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Identification of Cyclase-Associated Protein-2 as a Novel Biomarker for Early-Stage Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is an aggressive deadly cancer with few therapeutic options mostly limited for early-stage HCC. Unfortunately, alpha-fetoprotein (AFP) has a limited performance, especially in early-stage HCC.

Objectives: to investigate plasma levels of Cyclase-associated protein-2 (CAP2) as a new biomarker and evaluate its role in detecting early-stage and AFP-negative HCC Egyptian patients.

Methods: Plasma CAP2 and AFP levels in 150 HCC, 150 cirrhotic patients, 150 healthy controls. Correlation with tumor behavior, the area under the curve (AUC), sensitivity, specificity, and diagnostic accuracy were analyzed.

Results: Plasma CAP2 and AFP levels were significantly elevated in HCC patients than liver cirrhosis and controls. Only plasma CAP2 levels significantly correlated with clinico-pathological characteristics of HCC (BCLC, histological and clinical stages) but not correlated with patient's age, gender, viral infection status or AFP levels. Compared to AFP, CAP2 had significantly higher AUC: 0.86 (0.79-0.93) vs. 0.75 (0.65-0.85), Sensitivity: 81.5% vs. 62% in all HCCs and significantly higher AUC: 0.80 (0.72-0.89) vs. 0.68 (0.58-0.79, Sensitivity: 80.5% vs. 43.1% in early-stage HCC. Moreover, the combined diagnostic value of both CAP2+AFP was statistically significantly better than either CAP2 or AFP alone. Also, CAP2 could predict 82.4% of AFP-negative HCCs [AUC: 0.85 (0.77-0.92)] and 73.5% of AFP-negative early-stage HCCs [AUC: 0.80 (0.72-0.88)].

Conclusion: Compared with AFP, CAP2 was significantly elevated in HCC patients with higher sensitivity and AUC especially for early-stage HCC. Moreover, CAP2 was significantly correlated with the clinico-pathological features of HCC. CAP2 could be a novel biomarker predicting early-stage, AFP-negative, and AFP-negative early-stage HCC patients.

Keywords: Alpha-fetoprotein-negative HCC; BCLC stage; Biomarkers; Cyclase-associated protein-2; Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is considered the fifth commonest cancer in men, the seventh most common cancer in women worldwide and the second leading cause of cancer death worldwide with more than 1.6 million annual deaths [1,2]. HCC is one of the most aggressive cancers with limited therapeutic options and more than half a million people worldwide achieve HCC diagnosis [3,4]. The commonest and strongest risk factor for HCC development is liver cirrhosis; more than 90% of HCCs develop on top of a cirrhotic liver due to chronic hepatitis B, hepatitis C, alcoholic steatohepatitis, nonalcoholic steatohepatitis, diabetes or obesity [5]. Patients with advanced HCC progressing to the terminal stage have less than 10% one-year survival rate. In last decades, despite the recent considerable advances in surgical and radiological interventions of HCC and the clinical implementation of many therapeutic modalities, only a poor improvement in the five-year survival has been observed worldwide [6]. Hepatocarcinogenesis is usually silent and HCC patients usually experience symptoms in advanced disease stage. HCC prognosis depends markedly on its stage at the time of diagnosis. Unfortunately, Sorafenib which is a multikinase inhibitor and the only FDA-approved drug for advanced HCC has limited survival benefits [7]. Moreover, the curative treatments of HCC are largely limited to early disease stage. The recent therapeutic strategies including surgical resection, transarterial chemoembolization, percutaneous intervention (radiofrequency ablation and ethanol injection) or even orthotopic liver transplantation are effective only at an early-stage of HCC with approximately 70% recurrence within five years [8]. Thus, the early detection of HCC is an important and

crucial goal for all researchers. The early diagnosis and prognostic prediction of HCC are much difficult due to the coexistence of liver cirrhosis and inflammation in HCC [9]. Unfortunately, alpha fetoprotein (AFP) which was the golden marker of HCC lacks both sensitivity and specificity, has limited performance especially in early-stage HCCs and is no longer recommended by international surveillance guidelines [10].

Given these data, much interest was given for developing new potential therapeutic strategies. Also, the discovery of ideal novel biological non-invasive diagnostic biomarkers with a high-performance and prognostic prediction for this aggressive deadly disease has become a major focus of cancer research. The ideal biomarker should be protein-, RNA-, DNA-, or antibody-based, measurable in serum or urine, affordable, ethnically-specific and practically deployable in both the developed and developing worlds. Recently, a wide variety of novel biomarkers has been suggested to reinforce the current surveillance methods and predict early-stage HCC in patient's at-risk [11].

Serum biomarkers of HCC includes

Alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3), des-gamma-carboxy prothrombin (also known as prothrombin induced by vitamin K absence II, PIVKA II), Alpha-l-fucosidase (AFU), Glypican-3 (GPC3), Vascular endothelial growth factor, Interleukin-8, Transforming growth factor-beta 1, Tumor-specific growth factor, serine protease inhibitor squamous cell carcinoma antigen-immunoglobulin M complex (SCCA-IGM), Heat shock proteins (HSP70), Annexin I (ANX1), cyclase-associated protein (CAP), microRNAs (miRNAs), Exosomes, Osteopontin (OPN), Eag1 channels and Serum metabolites (Lysophosphatidylcholines, Free Fatty Acids species, serum bile acids) Urinary biomarkers of HCC include: Nucleosides, TGFα and β, Neopterin, Polyamines, Urinary trypsin inhibitor, soluble urinary metabolites [12-14].

