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RESEARCH ARTICLE

Prognostic value of serum carbonic anhydrase IX in Egyptian patients with cirrhosis and/or hepatocellular carcinoma

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ABSTRACT

Introduction: Hepatocellular carcinoma (HCC) is the commonest type of primary liver cancers, and most patients are diagnosed in advanced or terminal stages with poor prognosis. Furthermore, cirrhosis, a chronic liver disease characterized by fibrosis and impaired liver function, is a major risk factor for the development of HCC. Therefore, it is crucial to establish new clinical markers for early HCC diagnosis and staging. **Aim:** Our study aimed to evaluate carbonic anhydrase IX (CA9) as an early serum biomarker for HCC diagnosis within cirrhotic Egyptian patients. **Material and methods:** Fifty-eight cirrhotic patients and sixty HCC patients as well as fifty-eight healthy control subjects were selected for the current study. Routine liver tests, CBC, C-reactive protein, alpha-fetoprotein (AFP), and serum CA9 were done for all the patients included. **Results:** Serum CA9 and AFP levels increased significantly in HCC and cirrhotic patients compared to controls. CA9 increased with the development of hepatic disease through the direct proportion of CA9 with BCLC staging, child classification, ascites, and encephalopathy in the HCC cohort and the direct proportion with child classification and ascites in the cirrhotic cohort. Our findings showed that CA9 has higher accuracy than AFP to differentiate between HCC (at cutoff value>85 pg/mL) or cirrhotic patients (at cutoff value>54.7 pg/mL) and control with greater sensitivity and specificity than AFP. On the other hand, CA9 showed lower sensitivity and specificity than AFP in discrimination between cirrhosis and HCC only 51.67% and 46.55%, respectively. **Conclusions:** CA9 could be used as a biomarker for early HCC diagnosis and there is a strong relationship between CA9 level and HCC´s worse prognosis suggesting its potential role in HCC development and disease progression.

Keywords: , Biomarker, Carbonic anhydrase IX (CA9), Hepatocellular carcinoma, Prognosis, Egyptians

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INTRODUCTION

Liver cancer is still a life-threatening disease, which is expected, by 2025, to affect annually more than one million individuals (GLOBOCAN, 2018; Llovet et al., 2021). Worldwide, hepatocellular carcinoma (HCC) is the commonest type of primary liver cancer that accounts for about 90% of cases and it is the third leading cause of cancer death (GLOBOCAN, 2018; Llovet et al., 2021). Suresh et al. (2020) reviewed the etiological risk factors contributing to HCC incidence including hepatitis C and B viruses, chemical carcinogens (ex: aflatoxins), metabolic syndromes (ex: diabetes mellitus and obesity), alcoholic, and non-alcoholic fatty liver diseases. However, HCC induced by hepatitis C virus (HCV) has been decreased substantially due to patients´ sustained response to antiviral drugs (Kanwal et al., 2017; Kouroumalis et al., 2023). Nonetheless, cirrhotic patients remain at high risk for HCC incidence even after clearance of HCV (Llovet et al., 2021). Hence, cirrhosis from any etiology is the strongest risk factor for HCC (EASL, 2018; Marrero et al., 2018). HCC represents a global health challenge, because most patients are diagnosed in advanced or terminal stages with poor prognosis (Hyuga et al., 2017).

Therefore, to prepare individual therapy plan for HCC patient, clinical markers should be identified to correlate with the ideal prognosis and stage.

Hypoxia is a common characteristic in metastatic tumors where hypoxic regions are characterized by low oxygen content in addition to an acidic extracellular pH (van Kuijk et al., 2016). Tumor cells are adapted to the hypoxia of tumor microenvironment by a shift in their metabolism from mitochondrial oxidative phosphorylation to aerobic glycolysis in the cytosol as a source of energy (Vander Heiden et al., 2009). This shift results in increased amounts of lactic acid which are exported from the cells, thus lowering the extracellular pH. Therefore, the role of carbonic anhydrase (CA) in this point is to help tumor cells to regulate their intracellular pH at or near the physiological pH=7.4 (Sadri and Zhang, 2013; Mahon et al., 2015).

