BIOCELL
2018 42(2): 55-60

ISSN 1667-5746 (on-line)
ISSN 0327-9545 (printed)

Associations between CD133, CK19 and G2/M in cirrhotic HCV (genotype-4) patients with or without accompanying tumor

Hoda M. EL-EMSHATY[1]; Entsar A. SAAD[2]; Mona S. GOUIDA[3]; Zahraa R. ELSHAHAWY[1,2]

[1] Gastroenterology Surgical Center, Faculty of Medicine, Mansoura University, Egypt
[2] Chemistry Department, Faculty of Science, Damietta University, Damietta, Egypt
[3] Mansoura Children Hospital, Faculty of Medicine, Mansoura University, Egypt

Key words: Cell cycle, Diagnostic performance, Liver, Panel

Abstract: Hepatitis C virus (HCV)-cirrhotic patients have the highest threat of developing hepatocellular carcinoma (HCC) and may be at risk of extra hepatic cancer. The present study was designed to investigate CD133 and CK19 in HCV (genotype-4)-cirrhotic patients with/without HCC or extra hepatic cancer, to assess the degree of their correlation with cell cycle abnormalities and finally to assess the role of their combination as diagnostic tool for discrimination of cirrhotic patients with HCC from those with extra hepatic cancer. The study included 77 HCV-cirrhotic patients and 20 healthy non-disease control group. Patients were categorized histo-pathologically into: 24 have only liver cirrhosis, 26 with HCC, and 27 patients with extra hepatic cancer. Cell cycle abnormalities, CD133 and CK19 were determined by flow cytometry technique. CD133 and CK19 showed marked elevation in HCC and extra hepatic cancer compared with liver cirrhosis and control subjects ($p<0.0001$). Positive associations were noted between CK19, CD133 and G2/M. They were gradually increased with progression from liver cirrhosis to HCC. Combination of the three showed the best AUC (0.978) and accuracy (92.5%) for discrimination of HCC from extra hepatic cancer. Combined CD133 with G2/M and CK19 comprises an excellent diagnostic panel for discrimination of HCV-cirrhotic patients with HCC from those with extra hepatic cancer.

Introduction

Many variable pathologic conditions may directly/indirectly cause liver damage such as viral infection (Saad *et al*., 2017a), accumulated body fat (Habib *et al*., 2015), diabetes (Saad *et al*., 2015a; Saad *et al*., 2017b), different tumors (Saad *et al*., (2017c,d,e), etc. Among these, chronic HCV is the chief cause of liver fibrosis, liver cirrhosis and liver cancer and, in fact, it is one of the major five causes lead to death (Saad, 2014; El-Emshaty *et al*., 2014; Saad *et al*., 2017a). Patients with cirrhosis have the highest threat of developing HCC and may be at hazard for extra hepatic cancer (Attallah *et al*., 2015; Bridgewater *et al*., 2014).

HCV was reported to play a direct oncogenic role through production of cirrhosis (Bosch *et al*., 2005). Cell cycle mis-regulation has a principal responsibility in hepatic cells carcinogenesis promotion in addition to invasiveness and metastasis (Bisteau *et al*., 2014). Unambiguous verification proposes that tumors contain a small group of cancer stem cells that produce tumor bulk (Yi and Nan, 2008). Cancer stem cells contribute to tumor enlargement, relapse and poor response to treatment (Yoon, 2012). Therefore, assessment of liver cancer stem cell-specific surface markers could be employed in liver cancer prognosis, diagnosis, and cancer treatment following-up (Qiu *et al*., 2018).

CD133, is an important cell surface marker for both normal stem cells (cells able to self-renew and to differentiate into variable phenotypic lineages) and cancer stem cells (Romano *et al*., 2015; Saad et al., 2018a). The role of CD133 as a cancer stem cell marker has been documented in many extra hepatic cancers and in HCC (Galizia *et al*., 2012; Sun *et al*., 2016). $CK19^+$ in HCC is also a stemness marker which revealed strongest correlation with invasion, increasing tumor size, decreasing tumor differentiation, metastasis, and micro vascular invasion and it is an important predictive factor for prognosis, patient survival and tumor recurrence (Kim *et al*., 2011). CK19 is reported as an indicator of HCC invasiveness (Lee *et al*., 2018), is correlated with clinicopathologic signs of tumor aggressiveness and has been related to mortality in HCC patients (Lee *et al*., 2018). Few research papers gathered studying CD133 and CK19 together in HCV patients. For

*Address correspondence to: Entsar A. Saad, entsarsaad@gmail.com

example, Bahnassy *et al.* (2014) who studied CD133 and CK19 in HCC patients associated with HCV of genotype-4. They showed significant association between levels of both CD133 and CK19 with HCC high grade and advanced stage. However, to our knowledge gathering between CD133, CK19 and G2/M cell cycle phase in HCV (genotype-4) patients with hepatic/extra hepatic cancer was not studied before. Thus, we aimed to investigate CD133 and CK19 in HCV (genotype-4)-cirrhotic patients with hepatic or with extra hepatic cancer and to assess the degree of their correlation with cell cycle abnormalities in preparation for the study of their combination performance as diagnostic tool in those patients.