The Cyclase-associated protein (CAP), an evolutionary highly conserved multifunctional actin-binding protein consisting of 474 to 551 amino acid residues, is present in mammals and a wide range of organisms including yeast, flies, and plants. It is involved in and plays a crucial role in species-specific signaling pathways [15,16]. CAP activates adenylyl cyclase, binds to G-actin mediating the dynamics of actin polymerization and is required for normal cellular morphology, locomotion, division, endocytosis, growth, and development [17]. In higher eukaryotes, two different homologs (CAP1 and CAP2), are present and share about 76% amino acid similarity. CAP2 is rarely present in a few of tissues and co-localized with actin in skeletal muscle cells [18,19]. During mice embryos' development, CAP2 was identifiable throughout cardiogenesis and its depletion led to dilated cardiomyopathy and other cardiac detects [20]. In human, CAP2 was detectable and differently expressed in many cancers. For example, CAP2 mRNA has been reported to be highly upregulated in thyroid, kidney, bladder and breast cancers and down-regulated in breast fibroadenoma [21].

Also, CAP2 was involved in hepatic carcinogenesis being over-expressed in HCC patients demonstrated by immunohistochemistry staining [22]. However, the data in literature describing its relevant prognostic implication in HCC are so far limited and elusive. Moreover, it is not clear if plasma level of CAP2 might be detected or not. The relationship between plasma levels of CAP2 and AFP levels in different stages of HCC is also not known. Thus, the objective of this study was to investigate the plasma levels of CAP2 and AFP in HCC, cirrhosis, healthy subjects. Also, we evaluated its role in detecting early-stage and AFP-negative HCC Egyptian patients.

Patients and Methods

Subjects

A total of 300 adult consecutive outpatient subjects with a confirmed diagnosis of liver cirrhosis of any etiology (150) and HCC of any stage (150) and receiving long-term follow-up were initially enrolled in this study. The age range was 30-65 years and male/female ratio was 1.36 (259/191). Another one hundred fifty (150) age- and sex-matched healthy control subjects were also enrolled. The study was initiated in the January 2015 and continued through 2017. The study was approved by the Ethical Commission and Institutional review board of Mansoura Faculty of Medicine in Egypt (MFM-IRB; Code No: R/16.02.91). A written informed conscious consent was obtained from all participants before their participation. The inclusion criterion was the diagnosis of liver cirrhosis and HCC. Exclusion criteria were an age below 18 years and over 70 years, a history of another cancer of any type within the last 5 years, a history of solid organ transplantation or previous bone marrow transplantation, and local or systemic tumor-specific treatment within the last month. Patients with chronic renal failure, bone disorders, thyroid disorders, cardiac failure (ejection fraction <50%), and systemic bacterial or fungal infection were excluded from the present study.

Methods

Initially, all participants completed a detailed questionnaire regarding diet and habits and submitted to thorough history taking with detailed physical examinations and relevant medical history. At the day of study inclusion, three milliliters of venous blood (by venipuncture of the antecubital vein) were obtained from all participants and the serum samples were centrifuged at 3000 rpm then aliquoted and stored at –70°C until assayed. Laboratory parameters, Ultrasound, CT scans and MRI imaging, the model of end-stage liver disease (MELD score) and Child–Pugh scores were assessed at the time of inclusion in the study [23].

Liver cirrhosis: Liver cirrhosis was diagnosed by ascites, esophageal varices, fundic varices, splenomegaly, jaundice, imaging and liver biopsies (if available, according to modified-knodell histological activity index) [24].

HCC: HCC was diagnosed by 4-phase multi-detector computed tomography (CT) scan, dynamic contrast-enhanced magnetic resonance imaging (MRI) [25,26]. Diagnosis of HCC was confirmed if there is one of the following three items:

- One or more of liver nodules > 1 cm in diameter in CT or MRI.
- Early arterial enhancement with α-fetoprotein ≥ 400 ng/mL.
- Typical features of dynamic imaging (arterial phase hypervascularity and washout in portal venous or delayed phases) regardless α-fetoprotein level.

Barcelona Clinic Liver Cancer (BCLC): The Barcelona Clinic Liver Cancer (BCLC) staging system was used to determine the stage of HCC. BCLC staging has been authenticated by liver expert groups (EASL and AASLD) as it allocates stage-specific management options, predicts survival and is likely to be updated. BCLC staging is best validated and suited for selection of early-stage HCC patients who could benefit from curative therapies [27].

- Very early-stage HCC or BCLC stage 0 refers to patients with single lesion ≤ 2 cm in diameter or carcinoma *in situ* without vascular involvement or metastasis.
- Early-stage HCC or BCLC stage A refers to patients with single or up to three nodules of ≤ 3cm in diameter each, without portal vein thrombosis or extra-hepatic metastasis.
- The intermediate stage HCC or BCLC stage B represents asymptomatic, large, or multifocal HCCs without evidence of vascular invasion or extra-hepatic metastasis.
- Advanced and Late stages HCC or BCLC stage Cand D constitutes symptomatic patients with vascular invasion or extra-hepatic metastasis.

All participants were assigned to the following groups:

- **Control group**: It comprised 150 healthy controls (Age: 56.3 ± 1.21 years; Males/ Females: 91/59).
- **Liver Cirrhosis group (LC)**: It comprised 150 cirrhotic patients (Age: 57.15 ± 1.02 years; Males/ Females: 88/62).
- **All HCC group**: It comprised 150 patients with HCC at any stage (Age: 62.0 ± 5.39years; Males/ Females: 33/35). This group included **(Table 3)**.
- Sixty-eight patients with early-stage HCC (Age: 59.3 ± 2.1 years; Males/ Females: 27/29)
- Fifty-six patients with AFP-ve HCC (Age: 59.3 ± 2.1 years; Males/ Females: 27/29) of which, fifty patients were AFP-ve early stage HCC (Age: 58.3 ± 2.2 years; Males/ Females: 24/26).

Collection of blood samples

At the day of study inclusion, three milliliters of venous blood (by venipuncture of the antecubital vein) were obtained from all participants and the serum samples were centrifuged at 3500 rpm for 15 minutes at room temperature then aliquoted and stored at –70°C until assayed for AFP and CAP2. Avoid repeated freeze/thaw cycles.

