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Role of annexin A2 and osteopontin for early diagnosis of hepatocellular carcinoma in hepatitis C virus patients

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Abstract

Background: Liver cancer is the fifth most common cancer and the second most frequent cause of cancer-related death globally. Early stages of hepatocellular carcinoma (0&A) can be treated with curative procedures. The aim of this work was to evaluate the role of annexin A2 and osteopontin for early diagnosis of hepatocellular carcinoma in hepatitis C virus patients.

Methods: The study was carried out on 80 patients classified into two groups. Group A had 40 chronic hepatitis C patients without hepatocellular carcinoma, while group B had 40 chronic hepatitis C patients with early hepatocellular carcinoma (stages; 0&A). All patients were subjected to thorough history taking, clinical examination, liver function tests, renal function tests, serum alpha-fetoprotein, serum osteopontin, and serum annexin A2.

Results: Serum alpha-fetoprotein was found to be statistically significantly higher in patients with the hepatocellular carcinoma group than the chronic hepatitis C group. The ROC curve for alpha-fetoprotein for detection of HCC was significant, its diagnostic performance was 0.818* ($p < 0.001$*), and the cutoff point for predicting the probability for HCC was 6.0 (ng/ml) with sensitivity of 77.50%, specificity of 82.50%, positive predictive value of 81.60%, negative predictive value of 78.6%, and accuracy of 80%. Serum osteopontin was found to be statistically significantly higher in patients from the hepatocellular carcinoma group than the chronic hepatitis C group. The ROC curve for osteopontin was significant, its diagnostic performance was 0.739* ($p < 0.001$*), the cutoff point was 13.2 (ng/ml) with sensitivity of 65.0%, specificity of 90.0%, positive predictive value of 86.70%, negative predictive value of 72.0%, and accuracy of 77.0%. Serum annexin A2 was found to be statistically significantly higher in patients from the hepatocellular carcinoma group than the chronic hepatitis C group. The ROC curve for annexin A2 was significant, its diagnostic performance was 0.927* ($p < 0.001$*), the cutoff point was 10.1(ng/ml) with sensitivity of 85.0%, specificity of 85.0%, positive predictive value of 85.0%, negative predictive value of 85.0%, and accuracy of 85.0%.

Conclusions: Osteopontin had better specificity but lower sensitivity than serum alpha-fetoprotein for early diagnosis of hepatocellular carcinoma. Annexin A2 had better diagnostic sensitivity and specificity than alpha-fetoprotein for early diagnosis of hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, Hepatitis C, Annexin A2, Osteopontin

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Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. In a recent study carried out by the National Population-Based Cancer Registry Program in Egypt, liver cancer ranked first among cancers in Egyptian males (33%), second after breast cancer in females (13.5%), and first in both sexes together (23.8%). The distribution of liver cancer followed the distribution of hepatitis C Virus (HCV) [1].

Chronic hepatitis C (CHC) is a major risk factor for the development of cirrhosis and subsequent hepatocellular carcinoma (HCC) [2]. Although the emergence of highly effective direct-acting antivirals for HCV is expected to reduce the incidence of HCV-related HCC, the achievement of a sustained virological response (SVR) does not eliminate the occurrence of HCC, in patients already having liver cirrhosis [3].

Early stage of HCC can be treated with potentially curative procedures, such as resection, percutaneous ablation, and transplantation. Thus, there is an urgent need to identify better tools for detecting and characterizing these lesions in order to improve clinical outcome of HCC patients [4]. Recent data have shown a 5-year survival in 80–90% of patients with solitary HCC smaller than 2 cm treated with resection [5]. Median survival of patients with early HCC reaches 50–70% at 5 years after resection, liver transplantation, or local ablation [6].

Because of the asymptomatic nature of early HCC as well as the lack of its effective screening strategies; most patients present with an overt advanced disease [7]. Approximately 30% of HCC cases with normal serum alpha-fetoprotein (AFP) levels are diagnosed before the appearance of clinical manifestations [8].