Material and Methods

The present study involved 97 individuals admitted to Gastroenterology Surgical Center, Mansoura University, Egypt from 2016 to 2017. Seventy seven (58 men and 19 women; mean age of 58.6±9.8 years) patients suffered from HCV (genotype-4)-chronic liver disease, confirmed clinically and biochemically, categorized histo-pathologically into: 24 (31.2%) patients diagnosed as liver cirrhosis, 26 (33.7%) patients diagnosed as HCC, and 27 (35.1%) patients diagnosed as extra hepatic cancer. Twenty (12 men and 8 women; mean age of 61.0±6.4 years) healthy individuals without a clinical history of hepatitis and without symptoms or signs of liver disease were included as control. A written informed consent to use the samples and clinical data for research purposes was obtained from each individual before the study according to the ethical guidelines of Helsinki Declaration (World Medical Association, 2014). The study protocol was approved by the Institutional Review Board of the Gastroenterology Surgical Center, Mansoura University, Egypt.

Blood samples were collected from all individuals and processed for laboratory investigations; serum albumin, total bilirubin (T. bilirubin), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) using Hitachi 750XRC Analyzer. AST/ALT ratio and non-invasive fibrotic indices APRI and FIB-4 were calculated.

Peripheral blood mononuclear cells were separated via a density gradient technique (Sigma Aldrich Co., USA). Flow cytometric analysis of DNA cell cycle was done using propidium iodide (Sigma Aldrich Co., USA) and analysis of cancer stem cell markers was performed using fluorescent-labeled antibodies CD133 (TMP4, eBioscience, Inc., Alfymetrix company, www.eBioscience) and CK19 pacifically binds to cytokeratins 14, 15, 16 and 19 (KA4, BD Biosciences, www.bdbiosciences.com) according to the manufacturer's protocols.

Statistical analysis

Data were expressed in continuous variables as mean±SD and categorical variables as frequencies and percentages. The significance in continuous variables was calculated in Kruskal Wallis and Mann-Whitney U test. Differences were considered significant at $p<0.05$. The association between variables was analyzed by Spearman correlation's coefficient. The area under receiver operating characteristic (ROC) curve (AUC) was utilized for assessment of the diagnostic performance of biomarkers.

Results

HCC patients compared to those with extra hepatic cancer showed significant higher increases in AST, ALT, INR, APRI, and FIB-4 but showed a significant higher decrease as regards to platelets count (PLT). Significantly, HCC patients compared to liver cirrhosis showed higher serum AST, albumin, APRI and FIB-4 with lower T. bilirubin, ALP and PLT. However, extra hepatic cancer patients compared to liver cirrhosis showed significant differences in only serum albumin (higher) and ALP (lower) (Tab. 1).

Analysis of DNA cell cycle abnormalities by flow cytometry showed significant increases in Sub G1 ($p<0.05$), S phase and G2/M ($p<0.0001$) cell populations with significant decrease ($p<0.0001$) in G0/1 population in all diseased groups compared to control. Patients with extra hepatic cancer showed non-significant increase in cells accumulated in G2/M compared to HCC patients and non-significant increase in S phase cells compared to liver cirrhosis (Tab. 2).

Flow cytometric analysis of CD133 and CK19 showed highly significant increases in all diseased groups compared with control. Extra hepatic cancer patients showed much higher increase in CK19 than those with HCC. There was no difference was noticed between HCC and extra hepatic cancer patients in CD133 (Tab. 3).

Correlations between DNA cell cycle abnormalities, CK19 and CD133 with fibrotic markers were examined in HCV-infected patients and the data was recorded in Tab. 4.

CD133 when used alone or combined with G2/M phase, it showed non-significant diagnostic performance for discrimination of HCC from extra hepatic cancer patients. Nevertheless, when CK19 and CD133 were combined or when they were gathered with G2/M phase the diagnostic performance turned into significant ($p<0.001$). Combined CK19 and CD133 showed AUC of 0.973, accuracy of 90%, sensitivity of 90%, specificity of 90%, PPV of 90% and NPV of 90%. Combined CK19, G2/M and CD133 showed AUC of 0.978, accuracy of 92.5%, NPV of 100%, specificity of 85% and PPV of 87% (Tab. 5 and Fig. 1).

