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Evaluation of diagnostic accuracy of serum calcium channel $\alpha 2\delta 1$ subunit in hepatocellular carcinoma-related cirrhosis

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the commonest malignancies worldwide that carries a bad prognosis particularly in Egypt due to the high prevalence of HCV burden. Late diagnosis of HCC especially in cirrhosis suffering-liver is one of the causes that worsen HCC outcome. Identification of molecular pathways of HCC will open the gate for early diagnosis and effective management. Oscillation of calcium controlled by the $\alpha 2\delta 1$ subunit has been proposed as one of the mechanisms in tumor-initiating cell properties of HCC. In this study, we aim to evaluate the serum $\alpha 2\delta 1$ subunit level as a biological marker for HCC. A total of 90 participants were enrolled, 40 patients with HCC, 40 patients with cirrhosis, and 10 healthy volunteers; serum level of $\alpha 2\delta 1$ was assessed in all participants with ELISA

Results: The mean serum levels of $\alpha 2\delta 1$ were significantly higher in HCC group (19.53 ± 6.87 ng/dL) than cirrhotic (6.24 ± 2.64 ng/dL) and control groups (0.67 ± 0.48 ng/dL) ($P = 0.001$). There was no significance between $\alpha 2\delta 1$ and etiology of liver disease as viral (HCV, HBV) or non-viral ($P = 0.14$).

Conclusion: $\alpha 2\delta 1$ subunit may serve as a potential non-invasive marker with excellent sensitivity for diagnosis of HCC regardless of the etiology of liver disease.

Keywords: Hepatocellular carcinoma, $\alpha 2\delta 1$ subunit, CSCs, HCV, HBV, Novel marker

Background

Liver malignancy is the sixth commonest malignancy worldwide with an incidence of approximately 841,000 new cases in 2018 [1], of which hepatocellular carcinoma (HCC) represents 80% [2], and considered the fourth leading cause of cancer-related death worldwide [3]. In Egypt, HCC is the fourth common cancer [4].

HCC has molecular subgroups reflecting the biological background and might have prognostic potential and selection criteria for therapy; among the subgroups there are two major subtypes each encompassing almost 50% of patients: one, *proliferation class*, which is poorly differentiated, had worse clinical outcome, expressing high alpha-fetoprotein (AFP) levels with more vascular

invasion, and commonly associated with HBV infection; the other class, *non-proliferation*, more commonly associated with alcohol-related HCC or HCV infection, had better outcome with moderate to well differentiation [5].

In 1994, Lapidot and his team introduced the concept of cancer stem cell (CSC) through transplantation of acute myeloid leukemia (AML) cells from recently diagnosed patients into severe combined immune deficient (SCID) mice [6]. This CSC can self-renew, differentiate into all tumor cell lines giving the criteria of tumor hierarchy and intra-tumoral heterogeneity, have the unique tumor-initiating capability, and own high resistance to chemotherapy [7]. They possess many names as CSCs, tumor-initiating cells (TICs), or cancer stem-like cells [8].

Many CSCs have been identified in HCC, one of them was isoform 5 of the cell surface voltage-gated calcium channel $\alpha 2\delta 1$ described by Zhao et al. [9], they inject

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$\alpha 2\delta 1+$ cells subcutaneously in (non-obese diabetic) NOD/SCID mice, there was higher tumorigenic potential in comparison with $\alpha 2\delta 1-$ cells. Also, they showed that $\alpha 2\delta 1$ regulates calcium signaling and intracellular calcium levels, which is vital for activating signaling cascades that regulate gene transcription and various cell functions; one of them is the phosphorylation of ERK1/2 which prevents cell apoptosis [9].

Calcium oscillations not only occur in excitable tissues spontaneously, such as muscle, SAN, and neuronal tissues [10], but also occur in pluripotent cells (embryonic stem cells), multipotent cells (mesenchymal stem cells), immature dendritic cells, and G0/G1-phase cells [11]. Zhao et al.'s study proposed that $\alpha 2\delta 1$ was involved in amplitude-encoding signals that maintain the properties of HCC TICs and showed that inhibition of this calcium signaling could be a therapeutic strategy for HCC [9].