CAs are a family of metalloenzymes that contain zinc ion in the active site. They catalyze the rapid reversible hydration of carbon dioxide into bicarbonate and H^+ (Aggarwal et al., 2013). In humans there are 15 isoforms of CAs of which carbonic anhydrase IX (CA9) is more common in solid tumor tissue than normal tissue (Frost, 2014). CA9 is a transmembrane isoenzyme with an extracellular catalytic domain, which is important in regulating the cellular pH through the hydrolysis of carbon dioxide, produced as a waste product during tumor cell glycolysis, to bicarbonate and H^+ (Helmlinger et al., 2002; Pastorek and Pastorekova, 2015). The bicarbonate anions are transported into the cell to slightly increase the intracellular pH and promote tumor cell proliferation. While the produced protons remain extracellularly thus increasing the acidic nature of the tumor´s extracellular environment (Benej et al., 2014; Pastorek and Pastorekova, 2015). Therefore, CA9 has gained a prognostic value, as a biomarker, in cancer (van Kuijk et al., 2016). The objective of this study was to evaluate the role of CA9 as a serum marker in the diagnosis and prognosis of Egyptian patients with cirrhosis and HCC, which has not been investigated previously.

MATERIAL AND METHODS

Ethical statement

The present study was carried out according to the guidelines for good clinical practice and approved by the institutional ethical committee of the National Liver Institute, Menoufia University, Egypt with approval ID (00367/2022). Moreover, informed written consents were taken from all subjects.

Study subjects

Fifty-eight cirrhotic patients, sixty HCC patients, and fifty-eight healthy control volunteers (control subjects were age and sex-matched with cases) were recruited from National Liver Institute, Menoufia University, Egypt in the period between May 2020 to June 2022. Exclusion criteria were any patient with metabolic, autoimmune, alcoholic, and fatty liver diseases, age below 18 years, HIV patients, or patients with cancer other than HCC within the last five years. Inclusion criteria were the proven HCC and cirrhotic patients according to the following guidelines: Ultrasound, magnetic resonance imaging (MRI), or computed tomography (CT) and confirmed histopathologically if needed (Song and Bae, 2012). Child-Pugh classification was calculated for all cirrhotic and HCC patients according to Pugh et al. (1973). HCC treatment was determined by the Barcelona Clinic Liver Cancer (BCLC) staging system (Llovet et al., 1999).

Blood sampling

Venous blood was withdrawn from all subjects and divided into three vacutainer tubes: one with sodium citrate, one with EDTA, and one without anticoagulant. The third tube was allowed to clot, then centrifuged to obtain clear supernatant sera, which were stored at -80 ºC for further measurements.

Methodology

EDTA mixed blood samples were used to detect complete blood picture (CBC) on an automatic cell counter; Sysmex XT 1800 (Germany). While citrated plasma samples were used to detect prothrombin time (PT) using BFT II operates depending on the optomechanical measuring principle.

The results of PT were expressed as the International normalized ratio (INR) utilizing the prothrombin ratio (PR) and international sensitivity index (ISI). The control value for PT was 11.5 seconds which equals 100% concentration and an international ratio INR of 1.

C-reactive protein (CRP), liver function parameters; including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total & direct bilirubin, albumin, and total protein; and kidney functions tests; urea and creatinine; were carried out in separated sera using Cobas 6000 (c 501 modules) auto analyzer, Roche diagnostics, Germany, according to manufacturer instructions for each parameter. Viral markers including HCV antibody and hepatitis B surface antigen (HBsAg) were done by the electrochemiluminescence immunoassay (ECLIA) using Cobas 6000 (e 601 modules) according to their manufacturer instructions.

Sandwich enzyme linked immune sorbent assay (Sandwich ELISA) was used for the detection of serum alpha-fetoprotein (AFP) using a solid phase ELISA kit from Sorin Biomedica (USA). Carbonic anhydrase IX (CA 9) was quantitatively evaluated in serum using ELISA kit (Bioneovan Co., Ltd No. 18, Beijing, China) according to its manufacturer´s instructions. Optical density proportional to CA9 concentration was measured at 450 nm using BiotekElx 800-UV microtiter plate reader (Murex Biotech S.A.(Pty) Ltd, Republic of South Africa).