Discussion

Liver cirrhosis is a common recognized threat for primary hepatic malignancy, 7% of cirrhotic people develop HCC, and raises the threat for extra hepatic cancers development (Bridgewater *et al.*, 2014; Saad *et al.*, 2017a). The efficacy in monitoring cancer patients may get better via utilizing the proper single or group of markers (Attallah *et al.*, 2015). Therefore, our aim was to correlate CD133 and CK19 with DNA cell cycle in chronic HCV (genotype-4) patients with hepatic or extra hepatic cancer as a starting step for the study of their combination as diagnostic tool in those patients.

HCV related complications such as cirrhosis or HCC are usually presented in older age (Sherman and Levy, 2002). Patient's age in our study was 58.7±5.5 years and they were mainly men (82.2%) indicating that men are more susceptible as they have greater prevalence of viral hepatitis than women do. In addition, hormonal association including elevated testosterone serum levels declared men would suffer more vigorous inflammatory reactions in association with viral hepatitis C than women (Haj *et al.*, 2016).

TABLE 1

Clinical characteristics and laboratory investigations according to pathological diagnosis

Groups	HCC	Extra hepatic cancer	Liver cirrhosis	Control
T. bilirubin (mg%)	3.24±3.1*,†	5.026±8.49*,†	5.96±8.9*	0.976±0.44
Albumin (g%)	3.58±0.43†	3.74±0.307*,†	3.04±0.529*	3.6±0.516
ALP (K.A.U)	6.03±2.389*,†	8.038±5.28*,†	15.8±9.38	19.8±22.58
AST (U/mL)	80.6±24.7*,†,‡	65.9±48.02*	74.6±56.9*	33.7±5.15
ALT (U/mL)	72.0±41.06*,‡	52.6±42.5	58.18±35.02	35.0±7.36
AST/ALT	1.4±0.808*	1.45±0.78	1.36±0.56*	0.95±0.138
INR	1.2±0.15*,‡	1.08±0.099	1.209±0.306	1.18±0.32
PLT	120.15±58.87*,†,‡	244.19±91.3	247.9±85.3	262.2±123.5
APRI	2.147±1.78*,†,‡	1.67±1.76*	1.007±1.19*	0.406±0.213
FIB-4	5.845±2.8*,†,‡	3.65±4.64	2.92±2.246*	1.65±0.78

Data expressed as mean±SD. Hepatocellular carcinoma (HCC). *represents $p<0.05$ *vs.* control, †represents $p<0.05$ *vs.* liver cirrhosis, and ‡represents $p<0.05$ *vs.* extra hepatic cancer.

TABLE 2

Flow cytometry percentages of cells positive for CD133 and CK19

Groups	HCC	Extra hepatic cancer	Liver cirrhosis	Control
CK19	22.9±10.8*,†,‡	62.65±15.1*,†	13.88±4.2*	10.76±1.6
CD133	72.72±14.35*,†	72.35±17.7*,†	35.86±4.15*	6.62±1.03

Data expressed as mean±SD. Hepatocellular carcinoma (HCC). *represents $p<0.05$ *vs.* control, †represents $p<0.05$ *vs.* liver cirrhosis, and ‡represents $p<0.05$ *vs.* extra hepatic cancer.

TABLE 3

Flow cytometric analysis of cell cycle abnormalities

Groups	HCC	Extra hepatic cancer	Liver cirrhosis	Control
Sub G1	8.307±4.4*,††	9.54±4.12*,††	12.39±1.15*	5.18±1.25
G0/1	70.88±4.03***,‡‡‡	58.8±7.8***,††	63.03±2.08***	78.2±3.86
S phase	15.4±1.86***,†††,‡‡	13.35±4.76***	10.79±1.26***	6.1±0.84
G2/M	15.8±3.54***,†††	17.04±4.97***,†††	11.06±1.98***	5.76±2.8

Data expressed as mean±SD. Hepatocellular carcinoma (HCC). $^{*\text{ and }***}$represent $p<0.05$ and $p<0.0001$ *vs.* control, $^{\dagger\dagger\text{ and }\dagger\dagger\dagger}$represent $p<0.01$ and $p<0.0001$ *vs.* liver cirrhosis, and $^{\ddagger\ddagger\text{ and }\ddagger\ddagger\ddagger}$represent $p<0.01$ and $p<0.0001$ *vs.* extra hepatic cancer.