Other authors showed that there were other types of cancer that contain $\alpha 2\delta 1+$ cells such as small-cell lung cancer, laryngeal squamous cell carcinoma, and gastric cancer, with nearly the same stem cell-like properties [12], resistant to chemotherapy [13], and overexpression of ERK1/2 [14]. Also, they showed that inhibition of $\alpha 2\delta 1$ may serve as a new promising therapeutic target for these CSCs [15, 16].

The standard of care for surveillance of HCC is by ultrasound (US) with or without AFP every 6 (4–8) months in a cirrhotic patient [17] and the cornerstone in diagnosis is multiphasic CT and/or MRI. The problem of US is its low sensitivity (46%) especially in detecting small lesions [18] dropping to 33% in obese patients with BMI >30 [19]; also, AFP had a sensitivity of around 60% [17]. However, CT and MRI had higher sensitivity (69% and 84%, respectively) and specificity (94%), yet not recommended to be used as surveillance tools [17, 20]. That necessitates finding a new tool that can detect HCC early with high sensitivity and specificity non-invasively at low cost. From the aforementioned biological properties of $\alpha 2\delta 1$ subunit in tumor-initiating cells in HCC, we assume that it may have also a good diagnostic value.

Methods

The present study was conducted at the Ain Shams University Hospitals, Cairo, in the duration between December 2018 and December 2019. A case-control study was designed with a total of 90 subjects enrolled and divided into 3 groups as follow: 40 patients with HCC, 40 patients with cirrhosis with normal AFP and no radiological evidence of HCC, and 10 apparently healthy individuals as a control group, with no previous history of liver and other chronic disease or malignant diseases and negative for hepatitis viral markers. HCC patients were diagnosed according to definitive criteria in

multiphasic CT/MRI showing arterial enhancement and delayed venous washout; any subject that had a history of malignancy of other organs, inflammatory diseases, or hematological diseases was excluded. The study protocol was approved by the local ethics committee of Ain Shams University. Informed consent was taken from both the patients and control group subjects after explaining the aim and concerns of the study. For all subjects, the following were done: a collection of relevant clinical data, basic laboratory tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, serum albumin, AFP, INR, CBC, kidney function test, and hepatitis serology (HBsAg and HCV Ab) levels were measured using commercially available kits.

Blood sample collection, storage, and analysis

The serum sample was collected from all study subjects. Collected serum was allowed to clot for 10–20 min at room temperature. Centrifuge was at 2000–3000 rpm for 20 min. When the analysis was not performed immediately, the samples were frozen and stored at 80 °C until use. The serum concentration of the $\alpha 2\delta 1$ subunit was measured using an ELISA kit according to the manufacturer's guidelines (MyBioSource, USA).

Statistical analysis

IBM SPSS Advanced Statistics version 21 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The quantitative variables were presented as the mean \pm standard deviation or median percentile of the interquartile range (25th to 75th) for non-parametric data analysis done by the Mann-Whitney test. For parametric comparison between two/all study groups, Student *t* test or ANOVA was used. Spearman rho was used for correlation. The receiver operating characteristic (ROC) curve was applied to identify the best cut-off values for, the $\alpha 2\delta 1$ subunit. *P* values of <0.05 were considered significant.

Results

Baseline characteristics of the studied subjects

The mean age of the whole studied sample was 50.5 years (± 11.75) and the number distribution of gender among all subjects was 60 (66.6%) male and 30 (33.3%) female. The etiology of liver disease in the studied sample: 12 patients (15%) were HBV positive, where 45 patients (56.25%) were HCV positive, the remaining 23 patients (28.75%) were non-viral causes of liver disease (Table 1). Liver fibrosis was assessed by FIB-4 with a median value of 3.01 (IQR 2.03) across both groups. The number of smokers across both HCC and cirrhotic groups was 26 (32.5%); 54 (67.5%) were non-smokers. Regarding associated

Table 1 Socio-clinical criteria of the study population and relationship with $\alpha 2\delta 1$

