RESEARCH ARTICLE

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Diagnostic Performance of Circulating Tumor Cells for Predicting of Hepatocellular Carcinoma in Hepatitis C Virus-High Risk Patients: Role of Liquid Biopsy

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and a global health problem. It is often diagnosed at advanced stage where hopeless for effective therapies. Identification of more reliable biomarkers for early detection of HCC is urgently needed. circulating tumor cells (CTCs) represent a unique liquid biopsy carrying comprehensive biological information of the primary tumor. Herein, we sought to develop a novel score based on the combination of the most significant CTCs biomarkers with and routine laboratory tests for accurate detection of HCC. **Methods:** Cytokeratin 18 (CK18), Cytokeratin 19 (CK19), albumin, platelets count, and α-fetoprotein were assayed in HCC patients (42), liver cirrhosis patients (83) and healthy control (20). **Results:** Areas under receiving operating curve (AUCs) were calculated and used for construction on novel score. A novel score named HCC-CTCs = AFP (U/L) × 0.08 - Albumin (g/dl) × 84 + CK 18 % × 2.9 + CK19 × 3.1- Platelets count ($\times 10^9$)/L× 0.75– 510. HCC-CTCs score produce AUC of 1 for differentiate patients with HCC from those with liver cirrhosis with sensitivity and specificity of a cut-off 0. **Conclusions:** HCC-CTCs score could replace AFP during screening of HCV patients and early detection of HCC.

Keywords: Hepatocellular carcinoma- HCV- CTCs- Diagnosis

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent malignancy worldwide and is currently listed as the fourth leading cause of cancer-related death (Rashed et al., 2020) . HCC is a highly aggressive neoplasm; distant organ metastasis can occur at a very early stage (Bialecki and Di Bisceglie 2005). Thus, early detection of HCC is of great importance in the management of HCC. Surgical resection or liver transplantation remains the primary therapy for HCC patients. However, only approximately 20%-30% of patients are eligible for surgical intervention at the time of the first diagnosis, most patients have already reached an advanced cancer stage (Chen et al., 2020b). Currently, early detection or monitoring of HCC incidence mainly relies on imaging examinations and serum tumor biomarkers such as alpha-fetoprotein (AFP); however, their diagnostic sensitivity is limited and often fails to foresee the tumor metastatic potential (Pinero et al., 2020). Therefore, there is an unmet need for reliable biomarkers for early HCC detection and tumor recurrence monitoring. Recently, various "liquid biopsy" techniques have emerged and shown significant promise as novel biomarkers for HCC. Liquid biopsy offers a solution that can bypass the problems of invasive biopsy procedures, enabling repeated and real-time disease status monitoring (Michela 2021). Circulating tumor cells (CTCs) are the cells that derive from the primary or metastatic lesions and migrate into circulation and are regarded as the "seeds" of tumor metastasis (Ahn et al., 2021). CTCs represent a unique liquid biopsy form that is different from any of the existing cancer biomarkers, as they are a sampling of the patient's live tumor cells, carrying comprehensive biological information of the primary tumor, including genomic mutations, cancer subtypes, and drug sensitivity (Lim et al., 2019). Thus, CTCs therefore represent an interesting source of biological information to understand dissemination, drug resistance and treatment-induced cell death (Dianat-Moghadam et al., 2020). However, only a few studies have addressed the role of CTCs in HCC. This could be attributed to the paucity of CTCs in patient blood, which makes them difficult to detect, as well as the debate