TABLE 4

Correlations between CD133 and CK19 with DNA cell cycle abnormalities and fibrotic markers

	Sub G1	G0/1	S phase	G2/M	CK19	CD133	APRI	FIB-4
Sub G1	1	-0.281*	-0.291*	-0.308**	-0.254*	-0.189	0.112	0.091
G0/1	-0.281*	1	-0.71	-0.369**	-0.339**	-0.024	0.33**	0.346
S phase	-0.291*	-0.71	1	0.254*	0.004	0.330**	0.034	0.039
G2/M	-0.308**	-0.369**	0.254*	1	0.394**	0.585**	-0.118	-0.142
CK19	-0.254*	-0.339**	0.004	0.394**	1	0.490**	-0.028	-0.008
CD133	-0.189	-0.024	0.330**	0.585**	0.490**	1	0.290*	0.304
APRI	0.112	0.33**	0.034	-0.118	-0.028	0.290*	1	0.866**
FIB-4	0.091	0.346**	0.039	-0.142	-0.008	0.304**	0.866**	1

* and ** represent $p<0.05$ and $p<0.01$, respectively.

TABLE 5

Diagnostic performances of CD133, CK19 and G2/M phase for discrimination of HCC from extra hepatic cancer patients

Markers	AUC	*P* value	Cut off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
CD133	0.542	0.646	71.9	55%	50%	52.4%	52.6%	52.5%
CD133+G2/M	0.602	0.267	44.465	60%	65%	63.16%	61.9%	62.5%
CK19+CD133	0.973	0.001	56.685	90%	90%	90%	90%	90%
CK19+G2/M+CD133	0.978	0.001	40.041	100%	85%	87%	100%	92.5%

AUC; Area under ROC curve, PPV; Positive predictive value, NPV; Negative predictive value.

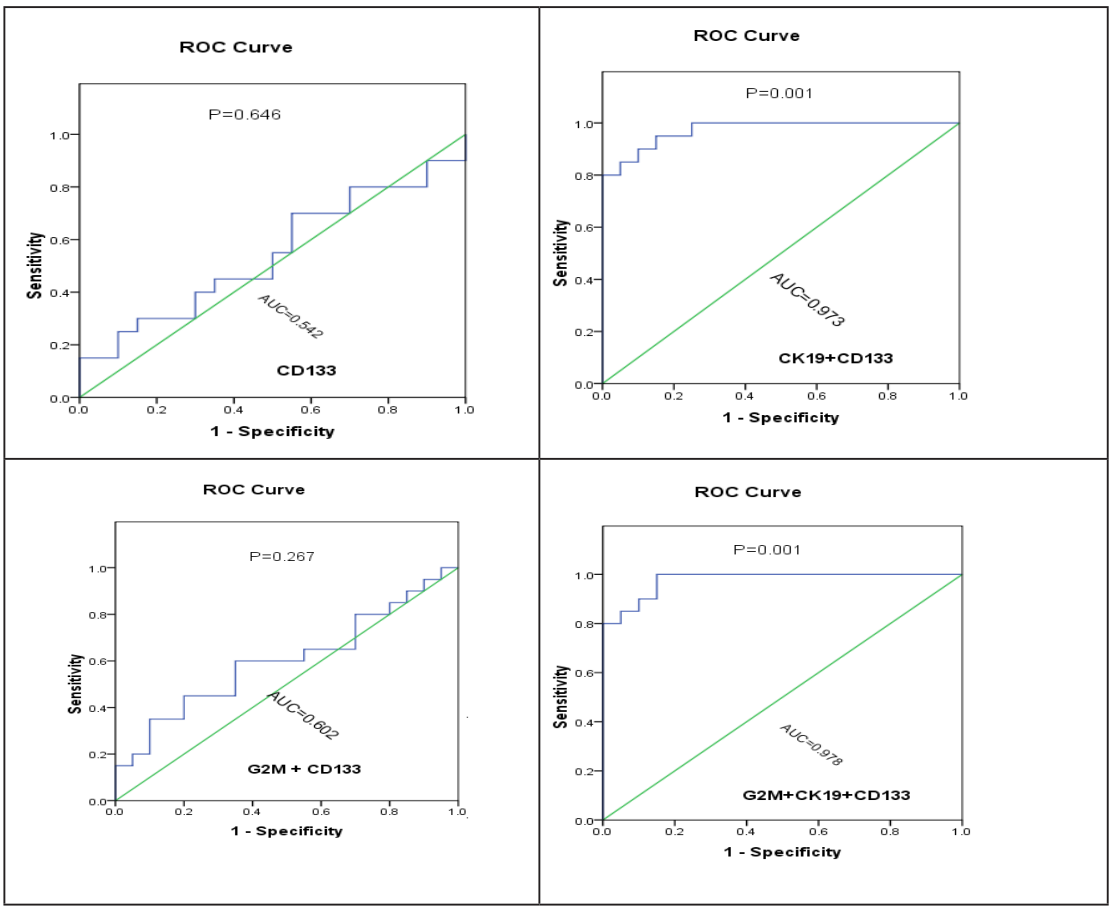

FIGURE 1. Receiver operator characteristic (ROC) curve of CD133, CK19 and G2/M phase for discrimination of HCC from extra hepatic cancer patients.