Unlike other solid malignancies, the coexistence of inflammation and cirrhosis makes an early diagnosis and prognostic assessment of HCC much more difficult [9]. In addition, the conventional tests of hepatic function do not distinguish HCC from cirrhosis, and thus they contribute little to the diagnosis of such tumor [10]. New sensitive and specific markers are needed for early identification to improve clinical outcomes of HCC patients.

Osteopontin (OPN) is an integrin-binding phosphoprotein secreted at low levels by biliary epithelial cells and is overexpressed in many cancers, including lung, breast, colon, and HCC. OPN interacts with integrin and CD44 family of receptors to mediate cell signaling that controls inflammatory processes (hepatitis), HCC tumor progression and development of metastasis. Plasma OPN levels are significantly higher in HCC patients than healthy controls and in patients with chronic liver disease [11, 12].

Annexin A2 (ANXA2) is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies and has emerged as an attractive candidate receptor for increased plasmin generation on the tumor cell surface [13]. ANXA2 is almost undetectable in the normal liver and in chronic hepatitis tissues [13].

The aim of this work was to evaluate the role of annexin A2 and osteopontin for early diagnosis of hepatocellular carcinoma in hepatitis C virus patients.

Methods

The study was carried out on 80 patients classified into two groups. Group A 40 CHC patients without HCC while, group B 40 CHC patients with early HCC (stages 0 and A). HCC is classified according to the Barcelona Clinic Liver Cancer (BCLC) staging system into five stages (O, A, B, C, and D). Patients with other malignancy, diabetes mellitus, renal failure, any bony lesions, other viral hepatitis, and stages B, C, and D (late HCC) are excluded. The study was approved by the Research Ethical Committee of Alexandria University and a written informed consent was obtained from all patients.

All patients were subjected to detailed history, thorough clinical examination, complete blood picture, fasting blood glucose, blood urea and serum creatinine, serum albumin, serum bilirubin, prothrombin activity, serum alanine transaminase (ALT), serum aspartate transaminase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), HCVAb, HCVRNA, HBsAg, HBcAb, serum AFP, serum level of OPN using ELISA, serum level of ANXA2 using ELISA, abdominal ultrasonography, and triphasic computerized tomography of the liver.

Blood samples from 40 HCC patients were collected at the time of HCC diagnosis and prior to therapy, and isolated plasma samples were stored at − 80 °C until measurements of OPN and ANXA2 were conducted. Blood samples from 40 patients with CHC but without HCC were obtained during the same time period as the blood samples from HCC patients.

Osteopontin assay: human OPN enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Co., USA); this kit was based on sandwich ELISA technology. Anti-OPN antibody was pre-coated onto 96-well plates, and the biotin conjugated anti-OPN antibody was used as detection antibodies.

Annexin A2 assay: human ANXA2 enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Co., USA); this kit was based on sandwich ELISA technology. Anti-ANXA2 antibody was pre-coated onto 96-well plates, and the biotin conjugated anti-ANXA2 antibody was used as detection antibodies.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY, IBM Corp) Qualitative data were described using number and

percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation, and median. Significance of the obtained results was judged at the 5% level.

Results

The demographic features of the included patients were as follows: 50% were males and 50% were females with an age range from 45 to 64 years in group A and 80% were males and 20% were females with an age range from 50 to 70 years in group B. There was significant difference between the two groups regarding age and sex. All patients were cirrhotic, 90% of patients were child A and 10% were child B in both groups. There was no significant difference between the two studied groups as regards the child-Pugh score ($^{FE}p = 1.000$). As regards HCC staging in group B, 50% of patients were stage 0 and the other 50% were stage A.

As regards clinical manifestations, there was statistically significant difference between the two groups as regards anorexia ($p < 0.001^*$), fatigue ($p = 0.002^*$), dyspepsia ($p < 0.001^*$), and cachexia ($p = 0.001^*$); all these parameters were higher in group B than group A (Table 1).