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concerning detection methods and the relative lack of specific HCC markers (Chen et al., 2020a). Cytokeratins (CKs) are the major filament proteins in the liver where any hepatocyte membrane integrity damage causes their release into the circulation (Bateman and Hubscher 2010). Moreover, CKs have been known as cellular integrators in several neoplastic changes. Characteristic combinations of CKs are expressed by different epithelia according to the organ of origin and differentiation (Turley et al., 2008). Cytokeratine-18 (Ck18) is a type 1 cytokeratin that represents about 5% of proteins in the liver and has potential role in hepatocyte apoptosis which mediates hepatocytes damage and morphological changes through a family of intracellular cysteine proteases (Caspases). These caspases react on different substrates including cytokeratin-18, with a consequence of apoptotic changes and characteristic damage. It has been confirmed that CK18 secretion occurs in parallel with DNA synthesis, protein synthesis, and cell division and this suggests an important role of Ck18 in carcinogenesis (Ismail et al., 2017). Recently, it was reported that elevation level of CK18 in patients with hepatocellular carcinoma (Menz et al., 2021). Cytokeratine-19 (Ck19) is an intermediate filament with a molecular weight of around 40 kDa. During the embryonic development, CK19 was detected in the primitive hepatic progenitor cells at the 4-10 weeks' gestation. CK19-positive HCC cells showed strong association with invasion, epithelial-mesenchymal transition (EMT) and angiogenesis. Moreover, knockdown of CK19 successfully inhibited the invasive capacity in human HCC cells (Zhuo et al., 2020). Therefore, in the present study, we assess the contribution of CTCs in patients with HCV-associated chronic hepatitis (CH) and HCC via determination of CK 19 and CK 18. We also developed and evaluated the sensitivity and specificity of a multivariate discriminate analysis (MDA) function based on five blood biochemical markers (CK18, CK19, AFP, Albumin, Platelets) to predict hepatocellular carcinoma among HCV-high risk patients.

Materials and Methods

Patients

The current study includes two groups of patients; the first one involved 42 patients with liver cirrhosis who has developed hepatocellular carcinoma (HCC). HCC was histologically diagnosed whenever surgical specimens were available. Otherwise, diagnosis of HCC was based on computed tomography and elevated AFP levels. HCC patients were classified according to the sixth edition of the International Union against Cancer tumor-node-metastasis staging system (Edge and Compton 2010). The second group comprises 83 of chronic hepatitis (CH) patients with liver cirrhosis and bearing no evidence of malignancy, which is confirmed by golden standard tests. All patients were recruited from Helwan University Hospital, Badr City, Cairo, Egypt from November 2017 till April 2019. Patients were subjected to full clinical examinations, radiologic investigations (including abdominal ultrasonography and triphasic computed tomography) and laboratory investigations. All studied cases (HCC and CH) were HCV-positive as confirmed by polymerase chain reaction (PCR) and serologic tests. Moreover, all participants were sero-negative for hepatitis B surface markers (HBsAg, HBeAg and HBcAb) and HBV antibodies. In addition, a control healthy group which involved 20 individuals with matched age and sex and have no evidence of any hepatitis markers. 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.

Blood Samples

Peripheral blood samples (Two samples, 7.5 mL each) were collected from patients and control subjects in Cell-Save blood collection tubes (Immunicon Inc., Huntingdon Valley, PA, United States) containing EDTA and a cellular preservative. From each subject, one tube was used for assessment of CTCs and the other was used for RNA and DNA extraction. Platelet's count was performed on a D-cell 60 Automated Hematology Analyzer (Diagon Ltd, Budapest, Hungary). Liver functions tests (albumin, total bilirubin, AST and ALT) were all measured on an automated Biochemistry Analyzer (A15; Biosystem, Barcelona, Spain). AFP level was performed by chemiluminescence, with Immulite AFP (1000) kit (Mini-Vidas, France).

Detection of HCV and HBV Total viral

DNA/RNA isolation was performed using QIAamp MinElute Virus Spin Kit (Qiagen, Venlo, Limburg, Germany). HBV-DNA was analyzed by PCR as previously described (Kazemi-Shirazi et al., 2000). HCV-RNA detection and quantification were done using a StepOne Real-Time PCR system (Applied Biosystems of Thermo Fisher Scientific Inc., Waltham, MA, United States).

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from whole blood by a standard density gradient centrifugation procedure using Ficoll-Hypaque (Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany) (McGahon et al., 1995). For each subject, blood sample was collected in a 15 ml sterile falcon tube and allowed to stand with an equal volume of dextran/saline solution for 45 min at 20–25 °C. The leukocyte-rich plasma (buffy coat) was aspirated and centrifuged at 170 × g for 10 min. Pellets were then suspended in a volume of PBS (phosphate-buffered saline) to the starting volume of blood, placed on top of Ficoll solution and centrifuged at 400 × g at 20 °C for 40 min. The supernatant was discarded and the pellets were washed with 0.34 M sucrose to remove platelets. A few remaining erythrocytes were disrupted by hypotonic lysis with 10% ammonium chloride (cold 0.2% NaCl for 30 s). Isolation was restored by 1.6% NaCl. PMMCs were finally washed and suspended in PBS and fixed in ice-cold absolute alcohol at +4 °C until used for flow cytometry analysis (Sirchia et al., 1973).