Plasma AFP and CAP2 Protein Concentration Assay

Plasma levels of CAP2 and AFP were measured using enzyme-linked immunosorbent assay (ELISA) kits purchased from Rand D systems (Minneapolis, MN, USA). The human Adenylyl Cyclase associated protein 2 (CAP2) ELISA KIT is a ready-to-use microwell, strip plate ELISA Kit for analyzing the presence of the CAP2 in biological samples. The sensitivities of ELISA kit of CAP2 and AFP were 0.037 ng/mL and 0.046 ng/mL respectively. The assay range was 0.300-20 ng/mL for CAP2 and 0.312-20 ng/mL for AFP.

The Assay principle applied in the kits is Sandwich enzyme linked immunosorbent assay. The ELISA analytical biochemical technique of the kit is based on CAP2 antibody-CAP2 antigen interactions (immuno-sorbent) and a Horseradish Peroxidase (HRP) colorimetric detection system to detect CAP2 antigen targets in samples. The microtiter plate provided in the kit has been pre-coated with an antibody specific to Adenylyl Cyclase Associated Protein 2 (CAP2). Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to Adenylyl Cyclase Associated Protein 2 (CAP2). Next, Avidin conjugated to HRP is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain adenylyl cyclase associated protein-2 (CAP2), biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of adenylyl cyclase associated protein-2 (CAP2) in the samples is then determined by comparing the optical densities (OD) of the samples to the standard curve.

Assay procedure

Plasma CAP2 and AFP concentrations were measured according to manufacturer's recommendation. Briefly, 10 µL of plasma samples were mixed with 40 µL of sample dilution buffer and incubated in 96-well plates coated with antibodies for30 min at 37°C. The solutions were decanted followed by washing. Then, 50 µL HRP-conjugated secondary antibodies were added into the wells and incubated for 30 min. After washing, 50 µL of each chromogen solution A and chromogen solution B were added into the wells, and incubated for15 min at 37°C. The reaction was stopped by adding 50 µL of stop solution. The OD values were determined in 96-well plate reader (Bio-Rad Laboratories, Hercules, MA, USA) at 450 nm wave length. All tests were performed in duplicate, average OD values were calculated and the plasma levels of CAP2 and AFP were determined by standard curve. Data were collected and analyzed, intra-batch variation was controlled within 5%, and inter-batch variation was less 10%.

Statistical Analysis

Data were analyzed using SPSS software (Version 17.0, SPSS Inc., Chicago; IL). Quantitative (continuous) data were expressed as (Mean ± Standard Deviation) while qualitative data and categorical variables were expressed as number and percentage. Categorical variables were compared using the chi-square (χ^2) test or Fisher's exact test. Subgroups were compared using the Mc-Nemar test. Comparisons between the groups were performed using the Student's t-test whenever applicable. Mann-Whitney U-test was used for the continuous ordinal data between two qualitative variables. One Way Analysis of Variance (ANOVA) compares more than two groups. Correlations between markers and other variables were evaluated using the Spearman rank correlation coefficient test. Variables that achieved statistical significance with the univariate analysis were included in multiple regression analysis to differentiate HCC patients from cirrhotic patients and to evaluate the independent factors associated with high CAP2 or AFP. Receiver operating characteristic curves (ROC) was constructed to determine optimal cutoff values, diagnostic accuracy and performance of CAP2 and AFP. Area under the curve (AUC) calculations and their 95% confidence intervals were used to evaluate diagnostic values [28]. For all statistical studies, P<0.05 was considered to be statistically significant. Sensitivity, specificity, and predictive values were calculated to study the overall predictability according to the following equations: [29]

- Positive (+ve) predictive value=(No of true +ve cases/N0 of all +ve cases with screening test) × 100.
- Negative (-ve) predictive value= (No of true -ve cases/N0 of all -ve cases with screening test) × 100.
- Sensitivity=(No of true +ve cases/No of all +ve cases with reference test) ×100.
- Specificity=(No of true –ve cases/No of all –ve cases with reference test) × 100.
- Overall predictability (accuracy)=(No of true +ve and true -ve cases/ total No of all +ve and all –ve cases) × 100.

Results

The patient basic demographic, clinical and laboratory data in all studied groups and subgroups were shown in **Table 1**. When compared with control or LC groups, patients with HCC had a statistically significantly higher mean age, serum AST, serum ALT, serum Bilirubin, INR, plasma AFP and CAP2 levels and had a significantly lower serum albumin and platelet counts (P<0.05 for all). Nevertheless, patients with early-stage HCC had no significant differences in AFP levels when compared with LC patients (P<0.05). Chronic Hepatitis C was the commonest etiology of liver cirrhosis in either HCC or non-HCC patients (63.3% and 62% respectively) followed by Chronic Hepatitis B (20% and 18.7% respectively) however, there were no significant differences in plasma levels of either CAP2 or AFP in chronic hepatitis B or C.

Table 1: The demographic, clinical and laboratory characteristics of patients in the studied groups.

Variables	Control (150)	LC (150)	HCC (150)	Early HCC (68)	AFP–ve HCC (56)	AFP–ve early-stage HCC (50)	ANOVA
Age (Years)	56.3 ± 1.21	57.15 ± 1.02	62.0 ± 5.39	61.4 ± 4.39	59.3 ± 2.1	58.3 ± 2.2	0.041
Male /Female	80/70	91/59	88/62	33/35	27/29	24/26	0.347
Etiology: [HCV/HBC/Alcoholism/ others]	-	93/30/8/19	95/28/8/19	36/16/3/13	35/11/2/8	33/9/2/6	-
AST (U/L)	39.6 ± 1.9	99.8 ± 3.17	113.1 ± 2.08	109.1 ± 3.78	101.8 ± 3.2	103.8 ± 3.2	0.005
ALT (U/L)	41.45 ± 1.6	86.0 ± 1.7	117.65 ± 3.8	113.6 ± 5.8	89.0 ± 1.6	90.0 ± 1.7	0.006
S. bilirubin (mg/dL)	1.2 ± 0.1	1.3 ± 0.2	2.8 ± 0.8	2.4 ± 0.8	1.2 ± 0.3	1.3 ± 0.3	0.012
Serum Albumin (g/dL)	4.6 ± 1.1	2.9 ± 1.9	2.1 ± 2.1	2.2 ± 2.1	2.7 ± 1.3	2.6 ± 1.3	0.034
S. Creatinine (mg/dL)	0.9 ± 0.4	1.1 ± 0.5	1.2 ± 0.4	1.2 ± 0.2	1.1 ± 0.8	1.1 ± 1	0.071
Platelets (104/µL)	22.6 ± 0.23	14.49 ± 0.5	10.78 ± 0.6	11.78 ± 0.6	12.1 ± 0.6	11.1 ± 0.6	0.001
INR	1.1 ± 0.1	1.3 ± 0.2	1.7 ± 0.4	1.7 ± 0.4	1.2 ± 0.3	1.2 ± 0.5	0.023
AFP (ng/mL)	4.7 ± 3.1	40.8 ± 62.3	1016.8 ± 946.1	33.3 ± 7.1	12.8 ± 2.8	126 ± 2.6	<0.001
Plasma CAP2 levels	4.44 ± 3.3	7.53 ± 3.54	37.1 ± 8.8	34.1 ± 10.2	31.7 ± 10.1	31.9 ± 9.9	<0.001