	Cirrhosis (N, 40)	HCC (N, 40)	Control (N, 10)	Test
Age, mean (SD)	55.62 (\pm 4.67)	51 (\pm 11.62)	28.1 (\pm 3.38)	$r = 0.14^*$, $P = 0.19$
Gender				
Male (%)	29 (72.5%)	24 (40%)	7 (70%)	$t = 0.70$
Female (%)	11 (27.5%)	16 (40%)	3 (30%)	$P = 0.48$
FIB 4				
Min-max	1.48–6.33	1.33–16	-	$r = -0.03^*$
Median (IQR)	3.12 (1.52)	2.7 (2.55)	-	$P = 0.77$
HBV	1 (2.5%)	11 (27.5%)	-	
HCV	28 (70%)	17 (42.5%)	-	$F = 2.00^{**}$
Non-viral	11 (27.5%)	12 (30%)	-	$P = 0.14$

*Spearman Rho

**ANOVA test

comorbidities in both groups, 16 patients (20%) had hypertension and 29 (36.25%) had diabetes (Table 3). Child-Pugh A in this studied sample was 3 patients (7.5%) in the cirrhotic group and 12 patients (30%) in the HCC group. Child-Pugh B was 36 patients (90%) and 22 patients (55%) in cirrhotic and HCC groups, respectively. Child-Pugh C in the cirrhotic group was 1 patient (2.5%) while in the HCC group were 6 patients (15%). The median value AFP in the cirrhotic group was 4.9 IU/mL (IQR 2.7) while its median value in HCC group was 485 IU/mL (IQR 463). The mean value of the MELD score in cirrhotic

groups was 18.5 ± 2.3 , while in the HCC group the mean value was 16.6 ± 5.3 .

As regards radiological characteristics of HCC, considering size, 12 lesions (30%) were < 3 cm, 21 lesions (52.5%) between 3 and 5 cm, and 7 lesions (17.5%) > 5 cm. Considering the number of lesions, 22 lesions were single (55%) and 18 (45%) were multiple; no infiltrative HCC pattern nor portal vein invasion were present in the study group.

There were statistically significant differences between the cirrhotic group and HCC group as regard ALT,

Table 2 Lab results in the study sample and relationship with $\alpha 2\delta 1$

	Cirrhosis (N, 40)	r^* , P value	HCC (N, 40)	r^* , P value	Mann-Whitney#, P value
ALT IU/L		0.19		- 0.19	
Min-max	17–54	0.23	8–90	0.25	$U = 434.5$
Median (IQR)	24 (10)		49.5 (38.25)		$P < 0.01$
AST IU/L		0.28		- 0.25	
Min-max	19–46	0.08	10–118	0.12	$U = 490$
Median (IQR)	28.5 (10.75)		44 (33.5)		$P < 0.01$
T.BIL mg/dL		0.27		- 0.05	
Min-max	1.2–2.7	0.09	0.4–9.1	0.75	$U = 737.5$
Median (IQR)	2.05 (1.5)		2.1 (2.28)		$P = 0.53$
Albumin g/dL		- 0.02		0.19	
Min-max	1.7–3.9	0.89	2–4.9	0.24	$U = 347$
Median (IQR)	2.75 (0.7)		3 (0.28)		$P < 0.01$
INR		0.29		- 0.01	
Min-max	1.4–2.3	0.07	1.1–2.8	0.94	$U = 515$
Median (IQR)	1.89 (0.18)		1.7 (0.6)		$P < 0.01$
Platelet $10^3/\mu\text{L}$		- 0.31		- 0.01	
Min-max	56–156	0.05	45–198	0.94	$U = 795$
Median (IQR)	100 (39)		99 (42.75)		$P = 0.962$

*Spearman Rho test between lab and $\alpha 2\delta 1$

#Comparison between cirrhosis and HCC as regards labs

AST, serum albumin, and INR, while there were no statistically significant differences between the two groups as regard total bilirubin and platelet (Table 2).

$\alpha 2\delta 1$ subunit levels among studied subjects

The serum levels of $\alpha 2\delta 1$ subunit were significantly different across all 3 groups ($F = 99.65$, $P < 0.001$) with the highest value in HCC group (mean = 19.53 ± 6.87 ng/dL, 95% CI 17.33–21.72), then the cirrhotic group (mean = 6.24 ± 2.64 ng/dL, 95% CI 5.39–7.09) and the least value in control group (mean = 0.67 ± 0.48 ng/dL, 95% CI 0.32–1.02). Also, post hoc LSD test showed significant difference between control group and both the cirrhotic and HCC groups ($P = 0.002$ and 0.001 , respectively) and between the HCC and cirrhotic groups ($P = 0.001$).