Fluorescent-Activated Cell Sorting Analysis (FACS)

After at least 12 h of fixation, the sample was again centrifuged, and excessive ethanol was removed by twice

washing with phosphate buffer saline. The separated cells were suspended in RPMI-1640, and the cell count was adjusted between 10×10^6 and 50×10^6/ml. The cell suspension was centrifuged, and the supernatant was discarded, cell pellet resuspended in PBS, and the cell count adjusted between 10×10^6 and 20×10^6/ml. Fifty µl of the cell suspension (containing from 0.5×10^6 to 1×10^6 cells) were added to each Falcon tube. Ten µl of the monoclonal; CK18-FITC and CK19-FITC (MACS; Milteny Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocols. CK18 and CK19 well-known epithelial marker. Cells (≥ 30,000/sample) were acquired after flow cytometry and counted using the Cell Quest software. Three successive readings were recorded for each sample and the mean was calculated and expressed as the number of CTCs/7.5 mL of blood. A sample of normal lymphocytes was included in each run as a negative control. A cut-off of 4 ± 1 CTCs/7.5 mL was chosen to define the test as positive (Komeda et al., 1995).

Statistical analysis

Statistical analysis was performed with the Medcalc version 11.3.3.0 statistical software package. All data were presented as arithmetic mean ± standard deviation (X ± SD), and they were considered statistically significant if the two-sided p value was <0.05. Mann–Whitney U test was used for comparisons between independent groups. To assess and compare the diagnostic accuracy of biochemical markers for discriminating those with HCC from that with chronic hepatitis, we plotted receiver operating characteristic (ROC) curves. The best collection parameters were selected based on the significant difference between patients with CH versus HCC. The multivariant discriminate analysis (MDA) was carried out stepwise with the use of minimum Wilks' lambda. The discriminate model is designed by the standardized canonical discriminate coefficients. The sign (plus or minus) depicts whether it is a direct or inverse relation of the independent variables with the dependent variable (HCC or CH). In addition, sensitivity, specificity, and accuracy were calculated.

Results

Patient's characteristics

Clinico-pathological and demographic parameters for chronic hepatitis, hepatocellular carcinoma, and healthy control groups were illustrated in Table 1. Both platelets count and serum albumin showed significant decrease in HCC patients compared to CH group. In contrast; significant increase of INR, AST/ALT ratio, APRI, bilirubin and AFP in HCC patients compared to CH group. HCC patients were divided into 30 (71%) patients with stage I+II and 12 patients (29%) with stage III+IV. Non-tumor capsulation represented in 27 (64%) patients and 15 (36%) patients with complete encapsulation. As illustrated in Table 1, HCC patients with tumor grade I represent 22 (52%) of total patients and patients with high tumor grade (II+III) represent 20 (48%). According to tumor size, patients were classified into, 10 (24%) patients with small tumor size and 32 (76%) patients with high tumor size. Vascular invasion was represented in 30 (71%) patients. Patients with multiple lesions represent 27 (64%) of total HCC patients.

Circulating Tumor Cells (CTCs) Biomarkers

As shown in Table 2 and Figure 1, there was a significant increase in CK18% in patients with CH as

