RESEARCH ARTICLE

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Diagnostic Performance of Epithelial Cell Adhesion Molecule for Early Detection of Hepatocellular Carcinoma Among HCV High-Risk Patients

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Abstract

Objective: Hepatocellular carcinoma (HCC) 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 routine laboratory tests for accurate detection of HCC. **Materials and Methods:** Epithelial cell adhesion molecule (EpCAM), α-fetoprotein, albumin, and platelets count were assayed in HCC patients (98), liver cirrhosis patients (77). Areas under receiving operating curve (AUCs) were calculated and used for construction on novel score. **Results:** A novel score named EpCAM-HCC = AFP (U/L) × 0.11 - Albumin (g/dl) × 1.5 + EpCAM % × 2.9 - Platelets count ($\times 10^9$)/L× 0.75 – 93. EpCAM-HCC score produce AUC of 1 for differentiate patients with HCC from those with liver cirrhosis with sensitivity and specificity of a cut-off 1.7 (i.e., less than 1.7 the case is considered cirrhotic, whereas above 1.7 it is considered HCC. **Conclusion:** EpCAM-HCC score could replace AFP during screening of HCV patients and early detection of HCC.

Keywords: Hepatocellular carcinoma- HCV- CTCs- EpCAM

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Introduction

Hepatocellular carcinoma (HCC) ranks fourth in terms of cancer-related mortality and is the sixth most common cancer globally [1]. The majority of patients with HCC have already reached an advanced stage of cancer when they are first diagnosed, and only 20% to 30% of patients are eligible for surgical surgery [2]. Currently, imaging tests and serum tumor biomarkers like alpha-fetoprotein (AFP) are the main tools used for early diagnosis or monitoring of HCC incidence; nevertheless, their diagnostic sensitivity is low and frequently falls short of predicting the tumor's capacity to spread [3]. 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 [4]. 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 [5]. 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 [6]. Thus, CTCs therefore represent an interesting source of biological information to understand dissemination, drug resistance and treatment-induced cell death [7]. 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 concerning detection methods and the relative lack of specific HCC markers [8].

EpCAM (epithelial cell adhesion molecule; CD326 (cluster of differentiation 326)) was originally identified as a novel tumour-specific cell surface antigen after immunization of mice with cancer cells, and was later

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defined as a cell–cell adhesion molecule [9]. EpCAM is a type I transmembrane glycoprotein with an ectodomain, one transmembrane domain, and a cytoplasmic domain of 26 residues. This glycoprotein is specifically expressed in epithelial tissue and overexpressed in a large variety of human epithelial-derived neoplasms, including cancer of the tongue, thyroid, prostate, oesophagus, liver, colon, breast, ovary, pancreas, gallbladder, lung, stomach and kidney [10]. Recent studies have revealed that EpCAM is involved in cell signalling, migration, proliferation and differentiation, as well as in metastasis and cancer stem cells [11]. EpCAM-positive HCC cells display some of the liver cancer stem cell-like characters which include; self-renewal and differentiation, maintaining the capacity for malignant proliferation, invasion, metastasis, and tumor recurrence [12]. As most cancers are epithelial in origin, targeting EpCAM therefore became the most commonly used epithelial marker for the capture of CTCs in the blood circulation of carcinoma patients [13]. Recently, we developed a novel score-based CTCs surface markers including cytokeratins 18 and 19 for accurate diagnosis of HCC among HCV patients [14]. Therefore, in the present study, we assess the contribution of CTCs in patients with HCV-associated chronic hepatitis (CH) and HCC via determination of EpCAM. We also developed and evaluated the sensitivity and specificity of a multivariate discriminate analysis (MDA) function based on four blood biochemical markers (EpCAM, AFP, Albumin, Platelets count) to predict hepatocellular carcinoma among HCV-high risk patients.

Materials and Methods

Patients

The current study includes 98 patients with liver cirrhosis who have developed hepatocellular carcinoma (HCC). 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 [15]. In addition, 77 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, and Damietta Cancer Institute, Damietta 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. 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 patient 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 [16]. 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) [17]. 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 [18].

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; EpCAM-FITC (MACS; Milteny Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocols. 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 [19].