Data were expressed as M ± SD: Mean ± Standard Deviation; INR: International Normalized Ratio; LC: Liver Cirrhosis; HCC: Hepatocellular Carcinoma; AST: Aspartate Transaminase; ALT: Alanine Transaminase; AFP: Serum Alpha-Fetoprotein Level (ng/mL); CAP2: Adenylyl Cyclase-Associated Protein-2 (ng/mL); HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; P: Probability; ANOVA: One Way Analysis of Variance; (P<0.05 was considered to be statistically significant).

The clinico-pathological characteristics of patients with HCC and LC and their relations to the plasma CAP2 levels were demonstrated in **Table 2**. In HCC patients, Higher plasma CAP2 levels were significantly related associated advanced BCLC stages of HCC, large tumor nodules, the presence of either metastasis or portal vein thrombosis (P=0.001) but not with age, gender or etiological cause (P>0.05). Moreover, high plasma CAP2 levels were significantly associated with high Child Pugh scores in HCC patients with underlying cirrhosis (p=0.0001).

Table 2: Comparisons between plasma levels of CAP2 in different clinicopathological characteristics of patients with HCC and LC.

Variables		CAP2 HCC			CAP2 LC		
		N (%)	M ± SD	P-value	N (%)	M ± SD	P-value
Age	≥ 50 years	102 (68)	36.1 ± 7.5	0.059	92 (61.3)	7.6 ± 3.3	0.258
	< 50 years	48 (32)	34.4 ± 10.2		58 (38.7)	6.9 ± 3.4	
Gender	Male	88 (58.7)	34.1 ± 9.8		91 (60.7)	7.1 ± 3.6	
	Female	62 (41.3)	36.2 ± 7.9	0.092	59 (39.3)	7.6 ± 3.4	0.671
Etiology	HCV	95 (63.3)	34.2 ± 9.8		93 (62)	7.4 ± 3.5	
	HBV	28 (18.7)	34.6 ± 8.8	0.607	30 (20)	7.7 ± 3.4	0.302
	Alcoholism	8 (5.3)	30.8 ± 10.6		8 (5.3)	10.2 ± 3.3	
	Others	19 (12.7)	35.3 ± 7.1		19 (12.7)	5.9 ± 3.7	
Metastasis	No	122 (81.3)	31.8 ± 9.1	0.001a	-	-	
	Yes	28 (18.7)	40.6 ± 5.2		-	-	
PVT	No	108 (72)	30.9 ± 8.4	0.001a	-	-	
	Yes	42 (28)	41.5 ± 5.8		-	-	
Tumor size	≥2 cm	54 (36)	28.6 ± 8.9	0.001b	-	-	
	>2, ≤ 5 cm	56 (37.3)	37.2 ± 8.1		-	-	
	>5 cm	40 (26.7)	36.8 ± 7.6		-	-	
BCLC stage	0, A	68 (45.3)	35.1 ± 10.2	0.001b	-	-	
	B, C	70 (46.7)	37.1 ± 7.2		-	-	
	D	12 (8)	38 ± 7.5		-	-	
Child Pugh	No LC	49 (32.7)	29.2 ± 9.5	0.0001b	-	-	-
	A	59 (39.3)	34.3 ± 9.1		70 (46.7)	7.8 ± 2.8	0.206
	B	30 (20)	36.8 ± 8.3		51 (34)	7.6 ± 3.9	
	C	12 (8)	39.1 ± 8.7		29 (19.3)	7.9 ± 4.1	

Data were expressed as M ± SD: Mean ± Standard Deviation; CAP2: Adenylyl Cyclase-Associated Protein-2 (ng/mL); HCC: Hepatocellular Carcinoma; LC: Liver Cirrhosis; AFP: Serum Alpha-Fetoprotein Level (ng/mL); -ve: Negative; +ve: Positive; N: Number; PVT: Portal Vein Thrombosis. BCLC: Barcelona Clinic Liver Cancer Staging System; HBV: Hepatits B Virus; HCV: Hepatits C Virus; Amann-Whitney U-Test Was Used. B One-Way Analysis of Variance (ANOVA) Test was Used. P: Probability; P<0.05 was considered to be statistically significant.

Also, the plasma CAP2 and AFP levels in early and advanced disease stages of both HCC and LC groups were demonstrated in **Table 3**. Only the plasma levels of CAP2 (but not plasma AFP levels) were significantly elevated in early-stage HCC and AFP–ve early-stage HCC than LC patients (P<0.01).

The Correlations between plasma CAP2 and AFP levels and the clinico-pathological characteristics in patients with HCC and LC were demonstrated in **Table 4**.

Only the plasma CAP2 levels (but not plasma AFP levels) were significantly and positively correlated with BCLC stages of HCC, tumor size, the presence of either metastasis or portal vein thrombosis (P<0.01) but not with age, gender. Moreover, and as shown in **Figure 1**, no significant correlations were found between plasma levels of CAP2 and AFP in HCC patients (r= -136-, P=0.156).