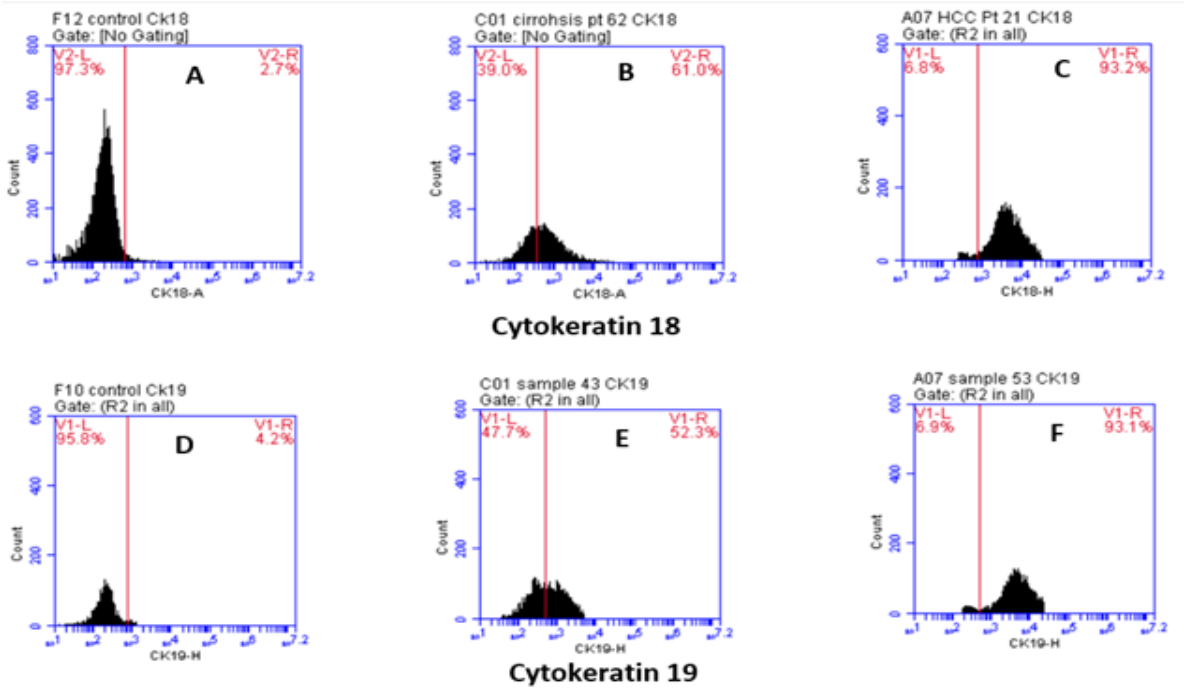

Figure 1. Flow Cytometry Histogram for Frequency of CTCs Biomarkers (CK18 and CK19): A. CK18+ for control, B. CK18+ for patients with chronic hepatitis, C. CK18+ for patient with hepatocellular carcinoma, D CK19+ for control, E. CK19+ for patient with chronic hepatitis, F. CK19+ for patient with hepatocellular carcinoma.

well as HCC patients when compared with corresponding control (P < 0.0001 for both). Moreover, CK18% was significantly increased in patients with HCC, (P < 0.001) when compared with those with CH. In addition, CK19% was significantly increased in both HCC and LC patients when compared with healthy group (P < 0.0001). Consequently, PBMCs subpopulation CK19% was significantly increased in patients with HCC when compared with those with CH (P < 0.0001).

Diagnostic performance using area under the ROC curves

ROC curve analysis was performed to assess and compare diagnostic utility of multiple biomarkers in order to find the best biomarkers to chosen in our combination for the best and accurate differentiation between CH and HCC patients. Our candidate parameters were including AST/ALT, albumin, total bilirubin, platelets count, AFP, CK18 and CK19.The most effective biomarkers with high area under curves were in order of CK19 (0.943) > AFP (0.827) > CK18 (0.803) > platelets count (0.748) > Albumin (0.675). On other hand, AST/ALT and bilirubin had a low AUCs and so was excluded from our combination (0.575 and 0.652 respectively) (Figure 2).

Multivariate analysis and predictive model

A predictive model was constructed using multivariate discriminant analysis. In order to enhance the diagnostic performance of AFP to able to differentiate HCC patients from those with CH we combined AFP with the most other biomarkers with high AUC. Simply, we start combination with two biomarkers (AFP and CK19), then three biomarkers (AFP, CK19 and CK18), then four biomarkers (AFP, CK19, CK18 and Platelets count) then five biomarkers (AFP, CK18, CK19, platelets count and Albumin). Multivariate discriminate analysis selects a most potent model for early prediction of HCC among hepatitis C virus patients. The proposed model named CTCs-HCC = AFP (U/L) × 0.08 - Albumin (g/dl) ×

Table 1. Clinico-Pathological Data of Healthy Individuals and Patients with Chronic Hepatitis and Hepatocellular Carcinoma