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 and hepatocellular carcinoma, 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 (31%) patients with stage I+II and 68 patients (69%) with stage III+IV. Non-tumor capsulation represented in 27 (28%) patients and 71 (72%) patients with complete encapsulation. As illustrated in Table 1, HCC patients with tumor grade I represent 22 (23%) of total patients and patients with high tumor grade (II+III) represent 76 (77%). According to tumor size, patients were classified into, 10 (10%) patients with small tumor size and 88 (90%) patients with high tumor size. Vascular invasion was represented in 30 (31%) patients. Patients with multiple lesions represent 61 (62%) of total HCC patients.

Table 1 added that, there was a significant increase in EpCAM percentage in patients with HCC patients when compared CH patients (P < 0.0001), (Figure 1).

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 included AST/ALT, albumin, total bilirubin, platelets count, AFP, and EpCAM. The most effective biomarkers with high area under curves were in order of EpCAM (0.961) > AFP (0.773) > platelets count (0.701) > 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. 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 EpCAM), then three biomarkers (AFP, EpCAM and platelets count) and finally, four biomarkers (AFP, EpCAM, platelets count and albumin). Multivariate discriminate analysis selects the most potent model for early prediction of HCC among hepatitis C virus patients. The proposed model named EpCAM-HCC = AFP (U/L) × 0.11 - Albumin (g/dl) × 1.5 + EpCAM % × 2.9 - Platelets count ($\times 10^9$)/L× 0.75 – 93. The score had a range from 1.1 to 3.5 and showed highly significant (P< 0.001. Figure 3) for differentiate patients with HCC from those with liver cirrhosis. EpCAM-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

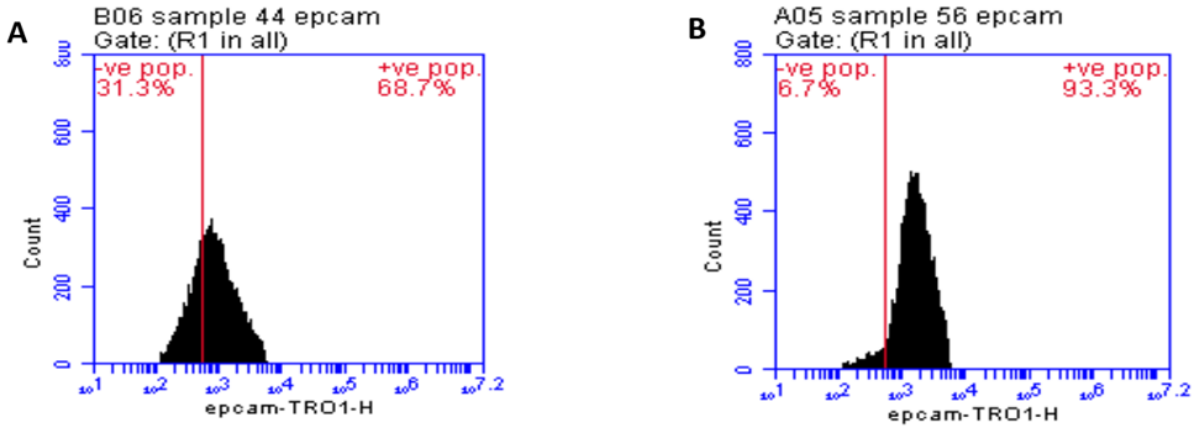

Figure 1. Flow Cytometry Histogram for Frequency of CTCs Biomarker, (EpCAM): A. EpCAM+ for Patients with Chronic Hepatitis, B. EpCAM+ for Patient with Hepatocellular Carcinoma