Statistical Analysis

Data were analyzed statistically using SPSS 22.0 (IBM/SPSS Inc., Chicago, IL). Normally distributed quantitative data was expressed as mean ± standard deviation (SD), while not normally distributed data was expressed as median and interquartile range (IQR). Chi-square test was used to measure the association between qualitative variables as appropriate. Mann-Whitney test was used for quantitative variables to compare between two groups of skewed data and when comparison was among more than two groups Kruskal-Walli's test followed by Dunn-Sidak post hoc test was used. In addition, correlation analysis using Spearman's correlation coefficient (rs), and receiver operating characteristic (ROC) analysis were used. In all applied tests, the *p*-values < 0.05 was considered significant, and *p*-values < 0.01 was considered highly significant.

RESULTS

Demographic data and subjects' characteristics

In the present study 60 HCC patients (50 males, 83.3% and 10 females, 16.7%) with median ages 57.00, 58 liver cirrhotic patients (50 males, 86.2% and 8 females, 13.8%) with median ages 58.00, and 58 control subjects (48 males, 82.8% and 10 females, 17.2%) with median ages of 55.00, were included. The statistical analysis revealed that there was no significant ($p>0.05$) difference in age and gender among the three studied groups as demonstrated in Table (1). In addition, data presented in Table (1) showed that cirrhotic and HCC patients suffered from highly significant ($p<0.05$) increases in ALT, AST, ALP, GGT, total and direct bilirubin, INR, urea, and creatinine accompanied with highly significant ($p<0.01$) decrease in hemoglobin concentration, platelet count, and serum albumin as compared to healthy control cases. While only cirrhotic patients showed a highly significant ($p<0.01$) decrease in total protein and a highly significant ($p<0.01$) rise in WBCs count compared to control group. Moreover, HCC patients showed a highly significant ($p<0.01$) elevation in albumin, total protein, and hemoglobin and significant ($p<0.05$) reduction in ALP, GGT, direct bilirubin, urea, creatinine, WBC, and INR in comparison with cirrhotic group.

The observed clinico-pathological parameters for both cirrhotic and HCC patients, as demonstrated in Table 1, showed that HCC patients were divided according to child classification into 46.7% (child A), 16.7% (child B) and 36.7% (child C), while cirrhotic patients represent 13.8% (child A), 41.4% (child B) and 44.8% (child C). Another classification for HCC patients according to BCLC score illustrates that 36.7% (A/B), and 63.3% (C/D). There was significant ($p<0.01$) difference between the groups in, child score, child classification, jaundice, and the proportion of blood transfused cases.