Variable	Healthy control (n= 20)	CH patients (n= 83)	HCC patients (n = 42)	*P value
Age (years)	41.3 ± 6.2	39.1 ± 8.7	50.4 ± 10.7	< 0.0001
AST (U/L)	14.4 ± 9.6	43.2 ± 11.4	91 ± 12.8	< 0.0001
ALT (U/L)	16.7 ± 10.1	51.4 ± 13.1	105 ± 11.4	< 0.0001
AST/ALT (AAR)	0.33 ±0.06	0.82 ± 0.06	0.91 ± 0.09	0.005
Albumin (g/dl)	4.8 ± 0.41	4.2 ± 0.63	2.7 ± 0.57	< 0.0001
Total Bilirubin (mg/dl)	0.82 ±0.34	1.01 ± 0.31	3.1 ± 0.31	< 0.0001
Platelets count ($\times 10^9$)/L	311 ± 66	141 ± 57	48 ± 14	< 0.0001
INR	0.78 ± 0.21	1.7 ± 0.34	2.6 ± 0.55	< 0.0001
AFP (U/L)	1.2 ± 3.02	8.5 ± 2.1	231 ± 23	< 0.0001
APRI	0.31 ± 0.08	2.1 ± 0.31	2.8 ± 0.71	< 0.0001
Tumor stage, n (%)				
I + II			30 (71)	
III + IV			12 (29)	
Tumor encapsulation, n (%)				
Non			27 (64)	
Complete			15 (36)	
Tumor grade,n (%)				
I			22 (52)	
II + III			20 (46)	
Tumor size, n (%)				
< 5cm			10 (24)	
>5 cm			32 (76)	
Vascular invasion, n (%)				
Absent			30 (71)	
Present			12 (29)	
Number of Lesion, n (%)				
Single			27 (64)	
Multiple			15 (36)	

*P> 0.05 considered not significant (NS), P< 0.05 considered significant. The reference group of p value were hepatocellular carcinoma (HCC) group and chronic hepatitis (CH) group. INR, international normalized ratio; AFP, alpha fetoprotein, APRI= [AST(U/L)/(40)]/[Platelet count $\times 10^9$/L]×100; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Variables were expressed as mean ± SD.

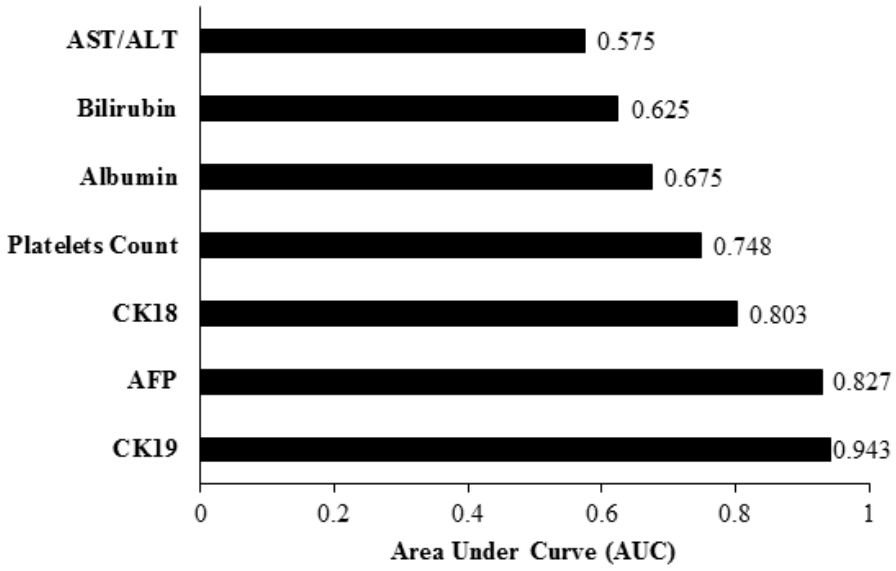

Figure 2. Area under the ROC Curves of Candidate Biomarkers for Discrimination between Chronic Hepatitis and Hepatocellular Carcinoma Patients

Table 2. Cytokeratin 18 and Cytokeratin 19 Percentage in Chronic Hepatitis, Hepatocellular Carcinoma and Corresponding Healthy Control.