Marked elevations of ALT and AST were found in viral hepatitis (Saad, 2014; Saad *et al.*, 2017a), and other hepatic disorders (Chung *et al.*, 2014; Saad, 2013; Saad *et al.*, 2015b; Toson *et al.*, 2014). They considered an index of liver cell injury (Saad *et al.*, 2018b). In the current study, significant increase in serum AST and ALT activity was recorded in all diseased groups compared to control. HCC showed elevated AST compared to liver cirrhosis in consistence with other reports and as explained previously cancer tissue seems to release AST into circulation at a faster rate than non-cancerous parenchyma (Attallah *et al.*, 2015). Clinical utilization of AST/ALT ratio is more than utilization of individual elevated levels (Parkes *et al.*, 2006; Saad, 2014). Ratios above 1 showed advanced liver fibrosis and chronic hepatitis C infection as reported by Parkes *et al.* (2006) and agrees with our results that AST/ALT ratio was above 1 in HCV (genotype-4)-associated liver diseases. This may be due to enhanced flow of mitochondrial AST, diminished AST clearance with/without arrested ALT synthesis in progressive hepatic disease (Saad *et al.*, 2018c).

Estimation of combined parameters, such as fibro test or AST to platelet ratio panels, has been evaluated to replace liver biopsy and to relate to the efficacy of diagnosis in patients with HCV-associated liver disease (Saad, 2014). In the current study, HCV-associated liver disease highly elevated APRI index and FIB-4 score compared with control and the elevation was progressively detected in the following order: HCC, extra hepatic cancer then liver cirrhosis. Therefore, these indices have high specificity for identifying cirrhosis and HCC.

In chronic HCV, hepatic cell proliferation alterations are reported (Williams and Stoeber, 2012). In the present work, substantial abnormalities in cell cycle phase distribution were noted especially in HCC and extra hepatic cancer compared with liver cirrhosis and control as reported previously by (Bisteau *et al.*, 2014) confirming that cell cycle for cancer cases has cells proliferate at high rate compared to healthy cells. This was supported further by insignificant difference recorded between HCC and extra hepatic cancer in G2/M. This agrees with Michalopoulos (1990) who have shown that most neoplastic cells were not able to complete cell cycle and as a result, they kept in an extended G1 phase or arrested state. In addition, no significant difference was detected in S phase of extra hepatic cancer patients compared with liver cirrhosis. This can be explained as reported by Kato *et al.* (2005) who stated that in cirrhosis, there is a highly suggestive chromosomal instability attributed to DNA damage and arrest suggesting that, liver cirrhosis is associated with alterations in cell cycle related proteins, which is responsible for hepatocyte regeneration in damaged liver and may be involved in liver carcinogenesis. Furthermore, our results, in consistence with Sasaki *et al.* (2010) showed that fibrotic markers APRI and FIB-4 were highly associated with G0/1 thereby severity of liver fibrosis is considered to be related with impaired regenerative capacity suggesting the arrest of cell cycle progression.

CD133 is a valuable marker expressed in human HCC, while absent in normal liver cells. CD133 was highly expressed in HCC and extra hepatic cancer compared with liver cirrhosis and control in our study suggesting that $CD133^{+}$ cells are responsible for the origin of cancer (Romano

et al., 2015). In our study, CD133 was expressed in liver cirrhosis cases and in both HCC and extra hepatic cancer by > 5-folds and >10-folds, respectively than those in controls reflecting gradual increase in level with progression of disease from cirrhosis to HCC or to extra hepatic cancer. Significant positive correlations found between CD133 and both APRI and FIB-4 in the current study support this assumption. In the same line, Xiang *et al.* (2016) showed that cytoplasmic expression of CD133 in HCC is associated with high-grade tumor and tumor invasion to portal vein. These results support CD133 prognostic value in HCC/extra hepatic cancer and emphasize CD133 importance in cancer early stage. However, CD133 expression was equally elevated (>2-folds) in both extra hepatic cancer and HCC patients compared with liver cirrhosis cases i.e. no significant difference was noted between HCC and extra hepatic cancer patients in CD133 expression accordingly it can't be used alone for differentiating liver cancer from extra hepatic cancer. CD133 was associated with cell cycle phases S and G2/M, which could be explained on the basis that deregulation of cell cycle underlies the abnormal cell proliferation that characterizes cancer wherever it located and loss of cell cycle checkpoint control promotes genetic instability (Michalopoulos, 1990).