Evaluation of serum $\alpha 2\delta 1$ subunit in-clinic- laboratory feature and etiology of liver disease

The mean levels of $\alpha 2\delta 1$ in males were 11.04 ± 8.45 ng/dL while in females were 12.51 ± 9.67 ng/dL; this showed no statistically significant differences across all study subjects. Also, there was no correlation with age or FIB-4 (Table 1). The mean value of $\alpha 2\delta 1$ in HCV patients was 11.57 ± 8.96 ng/dL and in HBV patients was 16.93 ± 6.04 ng/dL, while in the non-viral group was 13.35 ± 8.09 ng/dL; this was nonsignificant (Table 1). There were no statistically significant differences between the mean levels of $\alpha 2\delta 1$ as regarding smoking status, diabetes, and hypertension within each group (cirrhotic and HCC) (Table 3). AFP in both the HCC and cirrhotic groups showed strong positive correlation with $\alpha 2\delta 1$ ($r = 0.85$, $P < 0.001$). However, there was no statistically significant difference between the $\alpha 2\delta 1$ subunit as regards laboratory tests (ALT, AST, total bilirubin, albumin, and INR) within each group (Table 2). Despite platelets showing significant correlation in the cirrhotic group, this correlation was a very weak negative correlation ($r = -0.31$, $P = 0.05$).

Evaluation of serum $\alpha 2\delta 1$ subunit as a potential diagnostic marker for HCC

To further investigate the diagnostic value of serum $\alpha 2\delta 1$ subunit in HCC, ROC curves were constructed revealed excellent diagnostic value with AUC = 0.974 and $P < 0.0001$. The serum level of the $\alpha 2\delta 1$ subunit at the cut-off value ≥ 8.75 ng/dL showed a sensitivity of 95%, a specificity of 80%, a positive predictive value (PPV) of 82.6%, and a negative predictive value (NPV) of 94.1% with accuracy = 87.5%. However, its level at a cut-off 12 ng/dL showed a sensitivity of 85%, a specificity of 100%, PPV of 100%, and NPV of 87% with accuracy = 92.5%

Discussion

Many studies showed an expression of $\alpha 2\delta 1$ subunit in different types of malignancies such as pancreatic [21], ovarian [22], and lung tumors [14] and demonstrated a potential resistance to chemotherapy and may carry bad prognosis. Han et al. as well identified a subpopulation of TICs expressing $\alpha 2\delta 1$ subunit, which is essential for the activation of calcium influx that controls the TIC ability of HCC by an antibody against Hep-12 cells [23]. Since the liver cancer is one of the highest malignancies in Egypt and carries a bad prognosis, early diagnosis and management are essential [24].

In the present study, the serum level of $\alpha 2\delta 1$ subunit was significantly higher in the HCC group than both the cirrhotic and control group, in agreement with the study conducted by Zhao et al. who first reported $\alpha 2\delta 1$ subunit in HCC; on 86 freeze resected HCC tumor sample compared to the nearby para-cancerous area for the presence of 1B50-1 positive staining (a monoclonal Ab against $\alpha 2\delta 1$ subunit), they found significantly higher percent in tumor cells (72.1%) than non-tumor cells (46.5%), $P = 0.0006$ [9].

The study conducted by Badr et al. was in agreement with our results as they showed significantly higher levels of serum $\alpha 2\delta 1$ subunit in HCC group than both the cirrhotic and control ($P < 0.05$) with mean values as

Table 3 Comorbidity distribution among the main two groups and level of $\alpha 2\delta 1$

	Cirrhosis				HCC			
	N, 40	Mean (SD)	t	P	N, 40	Mean (SD)	t	P
Smoking								
Yes, N (%)	15 (37.5)	6.88 (3.03)	-1.12	0.27	11 (27.5)	16.66 (7.31)	-1.57	0.13
No, N (%)	25 (62.5)	5.86 (2.37)			29 (72.5)	20.61 (6.50)		
Diabetes								
Yes, N (%)	6 (15)	7.00 (2.92)	-0.75	0.46	23 (57.5)	20.74 (6.39)	-1.31	0.20
No, N (%)	34 (85)	6.11 (2.62)			17 (42.5)	17.89 (7.36)		
Hypertension								
Yes, N (%)	1 (2.5)	5	0.47	0.64	15 (37.5)	19.51 (7.35)	0.02	0.99
No, N (%)	39 (97.5)	6.28 (2.68)			25 (62.5)	19.54 (6.73)		

20.12 ± 3.7 ng/mL, 10.41 ± 3.4 ng/mL, and 10.2 ± 2.98 ng/mL, respectively. However, they did not find a significant difference between the cirrhosis and control groups and this was against our finding as we found significantly lower levels in the control than cirrhosis ($P = 0.002$); this may be attributed by the sample size used in their study [25].