	Cytokeratin 18 (%)	Cytokeratin 19 (%)
Healthy control (n= 20)	11.3 ± 6.4	9.2 ± 3.9
CH patients (n= 83)	27.1 ± 15.2*	22.3 ± 14.8*
HCC patients (n = 42)	66.5 ± 19.1**	56.2 ± 27.6**

P< 0.05 considered significant, (*) compared to healthy control group, (**) compared to chronic hepatitis group; CH, Chronic Hepatitis; HCC, Hepatocellular carcinoma

84 + CK 18 % × 2.9 + CK19 × 3.1- Platelets count ($\times 10^9$)/L× 0.75– 510. The score had a range from 0 to 1.0 and showed highly significant (P< 0.001. Figure 3) for differentiate patients with HCC from those with liver cirrhosis. CTCs-HCC score was calculated for everyone; in the current study and produce the highest AUC for differentiate HCC patient from those with liver cirrhosis (1.00) compared to AFP (0.827). The highest sensitivity (100%) and specificity (100%) was taken at a cut-off 0, where above 0 patient considered with HCC and below 0 patients considered with liver cirrhosis. In addition, sensitivity of AFP for detection of HCC after implantation to the new developed score was shifted from 84% to 100%.

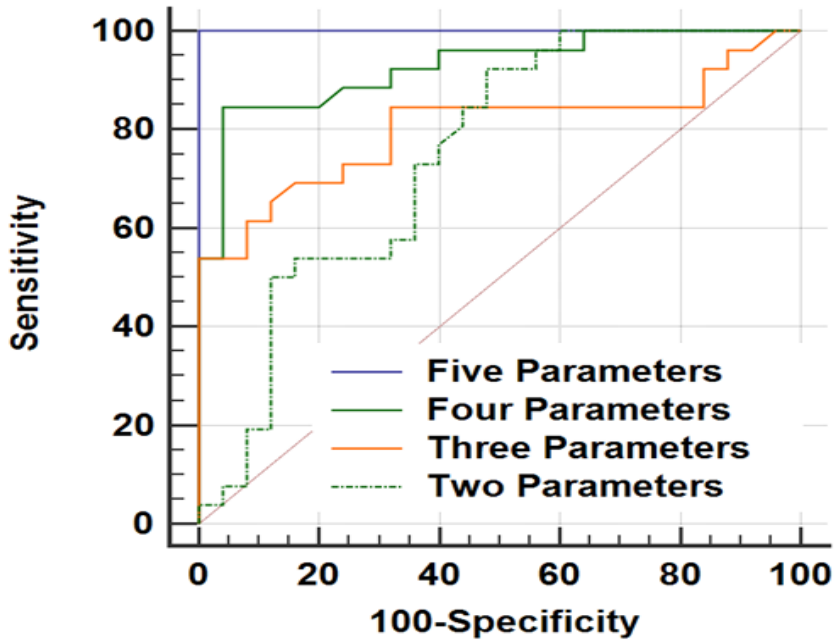

Figure 3. Area under the ROC Curve for CHCs-HCC Score for Two Parameters (AFP and CK19), three parameters (AFP, CK19 and CK18), four parameters (AFP, CK19, CK18 and Platelets count) and five biomarkers (AFP, CK18, CK19, platelets count and Albumin).

Table 3. Diagnostic Performance of CTCs-HCC Score against Tumor Burden in Hepatocellular Carcinoma

Clinical data	CTCs-HCC score			
	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC
Tumor stage				
I + II	82	83	73	0.864
III + IV	79	81	72	0.773
Tumor encapsulation				
Non	84	78	71	0.868
Complete	78	79	86	0.954
Tumor grade				
I	76	78	84	0.972
II + III	80	73	81	0.756
Tumor size				
< 5cm	82	79	88	0.801
> 5 cm	84	74	76	0.735
Vascular invasion				
Absent	78	88	83	0.821
Present	81	91	76	0.761
Number of Lesion				
Single	81	75	78	0.894
Multiple	78	71	84	0.807