As regards liver enzymes, AST and GGT were statistically significantly higher in the HCC group than the chronic hepatitis C group. Liver function tests showed serum albumin and prothrombin activity were statistically significantly lower in the HCC group than the chronic hepatitis C group. Serum bilirubin was statistically significantly higher in the HCC groups than the chronic hepatitis C group. The mean value of serum urea and serum creatinine were significantly higher in the HCC group than the chronic hepatitis C group ($p = 0.001^*$, $p = 0.001^*$).

As regards different tumor markers, serum AFP was significantly higher in group B than group A ($p < 0.001^*$), serum OPN was significantly higher in group B than group A ($p < 0.001^*$) and serum ANXA2 was significantly higher in group B than group A ($p < 0.001^*$) (Table 2).

As regards the correlation between the OSP level and the different variables in the HCC group, there was significant positive correlation between OPN level and serum bilirubin (total and direct) ($p = 0.046$*, 0.009*, respectively). However, no significant correlation was noted between OPN level and other variables.

As regards the correlation between the ANXA2 level and the different variables in the HCC group, there was significant positive correlation between ANXA2 level and alkaline phosphatase ($p = 0.008$*) and a significant relation was noted between ANXA2 level and fatigue, abdominal pain, and vomiting ($p = 0.032$, 0.033, and 0.023, respectively) (Fig. 1). However, no significant correlation was noted between ANXA2 level and other variables.

As regards sensitivity and specificity of AFP, the ROC curve was significant, its diagnostic performance was 0.818^* ($p < 0.001^*$), the cutoff point was 6.0 (ng/ml) with sensitivity of 77.50%, specificity of 82.50%, positive predictive value of 81.60%, negative predictive value of 78.6%, and accuracy of 80%. As regards sensitivity and specificity of OPN, the ROC curve was significant, its diagnostic performance was 0.739^* ($p < 0.001^*$), the cutoff point was 13.2 (ng/ml) with sensitivity of 65.0%,

Table 1 Comparison between the two studied groups according to clinical manifestations

Clinical manifestations	Group A ($n = 40$)		Group B ($n = 40$)		χ^2	P
	No.	%	No.	%		
Anorexia	21	52.5	38	95.0	18.660^*	$< 0.001^*$
Fatigue	24	60.0	36	90.0	9.600^*	0.002^*
Abdominal pain	20	50.0	20	50.0	0.000	1.000
Fever	6	15.0	8	20.0	0.346	0.556
Change in the color of urine and eyes	2	5.0	2	5.0	0.000	$^{FE}p = 1.000$
Dyspepsia	25	62.5	38	95.0	12.624^*	$< 0.001^*$
Vomiting	6	15.0	2	5.0	2.222	$^{FE}p = 0.263$
Weight loss	4	10.0	10	25.0	3.117	0.077
Cachexia	4	10.0	17	42.5	10.912^*	0.001^*
Jaundice	2	5.0	2	5.0	0.000	$^{FE}p = 1.000$
Splenomegaly	16	40.0	20	50.0	0.808	0.369
Hepatomegaly	9	22.5	8	20.0	0.075	0.785

FE Fisher Exact
χ^2 Chi square test
p *p* value for comparing between the studied groups
*Statistically significant at $p \leq 0.05$

Table 2 Comparison between the two studied groups according to different tumor markers

	Group A ($n = 40$)	Group B ($n = 40$)	U	p
AFP (ng/ml)				
Min–max	1.0–51.0	1.10–589.0	291.50*	< 0.001*
Mean ± SD	8.85 ± 14.51	90.49 ± 132.65		
Median	3.85	0.0		
OPN (ng/ml)				
Min–max	5.10–29.30	6.10–80.40	417.0*	< 0.001*
Mean ± SD	11.08 ± 4.20	22.87 ± 19.31		
Median	10.30	16.10		
ANXA2 (ng/ml)				
Min–max	5.40–16.60	8.90–40.80	116.50*	< 0.001*
Mean ± SD	8.34 ± 2.34	14.98 ± 5.93		
Median	8.05	13.45		