In work-related to Zhao et al.'s study, Han and his team performed the expression level of $\alpha 2\delta 1$ mRNA in 85 of the resected HCC and para-cancerous area found that there were no significant differences between its level and clinicopathological features (age, gender, and cirrhotic vs non-cirrhotic) [23]; this was as our findings.

Serum $\alpha 2\delta 1$ subunit level was not significantly different in associated comorbidity as smoking, DM, and HTN or viral (HBV and HCV) or non-viral etiology of liver disease.

Badr et al. who first describe serum level of $\alpha 2\delta 1$ subunit as a potential marker for HCC found that the sensitivity and specificity at level 14.22 ng/dL are 100% and 96%, respectively, with PPV 98%, NPV 100%, and accuracy 98.7% [25]. Per that, we found a sensitivity of 95% and specificity of 80% at a level of ≥ 8.75 ng/dL with PPV of 82.6%, NPV of 94.1%, and accuracy of 87.5%; this indicates that serum level of $\alpha 2\delta 1$ subunit may be used as a good biomarker for diagnosis of HCC with high sensitivity regardless of the different etiologies of liver disease as shown in our results. Besides, we demonstrated a higher level that yields more specificity and higher diagnostic potentially of $\alpha 2\delta 1$ subunit was that at a cut-off of 12 ng/dL; sensitivity and specificity were 85% and 100%, respectively, with 100% PPV, 87% NPP, and 92.5% accuracy.

Some authors found that the $\alpha 2\delta 1$ subunit may have a role in prognosis and recurrence of HCC. Zhao and his team showed that the presence of positive staining of anti $\alpha 2\delta 1$ subunit antibodies cells in the para-cancerous tissues did correlate significantly with hepatic very rapid recurrence, and a lower rate of 4-year overall survival post-surgery ($P = 0.00004$ and 0.00005 , respectively). Also, Han et al. identified that high level of $\alpha 2\delta 1$ mRNA is an independent risk factor of poor survival for HCC patients (relative risk = 2.66, $P = 0.005$) [9, 23]

Conclusion

The study showed serum $\alpha 2\delta 1$ subunit as a potential non-invasive marker with excellent sensitivity for diagnosis of HCC regardless of the etiology of liver disease and not affected by common comorbidity as smoking, diabetes, and hypertension.

Abbreviations

AFP: Alfa fetoprotein; ALT: Alanine aminotransferase; AML: Acute myeloid leukemia; ANOVA: Analysis of variance; AST: Aspartate aminotransferase; AUC: Area under the curve; CSCs: Cancer stem cells; CT: Computed

tomography; DM: Diabetes; ELISA: Enzyme-linked immunosorbent assay; ERK: Extracellular signal-regulated kinases; FIB-4: Fibrosis index based on four factors; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HTN: Hypertension; INR: International normalized ratio; IQR: Interquartile range; LSD: Least significant difference; MELD: Model for End-Stage Liver Disease; MRI: Magnetic resonant image; mRNA: Messenger RNA; NOD: Non-obese diabetic; NPP: Negative predictive value; PPV: Positive predictive value; ROC: Receiver operating characteristic; SAN: Sinoatrial node; SCID: Severe combined immune deficiency; TICs: Tumor-initiating cells; US: Ultrasonography

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Authors' contributions

All authors read and approved the final manuscript. AA had selected the idea and had made the final revision of data. EE did the data gathering. HE did data analysis and manuscript preparation. EB and AK did study design and revision of the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All individual included in the study signed an informed written consent to participate. All procedures performed in this study were in accordance with the standards of the Ethical Research Committee of Ain Shams University (reference number not applicable).

Consent for publication

Not applicable

Competing interests

None

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