75±0.03	0.91±0.23	2.27±0.39	14.54±2.54[f]
AFP (ng/ml)	5.98±0.5	4.69±0.84	5.93±0.69	8113.09±4313.24[f]

CBC: complete blood picture; AST: aspartate transaminase; ALT: alanine transaminase; K:serum potassium; Na: sodium; PT: Prothrombin time; INR: international normalization ratio (INR); AFP: Alfa-fetoprotein . [a]$p<0.001$ significant decrease than control and non-cirrhotic groups; [b]$p<0.001$ significant increase than cirrhotic group; [c]$p<0.05$significant decrease than healthy control and non-cirrhotic groups; [d]$p<0.01$ significant decrease than healthy control and HCC groups; [e]$p<0.001$ significant increase than healthy control and non-cirrhotic groups; [f]$p<0.001$ significant increase than healthy control, non-cirrhotic, and cirrhotic groups

Table 3 Percentage of cluster of differentiation 133 (CD133) and cluster of differentiation 44 (CD44) expression among different study groups

	Healthy control group	**Non-cirrhotic group**	**Cirrhotic group**	**Hepatocellular carcinoma group**	***P*-Value**
CD 133 (%)	5.59±1.32	9.43±1.39[a]	10.65±2.83[a]	18.19±4.27[b]	**<0.001**♦
CD 44 (%)	0.42±0.17	5.35±1.71[a]	11.91±3.58[a]	14.72±4.45[b]	**<0.001**♦

Numerical data are expressed as mean±SEM (standard error of the mean); ♦ ANOVA (Post Hoc test – Bonferroni); CD 133: Cluster of differentiation 133; CD 44: Cluster of differentiation 44; CHC: Chronic Hepatitis C; HCC: Hepatocellular Carcinoma; p-value: probability value, [a]p<0.001 significant increase than healthy control group; [b]p<0.001 significant increase than healthy control, non-cirrhotic, and cirrhotic groups.

Table 4 Plasma levels of Collagen Type XI Alpha I Chain (COLL11A1) and α-smooth muscle actin among (α-SMA) different study groups

Groups / **variable**	**Healthy control**	**Non-cirrhotic**	**Cirrhotic**	**Hepatocellular carcinoma group**	***p*-Value**
α-SMA (ng/L)	134.32±4.79	165.28±18.84[a]	99.84±3.75[b]	98.04±3.84[b]	**<0.001**♦
COLL11A1 (pg/ml)	0.70±0.47[a]	48.08±7.74[c]	51.28±6.74[c]	56.22±4.26[c]	**<0.001**♦

Numerical data are expressed as mean ± SEM (standard error of the mean); ♦ ANOVA (Post Hoc test – Bonferroni).
α-SMA: Alpha-smooth muscle actin; COLL11A1: Collagen Type XI Alpha I Chain; CHC: Chronic Hepatitis C; HCC: Hepatocellular Carcinoma; *p*-value: probability value, [a]p<0.001 significant increase than healthy control group; [b]p<0.001 significant decrease than healthy control, non-cirrhotic groups; [c]p<0.001 significant increase than *healthy* control, non-cirrhotic, and cirrhotic groups.

Table 5 Receiver operating characteristic curve (ROC) analysis for the predictive ability of hepatocellular carcinoma among (HCC) chronic hepatitis C (CHC) patients

Variable(s)	**Area under the curve**	**95% Confidence Interval**	**Sensitivity**	**Specificity**
CD133	0.962	0.930- 0.994	77.78%	88.24%
COLL11A1	0.785	0.699-0.872	83.33%	73.33%
AFP	0.754	0.647- 0.862	66.67%	81.82%
CD44	0.647	0.526-0.769	63.16%	71.43%
CD133/CD44	0.345	0.216-0.434	64.71%	73.58%
αSMA	0.229	0.131-0.328	61.54%	66.67%

CD 133: Cluster of differentiation 133; COLL11A1: Collagen Type XI Alpha I Chain; AFP: Alpha-fetoprotein; α-SMA: Alpha-smooth muscle actin; CD 44: Cluster of differentiation 44; AUC: Area under the curve; ROC: Receiver operating characteristic curve