Table 1. Demographic data and subjects' characteristics

	Healthy controls (n= 58)	Cirrhotic patients (n= 58)	HCC patients (n= 60)
Age (years)[a]			
Median (IQR)	55.0 (50.0-58.0)	58.0 (51.0-63.0)	57.0 (54.0-62.0)
Gender [n (%)][b]			
Male	48 (82.8)	50 (86.2)	50 (83.3)
Female	10 (17.2)	8 (13.8)	10 (16.7)
ALT (U/L) [a]			
Median (IQR)	26.00 (21.0-31.0)	36.5 (17.0-64.0)**	39.0 (25.0-56.0)**
AST (U/L) [a]			
Median (IQR)	22.0 (18.0-30.0)	68.0 (33-126)**	45.0 (31.0-76.0)**
ALP (U/L) [a]			
Median (IQR)	47.5 (41.0-52.0)	174.5 (112.0-242.0)**	74.5 (51.0-143.5)** ##
GGT (U/L) [a]			
Median (IQR)	40.00 (37.0-48.0)	67.5 (39.0-146.0)**	48.0 (38.5-71.0)*#
Total bilirubin (mg/dL) [a]			
Median (IQR)	0.73 (0.60-0.87)	2.55 (1.30-4.90)**	1.60 (0.90-4.60)**
Direct bilirubin (mg/dL) [a]			
Median (IQR)	0.16 (0.11-0.20)	1.70 (0.60-2.60)**	0.49 (0.30-2.20)**#
Albumin (g/dL) [a]			
Median (IQR)	4.00 (3.80-4.20)	2.55 (2.10-3.00)**	3.00 (2.10-3.80)**##
Total protein (g/dL) [a]			
Median (IQR)	6.90 (6.70-7.10)	6.40 (5.30-6.90)**	6.80 (6.50-7.00)##
Urea (mg/dL) [a]			
Median (IQR)	27.00 (22.0-29.0)	60.0 (35.0-127.0)**	29.50 (20.0-87.5)*##
Creatinine (mg/dL) [a]			
Median (IQR)	0.80 (0.7-1.0)	1.11 (0.85-2.6)**	1.02 (0.8-1.2)**#
Hemoglobin (g/dL) [a]			
Mean ± SD	14.67 ± 1.01	10.56 ± 1.65**	11.85 ± 2.10**##
WBCs (10^3 cell/µL) [a]			
Median (IQR)	5.85 (5.0-6.4)	7.45 (5.3-10.7)**	5.80 (4.3-10.3)#
Platelets (10^3 cell/µL) [a]			
Median (IQR)	287.5 (254.0-317.0)	95.5 (65.0-171.0)**	100.5 (65.5-157.0)**
INR [a]			
Median (IQR)	1.05 (1.0-1.1)	1.46 (1.2-1.8)**	1.28 (1.1-1.5)**#
Child score [c]			
Median (IQR)	-	9.00 (8.0-11.0)	7.00 (5.0-11.0)##
Child classification [n (%)][b]			##
A	-	8 (13.8)	28 (46.7)
B	-	24 (41.4)	10 (16.7)
C	-	26 (44.8)	22 (36.7)
BCLC score [n (%)]			
A/B	-	-	22 (36.7)
C/D	-	-	38 (63.3)
Ascites [n (%)][b]			
No	-	26 (44.8)	36 (60.0)
Yes	-	32 (55.2)	24 (40.0)
Encephalopathy [n (%)][b]			
No	-	36 (62.1)	38 (63.3)
Yes	-	22 (37.9)	22 (36.7)
Etiology of liver disease [n(%)] [b]			
Hepatitis C	-	48 (82.8)	52 (86.7)
Hepatitis B	-	10 (17.2)	8 (13.3)
Jaundice [n (%)] [b]			##
No	-	36 (62.1)	50 (83.3)
Yes	-	22 (37.9)	10 (16.7)
Blood transfusion [n (%)] [b]			##
No	-	20 (34.5)	38 (63.3)
Yes	-	38 (65.5)	22 (36.7)

Data are presented as median (IQR) or percent within group (%) or mean±standard deviation (SD), a: Kruskal-Walli's test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. b: Pearson chi-square test. c: Mann-Whitney test. *: Significant (p<0.05) compared to control, **: Highly significant (p<0.01) compared to control. #: Significant (p<0.05) compared to liver cirrhosis, ##: Highly significant (p<0.01) compared to liver cirrhosis. IQR: Interquartile range, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, WBCs: white blood cells, INR: International normalized ratio, BCLC: Barcelona Clinic Liver Cancer.

Changes in the measured serum biomarkers were illustrated in Table 2. The levels of CA9, AFP, and CRP were significantly (p<0.01) higher in cirrhosis and HCC cases than in controls. Although, no significant difference was detected between cirrhotic and HCC patients in CA9 and CRP. Noticeably, there was a highly significant (p<0.01) increase in the AFP concentration in HCC patients as compared to cirrhotic cases.

Correlation between CA9 and different parameters in cirrhosis and HCC cohorts

Spearman correlation coefficient was used to evaluate the correlation between CA9 and different parameters in cirrhosis and HCC cohorts. In HCC patients significant (p<0.05) positive correlation was detected between CA9 and AFP, ALT, AST, ALP, GGT, total and direct bilirubin, CRP, and child score. In cirrhotic patients a significant (p<0.05) positive correlation was detected between CA9 and ALT, AST, total and direct bilirubin, CRP, and child score. In contrast, there were significant (p<0.05) inverse correlations between CA9 and albumin among all patients' groups (Table 3).

Furthermore, statistical analysis using Mann-Whitney and Kruskal-Wallis tests demonstrated that, CA9 was directly proportional (p<0.01) with BCLC staging, child Pugh classification, ascites, and encephalopathy in HCC patients, proving that the level of CA9 was increased with the development of disease. No significant difference was detected between CA9 and the type of viral infection (Table 4). In cirrhotic patients, there was significant (p<0.01) increase in the level of CA9 in comparison with child Pugh classification and ascites, while no significant difference was detected in comparing CA9 with encephalopathy and type of viral infection (Table 5).