The receiver operating characteristic (ROC) curves for CAP2, AFP or combined (CAP2+AFP) levels were constructed to further evaluate the diagnostic role of CAP2 in All HCC patients, early-stage HCC, and AFP-ve early-stage HCC patients **(Figures 2A to 2D)**. Also, the diagnostic cutoff values, area under the curve (AUC), Sensitivity, Specificity, Likelihood ratio

and predictive values of CAP2 and AFP in HCC group and its subgroups were listed in **Table 5**.

Table 3: Plasma CAP2 and AFP levels in the different disease stages of HCC and LC groups.

HCC group		CAP2 (ng/mL)	AFP (ng/mL)	LC group		CAP2 (ng/mL)	AFP (ng/mL)
HCC stage	AFP status (N)	M ± SD	M ± SD	LC stage	AFP status (N)	M ± SD	M ± SD
Early	-ve (50)	31.7 ± 10.1	12.8 ± 2.8	Early	-ve (93)	6.3 ± 2.95	4.1 ± 3.5
	+ve (18)	36 ± 9.8	82 ± 47.9		+ve (6)	6.5 ± 2.7	25.5 ± 46.6
	Total (68)	34.1 ± 10.2	33.3 ± 7.1		Total (99)	6.75 ± 2.9	15.1 ± 35
	P1-value	<0.01	<0.001			NS	S
Advanced	-ve (6)	40.6 ± 5.4	13.2 ± 2.8	Advanced	-ve (31)	8.8 ± 3.43	17 ± 3.8
	+ve (76)	38.4 ± 10.3	1769.7 ± 726.2		+ve (20)	9.1 ± 4.4	106.5 ± 70.8
	Total (82)	39.6 ± 10.1	1713 ± 767		Total (51)	9 ± 4.2	90.7 ± 72.8
Total	-ve (67)	34.4 ± 9.8	11.7 ± 10.7	Total	-ve (124)	7.56 ± 3.1	6.1 ± 5.9
	+ve (83)	35.9 ± 9.3	1520.6 ± 892.4		+ve (26)	7.5 ± 3.8	62.1 ± 71.1
	Total (150)	37.1 ± 8.8	1016.8 ± 946.1		Total (150)	7.5 ± 3.5	40.8 ± 62.3
P2-value		<0.01	<0.0001			NS	S

Data were expressed as M ± SD: mean ± standard deviation; N: Number; LC: Liver Cirrhosis; HCC: Hepatocellular Carcinoma; AFP: Serum Alpha-Fetoprotein Level (Ng/Ml); CAP2: Adenylyl Cyclase-Associated Protein-2 (ng/mL); -ve: Negative; +ve: Positive; HCC Stage was according to the Barcelona Clinic Liver Cancer (BCLC) Staging System: Early HCC (Stages 0 & A), Advanced (Stage B, C, D); LC Stage Was According To Child–Pugh Scoring System: Early LC (Child A); Late LC (Child B, C); P: Probability; P1: Compared early HCC and early AFP –ve HCC; P2: Compared early HCC and total HCC; P<0.05 was considered to be statistically significant; S: Significant; NS: Non-Significant.

Table 4: Spearman's rho Correlation between plasma levels of both CAP2 and AFP and the clinico-pathological characteristics in patients with HCC and LC.

Variables	CAP2 HCC		AFP HCC		CAP2 LC		AFP LC	
	r	p	r	p	r	p	r	p
AFP HCC	-0.116-	0.156	--	--	--	--	--	--
AFP LC	--	--	--	--	0.078	0.528	--	--
Age	0.131	0.111	0.274	0.060	-0.231-	0.094	0.206	0.132
Gender	-0.135-	0.099	0.129	0.115	-0.202-	0.285	0.009	0.917
Etiology	0.041	0.622	-0.153-	0.061	-0.065-	0.427	-0.048-	0.563
Tumor size	0.294	0.000	0.224	0.056	--	--	--	--
Metastasis	0.342	0.000	0.099	0.229	--	--	--	--
Portal vein thrombosis	0.168	0.001	0.152	0.064	--	--	--	--
BCLC	0.286	0.000	0.151	0.066	--	--	--	--
Child Pugh scores	0.295	0.000	0.086	0.296	0.136	0.097	0.150	0.067

CAP2: Adenylyl Cyclase-Associated Protein-2 (ng/mL); HCC: Hepatocellular Carcinoma; LC: Liver Cirrhosis; AFP: Serum Alpha-Fetoprotein Level (ng/mL); -ve: Negative; +ve: Positive; BCLC: Barcelona Clinic Liver Cancer Staging System; r: Spearman's rho Correlation Coefficient; P: Probability; P<0.05 was considered to be statistically significant

CAP2 and AFP levels in All HCC patients (Table 5 and Figure 2A): CAP2 had a better diagnostic value than AFP as it had a significantly higher AUC (95%CI) at 0.86 (0.79-0.93) vs. 0.75 (0.65-0.85), Sensitivity of (81.5% vs. 62%), with a positive predictive value of 86.3% vs. 79.5% (P=0.001). Moreover, the combined diagnostic value of both (CAP2+AFP) was statistically significantly better than either CAP2 or AFP alone with highest AUC (95%CI) at 0.89 (0.83-0.95) and Sensitivity of 87.8% (P=0.012).

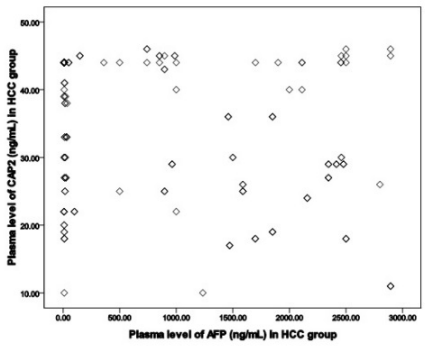

Figure 1: Correlation between the plasma levels of Cyclase-Associated Protein-2 (CAP2) and Alpha-fetoprotein (AFP) in Hepatocellular carcinoma (HCC) patients.