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

Variable	CH patients (n= 77)	HCC patients (n = 98)	*P value
Age (years)	44.1 ± 8.7	61.3 ± 12.7	< 0.0001
AST (U/L)	55.2 ± 13.4	101 ± 13.8	< 0.0001
ALT (U/L)	57.4 ± 11.1	120 ± 12.4	< 0.0001
AST/ALT (AAR)	0.98 ± 0.06	0.91 ± 0.09	0.005
Albumin (g/dl)	4.2 ± 0.63	2.7 ± 0.57	< 0.0001
Total Bilirubin (mg/dl)	1.01 ± 0.31	3.1 ± 0.31	< 0.0001
Platelets count (×109)/L	101 ± 57	55 ± 14	< 0.0001
INR	1.8 ± 0.34	2.9 ± 0.55	< 0.0001
AFP (U/L)	9.5 ± 2.1	345 ± 23	< 0.0001
APRI	3.1 ± 0.31	8.8 ± 0.71	< 0.0001
EpCAM (%)	27.8 ± 3.7	68.4 ± 12.8	< 0.0001
Tumor stage, n (%)			
I + II		30 (31)	
III + IV		68 (69)	
Tumor encapsulation, n (%)			
Non		27 (28)	
Complete		71 (72)	
Tumor grade,n (%)			
I		22 (23)	
II + III		76 (77)	
Tumor size, n (%)			
< 5cm		10 (10)	
>5 cm		88 (90)	
Vascular invasion, n (%)			
Absent		30 (31)	
Present		68 (69)	
Number of Lesion, n (%)			
Single		37 (38)	
Multiple		61 (62)	

*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 ×10^9/L]×100; AST, aspartate aminotransferase; ALT, alanine aminotransferase; EpCAM, Epithelial Cell Adhesion Molecules; Variables were expressed as mean ± SD.

(0.773). The highest sensitivity (100%) and specificity (100%) was taken at a cut-off 1.7, where above 1.7 patients considered with HCC and below 1.7 patients considered with liver cirrhosis. In addition, sensitivity of AFP for detection of HCC after implantation to the developed score was shifted from 81% to 100%.

Diagnostic performance of EpCAM-HCC score in comparison with AFP

The diagnostic utility of EpCAM-HCC score and AFP against tumor burden were illustrated in Table 2 and Table 3. The diagnostic power of EpCAM-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 0.773, respectively). The AUCs of EpCAM-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 (0.916, 0.924, 0.901, 0.842, 0.861, and 0.834, respectively) which were better than AFP (0.611, 0.604, 0.623, 0.558, 0.501, and 0.634, respectively).

Table 2. Diagnostic Performance of EpCAM-HCC Score against Tumor Burden in Hepatocellular Carcinoma

Clinical data	EpCAM-HCC			
	Sensitivity (%)	Specificity (%)	Accuracy (%)	AUC
Tumor stage				
I + II	82	83	73	0.916
III + IV	79	81	72	0.773
Tumor encapsulation				
Non	84	78	71	0.868
Complete	78	79	86	0.924
Tumor grade				
I	76	78	84	0.901
II + III	80	73	81	0.756
Tumor size				
< 5cm	82	79	88	0.842
> 5 cm	84	74	76	0.735
Vascular invasion				
Absent	78	88	83	0.861
Present	81	91	76	0.761
Number of Lesion				
Single	81	75	78	0.834
Multiple	78	71	84	0.807

Table 3. 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	0.611
III + IV	72	79	73	0.532
Tumor encapsulation				
Non	74	67	68	0.611
Complete	81	71	84	0.604
Tumor grade				
I	78	74	73	0.623
II + III	83	69	79	0.501
Tumor size				
< 5cm	79	73	81	0.558
> 5 cm	85	71	75	0.678
Vascular invasion				
Absent	74	87	75	0.501
Present	76	89	68	0.576
Number of Lesion				
Single	88	61	77	0.634
Multiple	79	83	81	0.522