CK19 is a liver progenitor, cholangiocyte, and early hepatoblast cells biomarker expressed in liver bile duct cells. It was shown dispersed in HCC and cirrhotic liver parenchymal cells (Xiang *et al*., 2016). CK19 in the current study is significantly and highly expressed in cirrhotic liver (p=0.006) and HCC (p<0.0001) and elevated in extra hepatic cancer (p<0.0001) patients also which agree with Cai *et al.* (2012) confirming CK19 release by viable epithelial tumor cells. Gradually increased CK19 was also noted with progression of liver diseases; CK19 was expressed in liver cirrhosis cases by 13.88±4.2% and in HCC by 22.9±10.8% (>2-folds) and in extra hepatic cancer cases by 62.65±15.1% (>5-folds) than those in healthy controls. Comparable findings were reported in Kim *et al*. (2011) study. These results indicate that CK19 is well associated with disease aggressiveness. Moreover, significant difference (p<0.0001) was noted between HCC and extra hepatic cancer patients in CK19 expression thus it could be used for differentiating HCC from extra hepatic cancer.

As presented in the current study, CD133 and CK19 were highly detected in all diseased groups (p<0.0001) indicating that these markers could be elevated because of HCV infection. Moreover, positive associations (p<0.0001) were recorded between CD133 and CK19, and between them and G2/M phase. These results could be explained on the basis that misregulation of cell cycle constituents may result in tumorigenesis; some mutated cellular genes such as the cell cycle suppressors may stimulate uncontrollable cell multiplication forming the tumor (Champeris Tsaniras *et al*., 2014).

CD133 alone failed to discriminate HCC from extra hepatic cancer patients since it showed bad AUC value (0.542) with poor accuracy (52.5%). Nevertheless, when CD133 was combined with G2/M phase the diagnostic performance was improved but still insignificant with poor AUC value (0.602) and somewhat better accuracy (62.5%). In harmony with Cai *et al*. (2012), Kim *et al*. (2011) and Lee *et al*. (2018), addition of CK19 to CD133 resulted in further improvement with considerable excellent AUC (0.973) and high accuracy (90%). Combination of the three markers together (CD133, G2/M and CK19) showed the best and excellent AUC (0.978) and accuracy (92.5%) with perfect sensitivity (100%). These results confirm that biomarkers accuracy can be improved when they are combined in diagnostic algorithms (Saad *et al*., 2017a). These findings suggested that utilization of G2/M phase or CK19 in specific with CD133 really improves its diagnostic performance but gathering the three make it excellent.

In conclusion, our results disclosed that CD133 and CK19 expressions, and accumulation of cells in G2/M phase as well were greatly positively associated (p<0.0001) in the studied cases and they were gradually increased with progression of liver disease-associated with chronic HCV of genotype-4 infection from liver cirrhosis to HCC. In this issue, it is worthy to state that CD133 was unique; more highly powerful compared to CK19. Concerning their clinical using for differentiating HCV-cirrhotic patients with HCC from HCV-cirrhotic patients with extra hepatic cancer, combined CD133 with G2/M cell cycle phase and CK19 is considered as an excellent diagnostic panel. Further future research on bigger sample size may validate these findings and support our suggestions.

Acknowledgements

The study originates from a master thesis. This work was done by all authors cooperatively. We thank Prof. Nabil A. Gadelhak, Professor of Digestive Surgery at Gastroenterology Surgical Center, Mansoura University for providing the facilities and support.

Conflict of interest: Authors have no conflict of interest regarding this study.

References

Attallah A, El-Far M, Abdel Malak C, Omran M, Farid K, Yahya R, Saad EA, Albannan MS, Attallah AA, El Basuni MA, Ali IS, Abed SB, El Naggar MA (2015). A simple diagnostic index comprising epithelial membrane antigen and fibronectin for hepatocellular carcinoma. *Annals of Hepatology* **14:** 869-880.

Bahnassy AA, Zekri A-R N, El-Bastawisy A, Fawzy A, Shetta M, Hussein N, Omran D, Ahmed *AA*, El-Labbody SS (2014). Circulating tumor and cancer stem cells in hepatitis C virus-associated liver disease. *World Journal of Gastroenterology* **20:** 18240-18248.