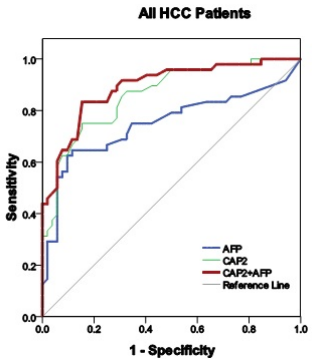

Figure 2A: The receiver operating characteristic (ROC) of Cyclase-Associated Protein-2 (CAP2) and Alpha-fetoprotein (AFP) in All HCC patients.

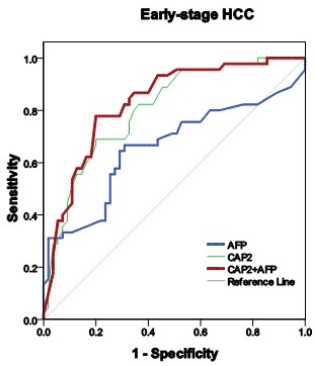

Figure 2B: The receiver operating characteristic (ROC) of Cyclase-Associated Protein-2 (CAP2) and Alpha-fetoprotein (AFP) in early-stage HCC patients.

CAP2 and AFP levels in Early-Stage HCC patients (Table 5 and Figure 2B): From all HCC patients only 68 patients had early-stage HCC. CAP2 had a better diagnostic value than AFP as it had a statistically significantly higher AUC (95% CI) at 0.80 (0.72-0.89) vs. 0.68 (0.58-0.79), Sensitivity of (80.5% vs. 43.1%), Specificity of 81.3% vs. 79.9% with a positive predictive value of 82.2% vs. 64.7% (P=0.001). Moreover, the diagnostic value of combined (CAP2+AFP) was statistically significantly better than AFP alone with the highest AUC (95% CI) at 0.82 (0.74-0.90), Sensitivity of 81.1% and Specificity of 80.4% with a positive predictive value of 81.8% (P=0.001). Nevertheless, the diagnostic value of combined (CAP2+AFP) was not significantly different from CAP2 alone (P=0.053). The cutoff value for combined (CAP2+AFP) was CAP2 (6.5 ng/mL) or AFP (10 ng/mL).

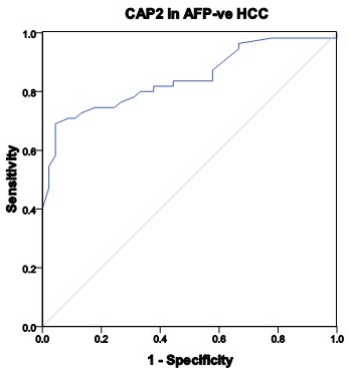

Figure 2C: The receiver operating characteristic (ROC) of Cyclase-Associated Protein-2 (CAP2) in Alpha-fetoprotein-negative HCC patients.

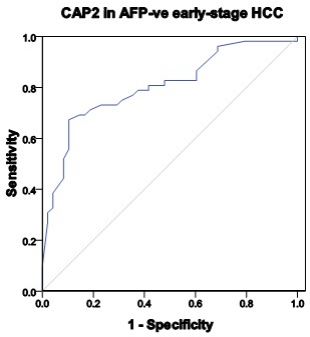

Figure 2D: The receiver operating characteristic (ROC) of Cyclase-Associated Protein-2 (CAP2) in Alpha-fetoprotein-negative early-stage HCC patients.

CAP2 levels in AFP-ve HCC patients (Table 5, Figures 2C and 2D): From all HCC patients only 56 patients had AFP-ve HCC, of which 50 patients had AFP–ve early-stage HCC. Using a cutoff value of 7.5 ng/mL when AFP levels were below 15 ng/mL, CAP2 was found to have a good diagnostic value for AFP-ve HCC patients **(Figure 2C)** and AFP-ve Early-stage HCC patients **(Figure 2D)** with AUC (95%CI) at 0.85 (0.77-0.92) and 0.80 (0.72-0.89) respectively, Sensitivity at 82.6% and 82.1% respectively, Specificity at 81.1% and 79.2% respectively and a

positive predictive value at 85.1% and 84.6% respectively **(Table 5)**.

Table 5: Diagnostic cutoff values, AUC, Sensitivity, Specificity, and predictive values of CAP2 and AFP in HCC group and its subgroups.

Variables		AUC (95%CI)	S. Error	Cutoff value (ng/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR	P-value
All HCC	AFP	0.75 (0.65-0.85)	0.052	15	62	83.9	79.5	68.7	3.9	0.001*
	CAP2	0.86 (0.79-0.93)	0.036	7.5	81.5	78.8	86.3	72.2	3.8	0.012**
	CAP2+ AFP	0.89 (0.83-0.95)	0.049	CAP2=6.5 or AFP=10	87.8	76.7	86.7	78.6	3.8	0.001***
Early HCC	AFP	0.68 (0.58-0.79)	0.053	15	43.1	79.9	64.7	63.3	2.2	0.001*
	CAP2	0.80 (0.72-0.89)	0.044	7.5	80.5	81.3	82.2	77.6	4.2	0.053**
	CAP2+ AFP	0.82 (0.74-0.91)	0.042	CAP2=6.5 or AFP=20	81.1	80.4	81.8	79.6	4.1	0.001***
AFP-ve HCC	CAP2	0.85 (0.77-0.92)	0.039	7.5	82.6	81.1	85.1	78.2	4.4	-
AFP-ve early HCC	CAP2	0.80 (0.72-0.88)	0.044	7.5	82.1	79.2	84.6	76	3.9	-

Abbreviations: AUC: Area Under The Curve; PPV: Positive Predictive Value; NPV: Negative Predictive Value; PLR: Positive Likelihood Ratio; CAP2: Adenylyl Cyclase-Associated Protein-2 (ng/mL); HCC: Hepatocellular Carcinoma; AFP: Serum Alpha-Fetoprotein Level (ng/mL); -ve: Negative; P: Probability (*AFP vs. CAP2; **CAP2 vs. Combined CAP2+AFP; ***AFP vs. Combined CAP2+AFP); P<0.05 was considered to be statistically significant

Discussion

Hepatocellular carcinoma is an aggressive deadly primary hepatic cancer with far limited therapeutic strategies which are effective only at an early-stage of HCC. Unfortunately, alpha fetoprotein (AFP) has a limited performance and no longer recommended by international surveillance guidelines [30]. So, the discovery of an affordable non-invasive deployable biomarker with a high-performance and prognostic prediction for this aggressive disease has become a major focus of cancer research.