U Mann Whitney test
p *p* value for comparing between the studied groups
*Statistically significant at $p \leq 0.05$

specificity of 90.0%, positive predictive value of 86.70%, negative predictive value of 72.0%, and accuracy of 77.0%. As regards sensitivity and specificity of ANXA2, the ROC curve was significant, its diagnostic performance was 0.927* ($p < 0.001$*), the cutoff point was 10.1 (ng/ml) with sensitivity of 85.0%, specificity of 85.0%, positive predictive value of 85.0%, negative predictive value of 85.0% and accuracy of 85.0% (Table 3) (Fig. 2).

As regards combination of different tumor markers, using a combination of OPN at 13.2 (ng/ml) and AFP at 6 (ng/ml), increased the specificity to 85%, but decreased the sensitivity to 70% and the accuracy to 77.50%. Using a combination of ANXA2 at 10.1 (ng/ml) and AFP at 6 (ng/ml) increased the sensitivity to 82.5%, the specificity to 92.5%, and the accuracy to 87.5%. Using a combination of OPN at 13.2 (ng/ml) and ANXA2 at 10.1 (ng/ml), increased the sensitivity to 92.5%, the specificity to 92.5%, and the accuracy to 92.5%. Using a combination of OPN at 13.2 (ng/ml), ANXA2 at 10.1 (ng/ml), and AFP at 6 (ng/ml), increased sensitivity to 87.5%, specificity to 92.5%, and accuracy to 90.0% (Table 4) (Fig. 3).

Discussion

In HCC, an elevated plasma level of OPN is regarded as a potential prognostic biomarker and overexpression of OPN is closely correlated with intrahepatic metastasis, early recurrence, and a worse prognosis [14]. ANXA2 is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies [13]. So the purpose of this study was to evaluate the role of ANXA2 and OPN for early diagnosis of HCC in hepatitis C virus patients.

In our study there were statistically significant differences in AST, GGT, serum bilirubin (total and direct), international normalized ratio, and serum albumin levels between the HCC group and the chronic hepatitis C group, all these parameters were higher in the HCC