Diagnostic performance of CA9 and AFP for discrimination between studied groups

Firstly, to discriminate between HCC and cirrhotic patients, data presented in Table 6 and Figure 1a showed that at the cutoff value > 6.3 ng/mL AFP level will be more specific to discriminate between stages of cirrhosis and HCC with the specificity of 75.86% and sensitivity of 80.0%.

While, at the cutoff value > 426 pg/mL CA9 has a sensitivity of 51.67% and specificity of 46.55%. On the other hand, in discrimination between HCC patients and healthy control group CA9 at cutoff value >85 pg/mL was more specific than AFP, with a specificity of 94.83% and a sensitivity of 95%, as presented in Table 6 and Fig. 1b. Finally, at a cutoff value > 54.7 pg/mL, CA9 recorded more diagnostic accuracy with higher sensitivity (93.1%) and specificity (82.76%) to differentiate between patients with cirrhosis and control group than serum AFP level (Table 6 and Figure 1c).

DISCUSSION

Hypoxia is a major stimulus of angiogenesis, fibrogenesis, and tumor progression (Rosmorduc and Housset, 2010). It is a known risk factor for poor prognosis and resistance to therapy in tumors. Several chemical substances are triggered intracellularly in malignant cells, under hypoxic conditions, which clear the way for cancer cell survival in harsh environments. These substances play crucial roles in the growth, invasiveness, and metastasis of tumor cells.

Hypoxia-inducible factor 1α (HIF1α), a key molecule in oxygen homeostasis, is generally induced by hypoxic stimuli (Wang et al., 1995) resulting in the production of several hypoxia-related molecules, such as glucose transporter1 (GLUT1), vascular endothelial growth factor (VEGF) and CA9 (Kim et al., 2002; Amann and Hellerbrand, 2009; Pastorek and Pastorekova, 2015). CA9 is a pH lowering enzyme (Pastorekova et al., 2008) that was reported as the most important member of its family for tumorigenesis and prognostication in various tumors (Korkeila et al., 2009; Woelber et al., 2011; Gigante et al., 2012; Fidan et al., 2013; Smith et al., 2016). Therefore, CA9 has gained interest as a biomarker in malignancies. An evaluation of circulating CA9 in Egyptian patients with HCC and cirrhosis has not yet been done to our knowledge, therefore, this study was conducted to evaluate the role of CA9 as a serum marker for those patients.

The present study revealed statistically significant difference in HCC cohort than cirrhotic group regarding ALP, GGT, direct

Table 2. Serum biomarkers (CA9, AFP and CRP) in control, liver cirrhosis, and HCC groups

	Healthy controls (n=58)	Liver cirrhosis (n=58)	HCC (n=60)
CA9 (pg/mL)[a]			
Median (IQR)	33.80 (21.8-53.0)	432.80 (167.2-751.1)**	490.60 (152.6-1264.8)**
AFP (ng/mL) [a]			
Median (IQR)	1.50 (1.1-2.0)	3.35 (1.9-6.3)**	116.00 (7.4-1121.5)**##
CRP (mg/dL) [a]			
Median (IQR)	2.45 (1.0-3.0)	32.70 (11.4-55.0)**	57.60 (14.5-99.2)**

Data are presented as median (IQR), a: Kruskal-Walli's test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test, **: Highly significant (p<0.01) compared to control, ##: Highly significant (p<0.01) compared to liver cirrhosis, IQR: Interquartile range, CA9: carbonic anhydrase IX, AFP: alpha-fetoprotein, CRP: C-reactive protein.