Diagnostic performance of CTCs-HCC score in comparison with AFP

The diagnostic utility of CTCs-HCC score and AFP against tumor burden were illustrated in Table 3 and Table 4. The diagnostic power of CTCs-HCC score was the better one for discriminating patients with HCC from those with liver cirrhosis compared to AFP alone (AUC were 1.00 and 0.827, respectively). The AUCs of CTCs-HCC for differentiating patients with low TNM stage, complete capsulation, low grade, small tumor size, absence of vascular invasion and single focal lesions from patients with non-malignancy (0.864, 0.954, 0.972, 0.801, 0.821, and 0.894, respectively) which were better than AFP (0.607, 0.599, 0.543, 0.612, 0.504, and 0.619, respectively). As illustrated in Table 4, AFP alone had a weak diagnostic power for differentiate HCC patients with small tumor size from those with liver cirrhosis where AUC was 0.612 versus CTCs-HCC which produced AUC

Table 4. Diagnostic Performance of AFP Score against Tumor Burden in Hepatocellular Carcinoma

Clinical data	AFP			
	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC
Tumor stage				
I + II	61	81	69	0.607
III + IV	72	79	73	0.532
Tumor encapsulation				
Non	74	67	68	0.611
Complete	81	71	84	0.599
Tumor grade				
I	78	74	73	0.543
II + III	83	69	79	0.501
Tumor size				
< 5cm	79	73	81	0.612
> 5 cm	85	71	75	0.678
Vascular invasion				
Absent	74	87	75	0.504
Present	76	89	68	0.576
Number of Lesion				
Single	88	61	77	0.619
Multiple	79	83	81	0.522

of 0.801. Noteworthy, AFP was unable to discriminate patients with low grade from those with high grade (AUC of 0.543) compared with high AUC of 0.972 produced by our CTCs-HCC score.

Discussion

Chronic HCV infection plays a major role for the development of cirrhosis and hepatocellular carcinoma that usually presents in a late stage with limited therapeutic options (Elgharably et al., 2017). AFP is recommended for surveillance of HCV patients even alone or combined with radiological imaging of the liver. Due to its low sensitivity for accurate diagnosis of HCC patients, many efforts were applied in order to improve its sensitivity for early detection of HCC among hepatitis high risk patients. Multiple indices were designed to improve diagnosis of HCC. Unfortunately, all these indices were not considered ideal because all parameters included in those scores were affected by other pathological state rather than development of HCC itself (Pinero et al., 2020). Accumulating evidence suggested that the aggressive behavior of hepatocellular carcinoma could be partially attributed to the presence of malignant hepatocytes that gained entry into circulation, either before or during surgery. Therefore, identification of these small populations of cells in patients' blood together with the search for sensitive biological biomarkers are highly recommended for better patient management (Bahnassy et al., 2014). In the present study, we validated the utility of flow cytometry for cell immuno-phenotyping as a rapid and highly sensitive technique for the follow-up of HCV infected patients at different disease stages. This was achieved through detecting the interaction of CK19 and CK18 antibodies with its antigens, which is present in the cytoplasm of hepatocytes. The possible prognostic and predictive values of CTC markers in monitoring HCV-infected patients was assessed by comparing their expression with standard prognostic factors, and their utility for early detection of HCC was also evaluated. Our data indicated that flow cytometry able to identify a significantly higher number of CTCs (CK18 and CK19) in the blood of HCC patients compared to CH and control groups. This confirms the utility of flow cytometry in enumerating CTCs, and thus it can be used to monitor CH patients for early detection of HCC, as it is sensitive and easy, relatively less expensive, and more rapid compared to the currently used techniques such as PCR or Cell Search system. In an attempt to identify sensitive diagnostic markers that can help to differentiate between CH and HCC in HCV-infected patients and thus permit early detection of HCC, we construct a simple score based on combination of CTCs biomarkers and routine available biochemical markers which associated with liver impairment. This provides evidence that biomarkers could be used as indicators to predict HCC in CH patients. In current study, CK19 was significantly elevated in HCC patients compared to CH patients that, which is in agreement with previous reports (Cai et al., 2016). Recently it was reported that, CK19 can predict HCC with high sensitivity (87%) and specificity (100%), and can thus be used as a prognostic factor which is associated with increased metastatic potential and early recurrence (Xu et al., 2021) so, it was chosen as the basic index for construction of our score. As a tumor marker, CK18 has been well studied in different cancers as esophageal squamous cell carcinoma, renal cell carcinoma, oral cavity carcinoma, lung cancer, human breast and colorectal cancer (Menz et al., 2021). Moreover, it was reported that both circulating and hepatic CK18 were significantly elevated in patients of chronic hepatitis compared to healthy controls which in line with our findings (Ismail et al., 2017). That elevation may be due to liver apoptosis and consequently could be useful for monitoring disease activity in chronic HCV and liver cirrhosis patients. In agreement with previous reports, our result showed a significant elevation of CK18 in HCC patient compared to CH patients and this suggests that CK18 measurement may improve non-invasive diagnosis of HCC (Waidmann et al., 2016, Gonzalez-Quintela et al., 2006). In addition, an in vitro study showed that cytokeratin-18 expression was significantly higher in six HCC cell lines examined than in the control cells using immunofluorescence staining and microscopic examination (Mu et al., 2014). Consequently, that CK18 may drive neoplastic transformation of glutathione S-transferase in rat hepatocytes, causing HCC (Yilmaz, 2009). So, we could suggest that CTCs play important roles in the development and progression of HCV-associated HCC. Enumeration of CTCs by flow cytometry using CK19 and CK18 has high sensitivity and specificity and is likely clinically useful in improving prognostic accuracy and monitoring therapeutic outcomes of HCV infected patients. In addition, aberrant expression of HCC-specific and CTCs markers (CK19 and CK18) contributes to poor prognosis in HCC patients and should be assessed to provide better management of those patients. In HCC patients, thrombocytopenia may occur due to a reduction in synthesis of thrombopoietin, which in turn increased slenic sequestration of platelets secondary to portal hypertension or the mylosuppressive action of HCV infection (Dai et al.,). In the current study, it was observed that platelets count was significantly reduced in HCC patients. Further activation of platelets ensues from the original tumor; triggering enhanced growth of the tumor as a result of the release of platelets granules (Nash et al., 2002). Release of the contents of the granules from platelets hinders the ability of the immune surveillance system against malignancy through cloaking tumor cells and protecting the tumor cells from natural killer (NK) cells by providing a physical barrier and also placing major histocompatibility complex (MHC) class I antigen into the vicinity of the tumor cell surface (Ntziachristos et al., , Sabrkhany et al.,). Thus, platelets count was taken in our consideration for monitoring liver status and development of malignancy. Liver is considered a huge reserve for albumin, so its decreased levels may reflect liver impairment and is considered main liver function monitor and thus albumin is used in liver assessment during development of HCC (Attallah et al.,). It was reported that, serum albumin is an important factor for use in several scoring systems, such as the Child–Pugh and CLIP score systems (Ishizuka et al.,). In the current