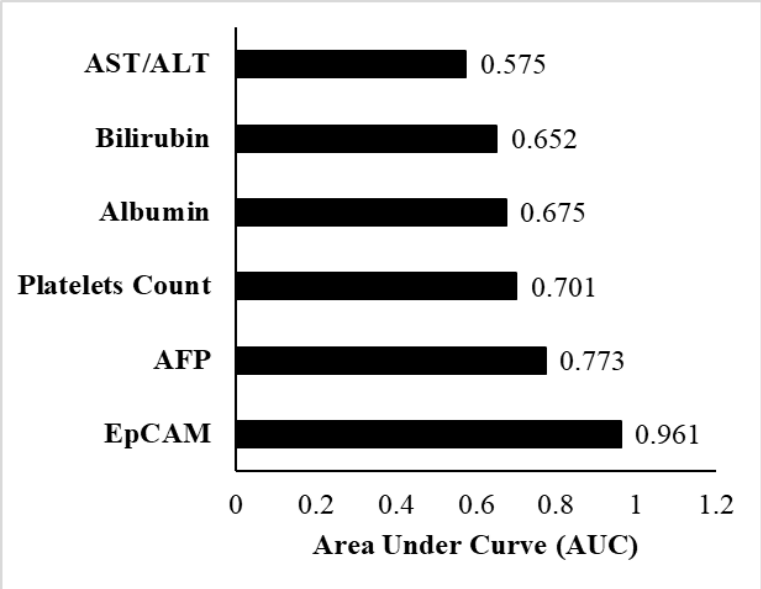

Figure 2. Area under the ROC Curves of Candidate Biomarkers for Discrimination between Chronic Hepatitis and Hepatocellular Carcinoma Patients.

Discussion

HCV plays a major role in liver damage and development of cirrhosis and hepatocellular carcinoma which usually presents in a late stage with limited therapeutic options [20]. Standard tumor marker for HCC, AFP is recommended for surveillance of HCV patients even alone or combined with other investigation including radiological imaging of the liver. Due to its low sensitivity for accurate diagnosis of HCC patients, many efforts were applied 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 [3]. 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 [21]. In the present study, we validated the utility of flow cytometry for cell immuno-

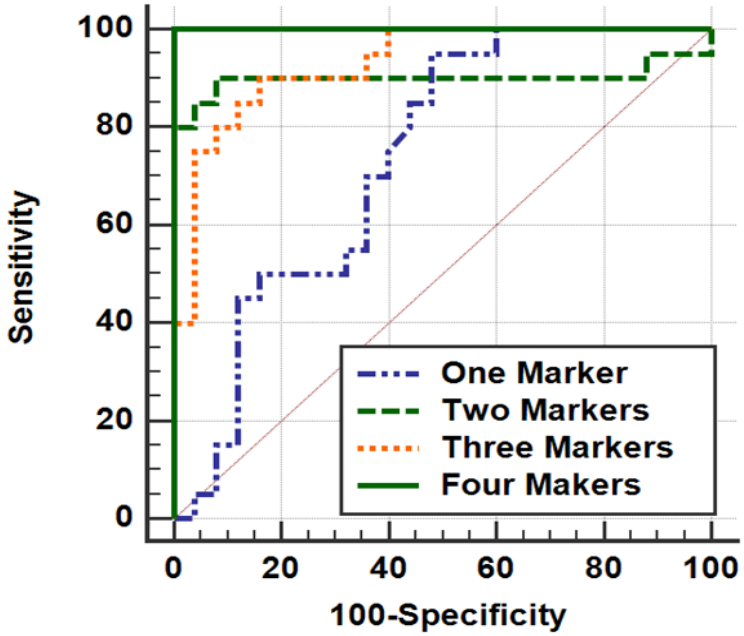

Figure 3. Area under the ROC Curve for EpCAM-HCC Score for One Marker (AFP), Two Parameters (AFP and EpCAM), Three Parameters (AFP, EpCAM and Platelets Count) and Four Parameters (AFP, EpCAM, Platelets Count and Albumin).

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 EpCAM antibodies with its antigens, which is present in the surface 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 was able to identify a significantly higher percentage of EpCAM 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 biomarker (EpCAM) 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, EpCAM was significantly elevated in HCC patients compared to CH patients that, which is in agreement with previous reports [22]. Recently it was reported that, EpCAM 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 [23]. As a tumor marker, EpCAM 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 [24]. So, it was chosen as the basic index for construction of our score. That elevation of EpCAM 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 EpCAM in HCC patient compared to CH patients and this suggests that EpCAM measurement may improve non-invasive diagnosis of HCC [25]. In addition, an in vitro study showed that EpCAM expression was significantly higher in six HCC cell lines examined than in the control cells using immunofluorescence staining and microscopic examination [26]. 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 EpCAM 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 (EpCAM) 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 [27]. 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 [28]. 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 [29, 30]. 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 [31]. 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 [32]. 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 (EpCAM, albumin and platelets count) in combination with AFP to improve the accuracy for diagnosis of HCC among hepatitis C high risk patients. EpCAM-HCC 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 against HCC from 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 EpCAM 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 RN collected and cleaned the data and obtained ethics approval and consent. MA and FM analyzed the data. HE and ME wrote the first draft of the paper. HE, ME, and RN 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.

Acknowledgements

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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