Table 3. Correlation between CA9 (pg/mL) and various parameters

Correlated parameters	CA9 (pg/mL)			
	Liver cirrhosis		HCC	
	r_s	p-value	r_s	p-value
Age (Years)	0.128	0.339^{NS}	0.213	0.102^{NS}
AFP (ng/mL)	0.171	0.200^{NS}	0.385	0.002^{HS}
ALT (U/L)	0.284	0.030^{S}	0.415	0.001^{HS}
AST (U/L)	0.273	0.038^{S}	0.506	<0.001^{HS}
ALP (U/L)	-0.013	0.922^{NS}	0.272	0.036^{S}
GGT (U/L)	-0.047	0.729^{NS}	0.337	0.009^{HS}
Total bilirubin (mg/dL)	0.345	0.008^{HS}	0.448	<0.001^{HS}
Direct bilirubin (mg/dL)	0.388	0.003^{HS}	0.392	0.002^{HS}
Albumin (g/dL)	-0.384	0.003^{HS}	-0.479	<0.001^{HS}
Total protein (g/dL)	-0.164	0.218^{NS}	-0.245	0.059^{NS}
INR	0.243	0.066^{NS}	0.140	0.286^{NS}
Urea (mg/dL)	0.082	0.542^{NS}	0.131	0.319^{NS}
Creatinine (mg/dL)	0.082	0.540^{NS}	0.004	0.974^{NS}
CRP (mg/dL)	0.381	0.003^{HS}	0.416	0.001^{HS}
Hemoglobin (g/dL)	0.156	0.241^{NS}	-0.195	0.139^{NS}
WBCs (10^3 cell/µL)	0.247	0.062^{NS}	0.223	0.087^{NS}
PLT (10^3 cell/µL)	-0.191	0.151^{NS}	0.179	0.172^{NS}
Child score	0.472	<0.001^{HS}	0.472	<0.001^{HS}

r_s: Spearman correlation coefficient, ^{NS} : Non significant at p-value ≥ 0.05, ^{S}: Significant at p-value < 0.05, ^{HS}: Highly significant at p-value < 0.01

Table 4. Comparison between CA9 and clinic-pathological parameters in HCC group

Parameters	CA9 (pg/mL)		
	no of cases (%)	Median (IQR)	p-value
BCLC staging			[a]=0.002 **HS**
A / B	22 (36.7)	241.2 (88.2-588.0)	
C / D	38 (63.3)	699.7 (337.1-2016.9)	
Child Pugh classification			[b]0.001 **HS**
A	28 (46.7)	294.8 (93.35-593.0)	
B	10 (16.7)	944.0 (336.1-1508.3)	
C	22 (36.7)	1264.8 (367.9-2192.5)	
Ascites			[a]<0.001**HS**
Absent	36 (60.0)	342.2 (104.2-699.7)	
Present	24 (40.0)	1343.8 (388.6-2109.7)	
Encephalopathy			[a]=0.010**S**
Absent	38 (63.3)	352.4 (106.7-911.2)	
Present	22 (36.7)	944.0 (367.0-2024.5)	
Etiology of liver disease			[a]=0.287**NS**
Hepatitis C	52 (86.7)	506.8 (195.6-1264.8)	
Hepatitis B	8 (13.3)	336.9 (85.3-1287.8)	

[a]: Mann-Whitney test, [b]: Kruskal-Walli's test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. ^{HS}: Highly significant at p-value < 0.01, ^{S}: Significant at p-value < 0.05, ^{NS}: Non-significant at p-value ≥ 0.05. IQR: Interquartile range, CA9: carbonic anhydrase IX, BCLC: Barcelona Clinic Liver Cancer.

Table 5. Comparison between CA9 and clinic-pathological parameters in liver cirrhosis group

Parameters	CA9 (pg/mL)		
	no of cases (%)	Median (IQR)	*p*-value
Child Pugh classification			[a]<0.001[HS]
A	8 (13.8)	62.24 (45.2-92.9)	
B	24 (41.4)	509.3 (338.2-859.4)	
C	26 (44.8)	699.0 (402.0-751.1)	
Ascites			[b] <0.001[HS]
Absent	26 (44.8)	403.5 (62.7-430.0)	
Present	32 (55.2)	713.6 (435.6-907.2)	
Encephalopathy			[b] =0.158[NS]
Absent	36 (62.1)	412.5 (63.7-824.2)	
Present	22 (37.9)	543.2 (414.5-722.8)	
Etiology of liver disease			[b] =0.629[NS]
Hepatitis C	48 (82.8)	488.2 (210.3-735.4)	
Hepatitis B	10 (17.2)	421.0 (54.8-826.2)	

[a]: Kruskal-Walli's test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. [b]: Mann-Whitney test, [HS]: Significant at *p*-value < 0.01, [NS]: Non-significant at *p*-value ≥ 0.05. IQR: Interquartile range, CA9: carbonic anhydrase IX.