study, serum albumin was significantly decrease in HCC patients compared to cirrhotic patents, so it was taken in our consideration during construction of our score. Herein, for the first time, we report the clinical validation of four biomarkers (CK19, CK18, albumin and platelets count) in combination with AFP to improve the accuracy for diagnosis oh HCC among hepatitis C high risk patients. HCC-CTCs score could potentially be used to diagnose HCC, especially early stages and will help to resolve the deficiencies of AFP in the testing of AFP negative patients. The possibility of discriminating HCC from healthy individuals and patients with cirrhosis offers hope for the early detection of HCC. Our score could be used as blood tests for the noninvasive diagnosis of HCC to reduce the need for the invasive liver biopsy. Applying our score on other large multicenter cohort to verify its effectiveness is needed to confirm our findings. However, further studies are still needed to confirm the utility of CTCs biomarkers in personalized medicine and targeted therapy as well as to clarify the possibility of using cytokeratins for early detection of HCC in HCV-infected patients, as it increased significantly with disease progression from CH to HCC.

Author Contribution Statement

HE conceived the study. All authors participated in the development of the protocol. ME and NG collected and cleaned the data, and obtained ethics approval and consent. FE and FM analyzed the data. HE and RZ wrote the first draft of the paper. HE, ME, and RZ edited the manuscript and provided expert advice based on their medical specialist knowledge. All authors prepared and revised the manuscript, including relevant scientific content. All authors approved the final version of the manuscript.

Acknowledgments

Compliance with Ethical Standards

All procedures performed in in the study followed the relevant ethical standards of the institutional or national research committee (Ethics Board of Helwan University with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interests

The authors declare that they have no conflicts of interests.

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