Recently, numerous biomarkers had been proposed for HCC diagnosis such as Glypican-3 (GPC3), Heat shock proteins (HSP70), Annexin A2 (ANXA2), microRNAs panel (miRNAs), Eag1 channels, Exosomes, Osteopontin (OPN), Neopterin and Cyclase-Associated Protein-2 (CAP2) [31]. The current study investigated CAP2 as a diagnostic biomarker for HCC patients in contrast to AFP and further evaluated its role in detecting early-stage and AFP-negative HCC Egyptian patients.

In contrast to previous studies [5], chronic Hepatitis C was the commonest etiology of liver cirrhosis in either HCC or non-HCC patients (63.3% and 62% respectively) followed by chronic Hepatitis B (20% and 18.7% respectively) however, there were no significant differences in plasma levels of either CAP2 or AFP related to chronic hepatitis B or C. This may be explained by the high endemicity of chronic HCV in Egypt.

The current data revealed that HCC patients had a significantly higher plasma AFP and CAP2 levels than control and LC patients. Nevertheless, Early-stage HCC patients had no significant elevation in AFP levels. This finding could imply a role of CAP2 in the prediction of HCC and coincided with a previous study in which the authors proposed that plasma CAP2 level is a promising biomarker complementary to AFP in diagnosing HCC [32].

In human, CAP2 was detectable and differently expressed in many cancers being highly upregulated in thyroid, kidney, bladder, breast cancers [21]. However, there is paucity in literature describing the relevant prognostic implication of plasma levels of CAP2 in HCC patients.

In line with current data, Fu et al. suggested CAP2 as a promising biomarker for HCC prognosis as it noticeably over-expressed in HCC tissues, compared with the non-cancerous tissues and significantly correlated with poor survival. Of interest, they proposed the prognostic implication of CAP2 in the subgroups of HCC patients [33].

The current study also demonstrated that the higher plasma levels of CAP2 in HCC patients were significantly positively correlated with disease severity, BCLC stages of HCC, tumor nodules, the presence of either metastasis or portal vein thrombosis (P=0.001) but not with age, gender or offending etiology of LC or HCC. Moreover, high plasma CAP2 levels were significantly associated with high Child Pugh scores in HCC patients with underlying cirrhosis. Nevertheless, there were no significant correlations between plasma levels of CAP2 and AFP in HCC patients. The results presented in this study could also imply a prognostic role of plasma CAP2 level in HCC

patients as well as HCC subgroups. Alpha fetoprotein (AFP) which was the golden marker of HCC lacks both sensitivity and specificity and has limited performance especially in early-stage HCCs and is no longer recommended by international surveillance guidelines [30].

Despite the advanced imaging methods, it is still difficult to detect early-stage HCC and AFP-ve HCC patients. Recently, numerous biomarkers had been proposed to predict early-stage HCC as well as AFP-negative HCC patients such as des-gamma-carboxy prothrombin (DCP) (also known as Prothrombin Induced by Vitamin K Absence II: PIVKA II), Lens culinaris-agglutinin-reactive fraction of AFP (AFP-L3) [34], Glypican-3 (GPC3) [35], fucosylated haptoglobin [36], fucosylated paraoxonase 1 (FUC-PON1) [37]; Heat shock proteins (HSP70) [38], Annexin A2 (ANXA2) [39], microRNAs panel (miRNAs) [40], Eag1 channels [41], Transforming Growth Factor-Beta [42], Osteopontin (OPN) [43] and CAP2 [32,33]. The combination of PIVKA-II, the sensitivity of which was 48.9% in HCC patients, with AFP or AFP-L3 significantly improved its diagnostic performance [34]. Haptoglobin which had an AUC at 0.76%, Sensitivity at 72.2% and Specificity at 70% was reported to complement AFP diagnostic performance [36].

Chen et al. suggested that CAP2 has a pivotal and superior role in differentiating HCC from LC being had a better sensitivity at 82.6% vs. 59.3%, higher AUC at 0.86 vs. 0.75 than AFP [32]. This evidence coincides with the current study in which, CAP2 had a better diagnostic value than AFP, a significantly higher AUC (95%CI) at 0.86 (0.79-0.93) vs. 0.75 (0.65-0.85), a significantly higher Sensitivity of (81.5% vs. 62%), and a higher positive predictive value of 86.3% vs. 79.5%. Moreover, the combined diagnostic value of both (CAP2+AFP) in HCC patients was statistically significantly better than either CAP2 or AFP alone with highest AUC (95%CI) at 0.89 (0.83-0.95) and Sensitivity of 87.8%. From the present data, it is evident that CAP2 alone or combined with AFP had higher AUC, Sensitivity, positive predictive value and overall diagnostic accuracy than those mentioned in other studies for CAP2 or for other biomarkers.

Additionally, the observed absence of a significant correlation between plasma levels of CAP2 and AFP in HCC patients supported the diagnosis of early-stage HCC, AFP –ve HCC, and AFP –ve early-stage HCC patients. Moreover, the diagnostic value of combined (CAP2+AFP) in detecting early-stage HCC was statistically significantly better than AFP alone with higher AUC (95%CI) at 0.82 (0.74-0.90), Sensitivity of 81.1% and Specificity of 80.4% with a positive predictive value of 81.8%. Using a cutoff value of 7.5 ng/mL when AFP levels were below 15 ng/mL, CAP2 was found to have a high diagnostic accuracy for both AFP-ve HCC patients and AFP-ve early-stage HCC patients with AUC (95%CI) at 0.85 (0.77-0.92) and 0.80 (0.72-0.89) respectively, Sensitivity at 82.6% and 82.1% respectively, Specificity at 81.1% and 79.2% respectively and a positive predictive value at 85.1% and 84.6% respectively.