Table 6. Diagnostic performance of CA9 and AFP for discrimination between different studied groups

	Cutoff value	AUC	*p*-value	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
HCC vs. cirrhosis								
CA9 (pg/mL)	> 426	0.553	0.319[NS]	51.67	46.55	50.0	48.2	49.15
AFP (ng/mL)	> 6.3	0.844	<0.001[HS]	80.0	75.86	77.4	78.6	77.97
HCC vs. control								
CA9 (pg/mL)	> 85	0.987	<0.001[HS]	95.0	94.83	95.0	94.8	94.92
AFP (ng/mL)	> 2.8	0.972	<0.001[HS]	90.0	89.66	90.0	89.7	89.83
Cirrhosis vs. control								
CA9 (pg/mL)	> 54.7	0.955	<0.001[HS]	93.1	82.76	84.4	92.3	87.93
AFP (ng/mL)	> 1.91	0.817	<0.001[HS]	72.41	70.69	71.2	71.9	71.55

AUC: Area under the curve, PPV: Positive predictive value, NPV: Negative predictive value, HCC: hepatocellular carcinoma, CA9: carbonic anhydrase IX, AFP: alpha-fetoprotein. NS: Non-significant at p-value > 0.05. HS: Highly significant at p-value< 0.01.

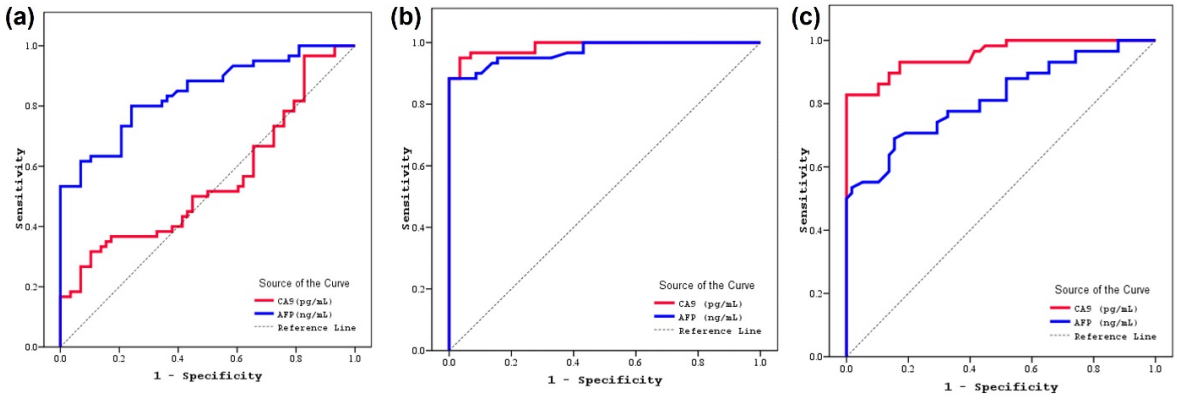

Figure 1. ROC curves of CA9 and AFP for discrimination between (a) HCC and cirrhosis (b) HCC and healthy control (c) cirrhosis and healthy control

bilirubin, albumin, total protein, INR, hemoglobin, platelets count, WBCs count, jaundice, child score and classification in addition to AFP level. These results agree with previous findings of Carr and Guerra (2017), Omar et al. (2020), El-Hawawshy et al. (2021), and Sharaf et al. (2022). According to Sharaf et al. (2022), these substantial alterations were linked to the damage of liver parenchyma caused by the growth of the tumor. Moreover, HCC patients showed significant differences from cirrhotic patients regarding urea, and creatinine. In their study, Bai et al. (2021) observed a noteworthy rise in urea levels among cancer patients, while the increase in creatinine levels was not statistically significant.

The findings suggest that elevated serum urea may be a prevalent occurrence in individuals with cancer.

Current study reported significant direct proportion ($p<0.01$) between CA9 and BCLC staging, child Pugh score and classification, encephalopathy, and ascites in HCC patients and significant direct proportion ($p<0.05$) between CA9 and child Pugh score and classification, and ascites in cirrhotic patients. These results indicate that the level of CA9 increased with the development of the disease. No significant difference was detected between CA9 and the type of viral infection of both HCC and cirrhotic cases. Previous data agree with Finkelmeier et al. (2018) who reported that CA9 levels did not significantly vary with the indicated etiologies of liver disease (HBV, HCV infection or , non-alcoholic steatohepatitis), but significantly changed in different stages of cirrhosis and HCC patients, with the highest levels found in Child C stage patients and BCLC C or D stage HCC patients.