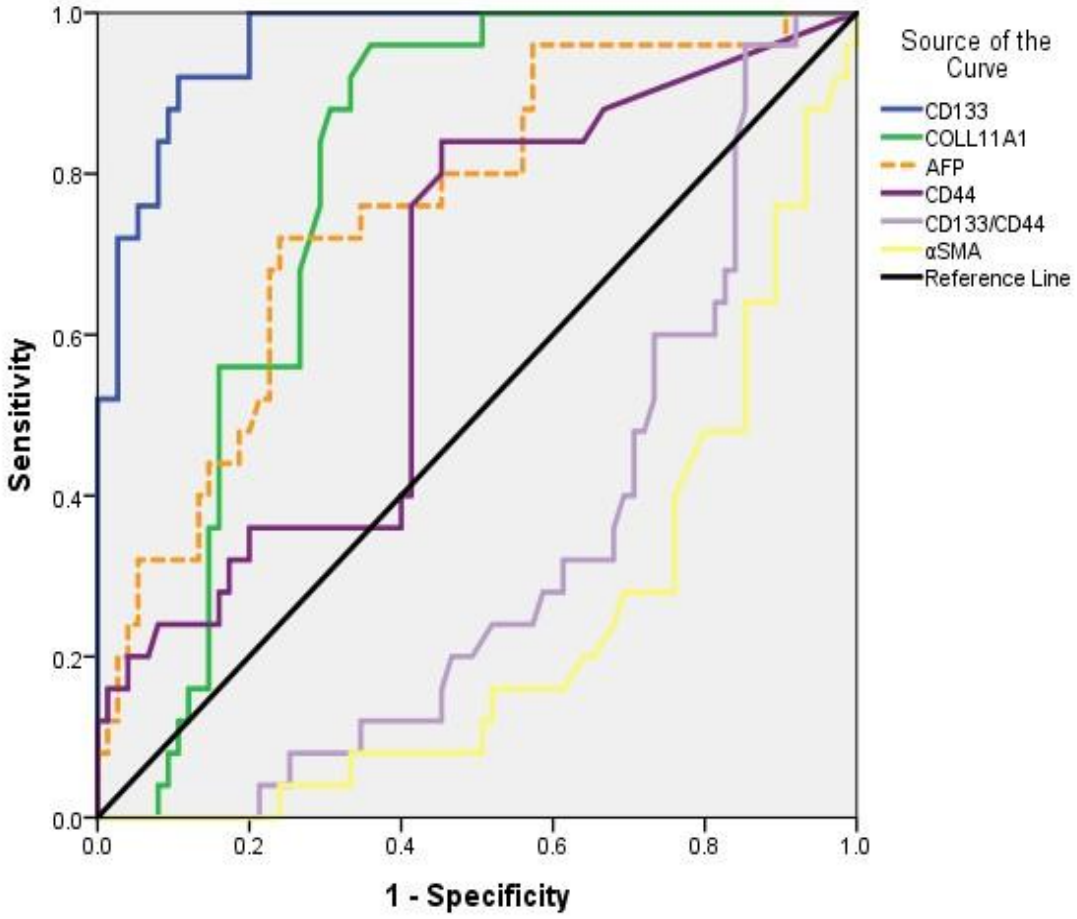

Fig. 1 The receiver operating curve (ROC) showing performance of CD133, CD 44, α-SMA and COLL11A1 for detecting hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC); CD 133: Cluster of differentiation 133; COLL11A1: Collagen Type XI Alpha I Chain; AFP: Alpha-fetoprotein; α-SMA: Alpha-smooth muscle actin; CD 44: Cluster of differentiation 44

Conclusion

We found that CD133 emerges as a promising diagnostic marker for HCC, followed by COL11A1. However, CD44 and α-SMA may have limited effectiveness as standalone markers for HCC diagnosis however using of combination may increase predictive value.

Abbreviations

AFP : Alpha feto-protein serum level
α-SMA : α-smooth muscle actin
AUC : Areas under the ROC curve
CAFs : Cancer-associated fibroblasts
CSCs : Cancer stem cells
CHC : Chronic hepatitis C
CD133 : Cluster of differentiation 133
CD44 : Cluster of differentiation 44
COL11A1 : Collagen Type XI Alpha I Chain
CCSCs : Circulating cancer stem cells
HCV : Hepatitis C virus
HCC : Hepatocellular carcinoma
HB : Hepatoblastoma
LCSCs : Liver cancer stem cells
TME : Tumor microenvironment
TAMs : Tumor-associated macrophages
ROC : Receiver operating characteristic
TBRI : Theodor Bilharz Research Institute

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