The mechanism of increased plasma CAP2 levels in HCC is still not clear. Shibata and colleagues evidenced that CAP2 was involved in and related to hepatic carcinogenesis being noticeably over-expressed and upregulated in HCC cells demonstrated by remarkably higher immunohistochemistry (IHC) Score. On the other hand, no or trivial CAP2 expression in normal hepatocytes was noticed [44]. *In-vivo* and *in-vitro* immunohistochemistry (IHC) staining showed that CAP2 was distributed in the nuclear and perinuclear areas suggesting that CAP2 might be produced by HCC cells of differentiated as well as early-stage HCC [22]. CAP is involved in regulating the adenylyl cyclase activity in yeast under the control of *RAS*. CAP2 is released from the nucleus by high salt concentrations and released into blood stream by vesicles formation and trafficking [45]. However, the detailed mechanisms for CAP2 release into the blood stream still elusive and necessitate future studies.

In this study, CAP2 had a better diagnostic value than AFP in detecting Early-stage HCC; with higher AUC (95%CI) at 0.80 (0.72-0.89) vs. 0.68 (0.58-0.79), Sensitivity of (80.5% vs. 43.1%), Specificity of 81.3% vs. 79.9% and a positive predictive value of 82.2% vs. 64.7%. Additionally, the plasma CAP2 level was higher in early-stage HCCs than in LC patients. Consequently, it could be proposed for Early-stage HCC diagnosis and distinguishing AFP-negative Early-stage HCC from LC.

In similar studies, some novel biomarkers were also proposed for Early-stage HCC diagnosis and differentiate AFP-negative Early-stage HCC from LC [46]. Fucosylated Paraoxonase 1 (Fuc-Pon1) was proposed as a promising biomarker for diagnosis of early-stage HCC and distinguishing AFP-negative early-stage HCC from LC patients [37]. Osteopontin (OPN) alone or in combination with AFP provided a significantly AUC, higher sensitivity, overall accuracy and performance than AFP in diagnosing LC, HCCs, early-stage HCC and AFP-negative HCC patients [43]. Fucosylated Haptoglobin (Hp) /Haptoglobin ratio alone or combined with AFP and the ELISA Index (optical density [OD] value of fucosylated Hp /OD value of Hp) had a better diagnostic performance in Early-stage HCC and AFP-negative HCC [36].

Similarly, the serum level of Annexin A2 (ANXA2) might be a good biomarker for detection of early of HCC and could differentiate between HCC and CLD as it is over-expressed and upregulated in HCC cells [47]. Unfortunately, the diagnostic performance of serum Glypican 3 (GPC3) which had a diagnostic value comparable to AFP in HCC diagnosis is still unsatisfactory for early-stage HCC [48]. However, both serum GPC-3 and GPC-3mRNA are promising diagnostic markers for early detection of HCC in Egyptian patients [35,49]. The diagnostic accuracy and performance of plasma CAP2 levels for early-stage HCC noted in the current study were much higher than that of previously studied biomarkers (such as AFP-L3, PIVKA II, GPC3, HSP70, OPN, FUC-PON1, ANXA2). Meanwhile, CAP2 was found to have a high diagnostic accuracy for both AFP-ve HCC patients and AFP-ve early-stage HCC patients with AUC (95%CI) at 0.85 (0.77-0.92) and 0.80 (0.72-0.89) respectively, Sensitivity at 82.6% and 82.1% respectively, Specificity at 81.1% and 79.2% respectively and a positive predictive value at 85.1% and 84.6% respectively.

Collectively, we could suggest that CAP2 might be a superior biomarker for detection of early-stage HCC, AFP-ve HCC patients and AFP-ve Early-stage HCC patients. Also, the combined diagnostic value of both CAP2+AFP (AUC: 0.89 (0.83-0.95)) in HCC patients was statistically significantly better than either CAP2 (AUC: 0.86 (0.79-0.93)) or AFP (AUC: 0.75 (0.65-0.85)) alone with the highest Sensitivity at 87.8% vs. 86.3% or 83.9% for CAP2 or AFP respectively. In addition, combined CAP2+AFP could greatly improve the detection rate of all HCCs and differentiate early-stage of HCC from liver cirrhosis. Nevertheless, the diagnostic value of the combined CAP2+AFP in early-stage HCCs was not significantly different from CAP2 alone [AUC: 0.82 (0.74-0.91 vs. 0.80 (0.72-0.89)].

To determine the factors that may affect the plasma CAP2 level, we further investigate if plasma CAP2 level is related to the clinico-pathological parameters of the tumor. The clinicopathological features of HCC patients such as tumor size, histological grade, metastasis, portal vein thrombosis, BCLC stage and clinical stage were proposed to be relevant prognostic factors of tumor progression [50]. Effendi et al. demonstrated that CAP2 levels were associated with the clinicopathological features of HCC patients [51]. Similarly, we found significant positive correlations between plasma CAP2 level and clinico-pathological features of HCC patients such as tumor size, BCLC stage, metastasis, portal vein thrombosis, and clinical stage. Zhang et al. also, suggested that BCLC stage B and C were associated with poor overall survival and tumor recurrence in AFP-ve HCC patients [52]. Taken together, CAP2 levels might be a predictor for HCC. However, this assumption should be validated by future large-scale surveillance studies.

Conclusion

In conclusion, plasma CAP2 levels alone or combined with AFP could be a promising superior biomarker for diagnosis of HCC, detection of early-stage of HCC, prediction of AFP-negative HCC and differentiating it from liver cirrhosis.

Conflict of Interest

The authors declare no conflicts of interest

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