Our findings showed that CA9 has higher accuracy than AFP to differentiate between HCC (at cutoff value>85 pg/mL) or cirrhotic patients (at cutoff value>54.7 pg/mL) and control with greater sensitivity and specificity than AFP. On the other hand, CA9 showed lower sensitivity and specificity than AFP in discrimination between cirrhosis and HCC only 51.67% and 46.55%, respectively. Previous studies reported that tumor stage is a crucial histopathological factor in determining prognosis (Poon et al., 2000; Peng et al., 2005). In other studies, they found that CA9 expression is an independent predictor for high-stage HCCs (Hyuga et al., 2017; Finkelmeier et al., 2018) which is consistent with the reports regarding various human cancers (Loncaster et al., 2001; Swinson et al., 2003; Måseide et al., 2004; Haapasalo et al., 2006; Hussain et al., 2007). Huang et al. (2015) showed positive CA9 expression in the HCCs correlated with high serum AFP levels and high tumor grade, but not liver cirrhosis. Importantly, high-stage (stage II-III) HCCs, which have vascular invasion and various degrees of intrahepatic metastasis, had significant CA9 expression as compared with low-stage HCCs (Huang et al., 2015).

Moreover, Yu et al. (2011) demonstrated that CA9 expressed in 30.4% of HCCs, but neither the extent nor the intensity of CA9 immune-reactivity correlates with clinic-pathological variables.

Studies on CA9 expression in HCCs are sparse. Luong-Player et al. (2014) found that CA9 expressed focally in only 15% of HCCs and its expression may be useful in differentiating HCC from intrahepatic cholangiocarcinoma. In the liver, it has been reported that 78% of cholangio-carcinomas show a positive reaction for CA9, whereas HCCs show a weak immune-reactivity in only 33% of cases (Saarnio et al., 2001). On the level of CA9 prognostic value, Yuan et al. (2014) reported that tumoral CA9 intensity was found to be inversely related to E-cadherin intensity; increases in E-cadherin intensity may predict a favorable prognosis. Furthermore, when CA9 expression was suppressed by siRNA, E-cadherin expression was increased. The ability of CA9 to adhere to β-catenin (Haapasalo et al., 2006) is consistent with the suggestion that hypoxia can promote tumor invasion by decreasing E-cadherin-mediated intercellular adhesion, offering the possibility that CA9 participates in epithelial-mesenchymal transition (Peng et al., 2004; Rosmorduc and Housset, 2010). Moreover, an alkaline intracellular pH and an acidic extracellular pH have been hypothesized to increase tumor growth (Martinez-Zaguilan et al., 1996). Thus, in CA9-expressing HCCs, CA9 inhibition could be therapeutically useful for reducing tumor survival or invasiveness and metastasis. The study of Yu et al. (2011) demonstrates that the inhibition of hypoxia-inducible CA9 enhances 3-BP-induced HCC cell apoptosis and that CA9 expression profiles may have prognostic implications in HCC patients. Thus, the blockage of CA9 in combination with hexokinase II inhibitor treatment may be therapeutically useful in patients with large or infiltrative hypovascular HCCs that are aggressively growing in a hypoxic environment (Yu et al. 2011).

CONCLUSIONS

From the previous findings we can conclude that CA9 can be a promising prognostic marker for Egyptian HCC cases. Further studies

targeting CA9 should be performed to test the possibility of inhibiting CA9 function for the treatment of HCC patients.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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AUTHOR CONTRIBUTION

All authors contributed equally and approved the manuscript.

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ABBREVIATION LIST

CA9: carbonic anhydrase IX, HCC: hepatocellular carcinoma, AFP: alpha-fetoprotein, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, CRP: C-reactive protein, BCLC: Barcelona Clinic Liver Cancer, HCV: hepatitis C virus, HBsAg: hepatitis B surface antigen, MRI: magnetic resonance imaging, CT: computed tomography, PT: prothrombin time, INR: International normalized ratio, ELISA: enzyme-linked immune sorbent assay, SD: standard deviation, IQR: interquartile range, ROC: receiver operating characteristic, WBCs: white blood cells.

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