Bisteau X, Caldez MJ, Kaldis P (2014). The complex relationship between liver cancer and the cell cycle: A story of multiple regulations. *Cancers* **6:** 79-111.

Bosch F, Ribes J, Cléries R (2005). Epidemiology of hepatocellular carcinoma. *Clinical Liver Disease* **9:** 91-211.

Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ (2014). Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *Journal of Hepatology* **60:** 1268-1289.

Cai X, Zhai J, Kaplan DE, Zhang Y, Zhou L, Chen X, Qian G, Zhao Q, Li Y, Gao L, Cong W, Zhu M, Yan Z, Shi L, Wu D, Wei L, Shen F, Wu M (2012). Background progenitor activation is associated with recurrence after hepatectomy of combined hepatocellular-cholangiocarcinoma. *Hepatology* **56:** 1804-1816.

Champeris Tsaniras S, Kanellakis N, Symeonidou IE, Nikolopoulou P , Lygerou Z, Taraviras S (2014). Licensing of DNA replication, cancer, pluripotency and differentiation: an

interlinked world? *Seminars in Cell and Developmental Biology* **30:** 174-180.

Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, Adcock IM, Bateman ED, Bel EH, Bleecker ER, Boulet L-P, Brightling C, Chanez P, Dahlen S-E, Djukanovic R, Frey U, Gaga M, Gibson P, Hamid O, Jajour NN, Mauad T, Sorkness RL, Teague WG (2014). International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *European Respiratory Journal* **43**: 343-373.

El-Emshaty HM, Saad EA, Toson EA, Abdel Malak CA, Gadelhak NA (2014). Apoptosis and cell proliferation: Correlation with BCL-2 and P53 oncoprotein expression in human hepatocellular carcinoma. Hepato-Gastroenterology **61**: 1393-1401.

Galizia G, Gemei M, Del Vecchio L, Zamboli A, Di Noto R, Mirabelli P, Salvatore F, Castellano P, Orditura M, De Vita F, Pinto M, Pignatelli C, Lieto E (2012). Combined CD133/CD44 expression as a prognostic indicator of disease-free survival in patients with colorectal cancer. *Archives of Surgery* **147:** 18-24.

Habib S, Saad EA, Elsharkawy AA, Attia ZR (2015). Pro-inflammatory adipocytokines, oxidative stress, insulin, Zn and Cu: Interrelations with obesity in Egyptian non-diabetic obese children and adolescents. *Advances in Medical Sciences* **60:** 981-971.

Haj Ali FA, Anber NH, El- Gayar AM, El-Shishtawy MM (2016). Diagnostic value of serum syndecan-1 in hepatocellular carcinoma associated with hepatitis C virus in Egyptian patients. *Journal of Biomedical and Pharmaceutical Research* **5:** 46-57.

Kato A, Bamba H, Shinoharaetal M (2005). Relationship between expression of cyclin D1 and impaired liver regeneration observed in fibrotic or cirrhotic rats. *Journal of Gastroenterology and Hepatology* **20:** 1198-1205.

Kim H, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, Cho JY, Yoo JE, Choi JS, Park YN (2011). Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. *Hepatology* **54:** 1707-1717.

Lee CW, Lin SE, Tsai HI, Su PJ, Hsieh CH, Kuo YC, Sung CM, Lin CY, Tsai CN, Yu MC (2018). Cadherin 17 is related to recurrence and poor prognosis of cytokeratin 19-positive hepatocellular carcinoma. *Oncology Letters* **15:** 559-567.

Michalopoulos GK. Liver regeneration: Molecular mechanisms of growth control. *The FASEB Journal* **4:** 176-187.

Parkes J, Guha IN, Roderick P, Rosenberg W (2006). Performance of serum marker panels for liver fibrosis in chronic hepatitis C. *Journal of Hepatology* **44:** 462-474.

Qiu L, Li H, Fu S, Chen X, Lu L (2018). Surface markers of liver cancer stem cells and innovative targeted-therapy strategies for HCC (Review). *Oncology Letters* **15:** 2039-2048.

Romano M, De Francesco F, Pirozzi G, Gringeri E, Boetto R, Di Domenico M, Zavan B, Ferraro GA, Cillo U (2015). Expression of cancer stem cell biomarkers as a tool for a correct therapeutic approach to hepatocellular carcinoma. *Oncoscience* **2:** 434-456.

Saad EA (2013). Kidney response to L-arginine treatment of carbon tetrachloride-induced hepatic injury in mice. *Natural Science* **5:** 1-6.

Saad EA (2014). Non-invasive assessment of liver fibrosis using serum markers. *Journal of Pharmaceutical, Chemical and Biological Sciences* **2:** 59-76.

Saad EA, El-Gayar HA, El-Demerdash RS, Radwan KH (2018b). Hepato-toxic risk of gum arabic during adenine-induced renal toxicity prevention. *Journal of Applied Pharmaceutical Science*. doi: 10.7324/JAPS.2018.8801.

Saad EA, El-Gayar HA, El-Demerdash RS, Radwan KH (2018c). Frankincense administration antagonizes adenine-induced chronic renal failure in rats. *Pharmacognosy Magazine*. doi: 10.4103/pm.pm_271_18.

Saad EA, Habib SA, Eltabeey M (2017a). Diagnostic performance of AFP, autotoxin and collagen IV and their combinations for non-invasive assessment of hepatic fibrosis staging in liver fibrosis patients associated with chronic HCV. *International Journal of Pharmaceutical Quality Assurance*. **8:** 165-173.

Saad EA, Habib SA, Refai WA, Elfayoumy AA (2017b). Malondialdehyde, adiponectin, nitric oxide, C-reactive protein, tumor necrosis factor-alpha and insulin resistance relationships and inter-relationships in type 2 diabetes early stage. Is metformin alone adequate in this stage?. *International Journal of Pharmacy and Pharmaceutical Sciences* **9:** 176-181.

Saad EA, Hassanien MM, El-Hagrasy MA, Radwan KH (2015a). Antidiabetic, hypolipidemic and antioxidant activities and protective effects of *Punica granatum* peels powder against pancreatic and hepatic tissues injuries in streptozotocin induced IDDM in rats. *International Journal of Pharmacy and Pharmaceutical Sciences* **7:** 397-402.

Saad EA, Hassanien MM, El-lban FW (2017d). Nickel(II) diacetyl monoxime-2-pyridyl hydrazone complex can inhibit Ehrlich solid tumor growth in mice: A potential new antitumor drug. *Biochemical and Biophysical Research Communications* **484:** 579-585.

Saad EA, Hassanien MM, El-mezayen HA, ELmenawy NM 2017e). Regression of murine Ehrlich ascites carcinoma using synthesized cobalt complex. *Medicinal Chemistry Communication* **8:**1103-1111.

Saad EA, Hassanien MM, Elneely EA (2017c). Iron(III) diacetylmonoxime-2-hydrazinopyridine complex: A new prospective antitumor drug. *Applied Organometallic Chemistry* **31**: e3684.

Saad EA, Marei HES, El-Magd MA, El-Fatiry HM (2018a). Molecular characterization of olfactory bulb neural stem cells during proliferation and differentiation. *Journal of Applied Pharmaceutical Science* **8:** 87-92.

Saad EA, Toson E, Ahmed G (2015b). Clove or green tea administration antagonizes khat hepatotoxicity in rats. *International Journal of Pharmacy and Pharmaceutical Sciences* **7:** 72-76.

Sasaki A, Kamiyama T, Yokoo H, Nakanishi K, Kubota K, Haga H, Matsushita M, Ozaki M, Matsuno Yand Todo S (2010). Cytoplasmic expression of CD133 is an important risk factor for overall survival in hepatocellular carcinoma. *Oncolology Reports* **24**: 537-546.

Sherman M, Levy (2002). Staging of hepatocellular carcinoma: assessment of the CLIP, Okuda, and Child-Pugh staging systems in a cohort of 257 patients in Toronto. *Gut* **50:** 881-885.

Sun JH, Luo Q, Liu LL, Song GB (2016). Liver cancer stem cell markers: Progression and therapeutic implications. *World Journal of Gastroenterology* **22:** 547-557.

Toson EA, Habib SA, Saad EA, Harraz NH (2014). Toxic and anti-fertility effects of *Alocasia macrorrhiza* and *Calotropis procera* ethanolic extracts on male mice. *International Journal* of *Biochemistry Photon* **195:** 328-338.

World Medical Association (2014). Declaration of Helsinki: Ethical principles for medical research involving human subjects. *Journal of the American Medical Association* **310**: 2191-2194.

Williams GH, Stoeber K (2012). The cell cycle and cancer. *Journal of Pathology* **226:** 352-364.

Xiang Y, Yang T, Pang B, Zhu Y, Liu Y (2016). The progress and prospects of putative biomarkers for liver cancer stem cells in hepatocellular carcinoma. *Stem Cells International* 2016.

Yi SY, Nan KJ (2008). Tumor-initiating stem cells in liver cancer. *Cancer Biology & Therapy* **7:** 1-6.

Yoon SK (2012). The biology of cancer stem cells and its clinical implication in hepatocellular carcinoma. *Gut Liver* **6:** 29-40.