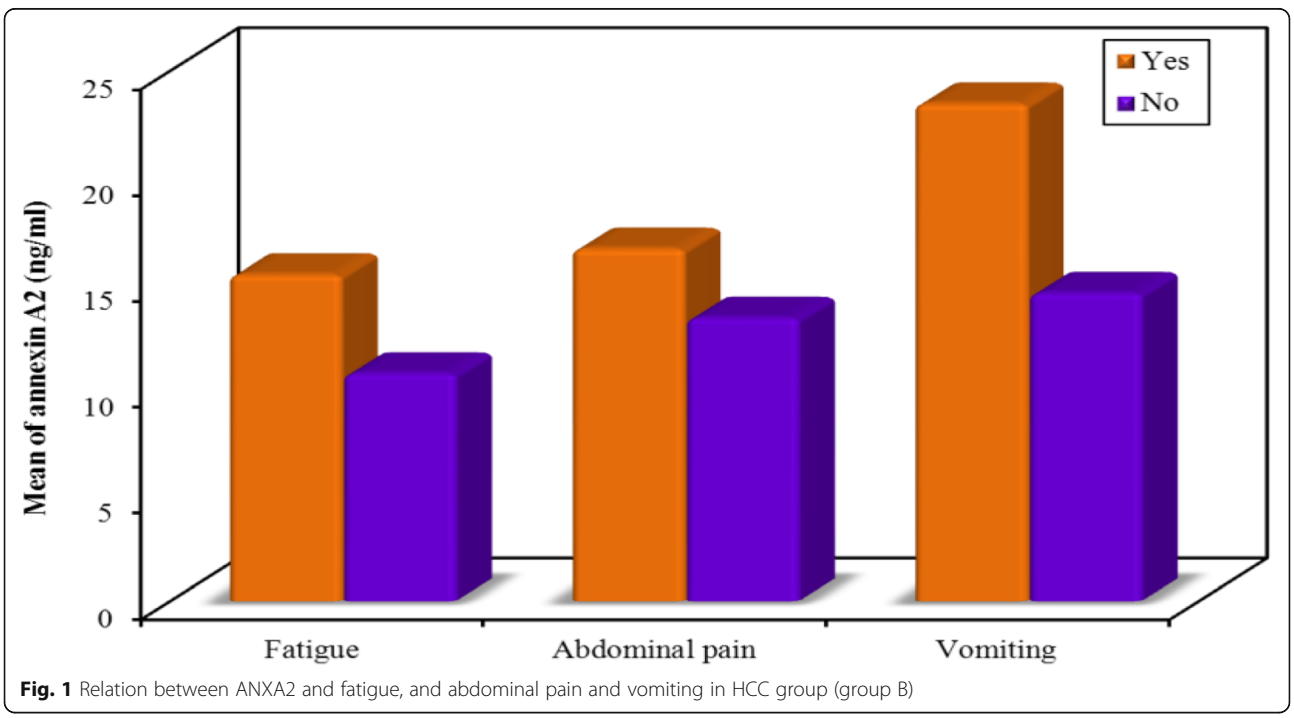

Fig. 1 Relation between ANXA2 and fatigue, and abdominal pain and vomiting in HCC group (group B)

Table 3 Agreement (sensitivity, specificity) for AFP, OPN, and ANXA2 to diagnose HCC

	AUC	*P*	95% C.I.	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP (ng/ml)	0.818*	< 0.001*	0.719–0.916	> 6#	77.50	82.50	81.6	78.6	80.0
OPN (ng/ml)	0.739*	< 0.001*	0.621–0.857	> 13.2#	65.0	90.0	86.7	72.0	77.50
ANXA2 (ng/ml)	0.927*	< 0.001*	0.872–0.982	> 10.1#	85.0	85.0	85.0	85.0	85.0

AUC area under a curve, *CI* confidence intervals, *NPV* negative predictive value, *PPV* positive predictive value
*Statistically significant at $p \leq 0.05$
#Cut off was done by using Youden index

group than the chronic hepatitis C group which is in agreement with the results by Fuoad et al. who explained this difference by the progression of the underlying liver cirrhosis caused by HCC with a subsequent decreased albumin and protein synthesis and poor utilization of vitamin K in advanced parenchymal liver disease [15].

Our results showed that serum AFP was found to be significantly higher in the HCC group in comparison to the chronic hepatitis C group and the cutoff point for predicting the probability for HCC was 6.0 (ng/ml) with sensitivity of 77.50%, specificity of 82.50%, positive predictive value of 81.60%, negative predictive value of 78.6%, and accuracy of 80%. These results are comparable with those of Marrero et al. who performed a large case-control study involving 836 patients. There was a significant difference between the early HCC and the cirrhotic patient group as regards AFP [16].

Also, these results were in agreement with that of El-Tayeh et al. who explained his results by an increase in the selective transcriptional activation of the AFP gene in malignant hepatocytes, which resulted in the increased secretion of AFP during the development of HCC [17]. On the other hand, these results are incompatible with El-Gezawy et al. who postulated that there was a similarity and no significant difference between the early HCC and cirrhotic groups as regards AFP [18].

In the present study, OPN level was found to be significantly higher in HCC patients than chronic hepatitis C patients. These results are compatible with the study performed by Shang et al. who reported that OPN was significantly higher in early HCC patients than cirrhotic patients [19].

In the HCC group, there was significant positive correlation between OPN level and serum bilirubin (total and direct); these results suggested that the OPN was correlated with the progression of liver disease. However, no significant correlation was noted between OPN level and other parameters. The correlation coefficient between serum OPN and AFP values was not significant. Hodeib et al. reported that OPN levels were significantly

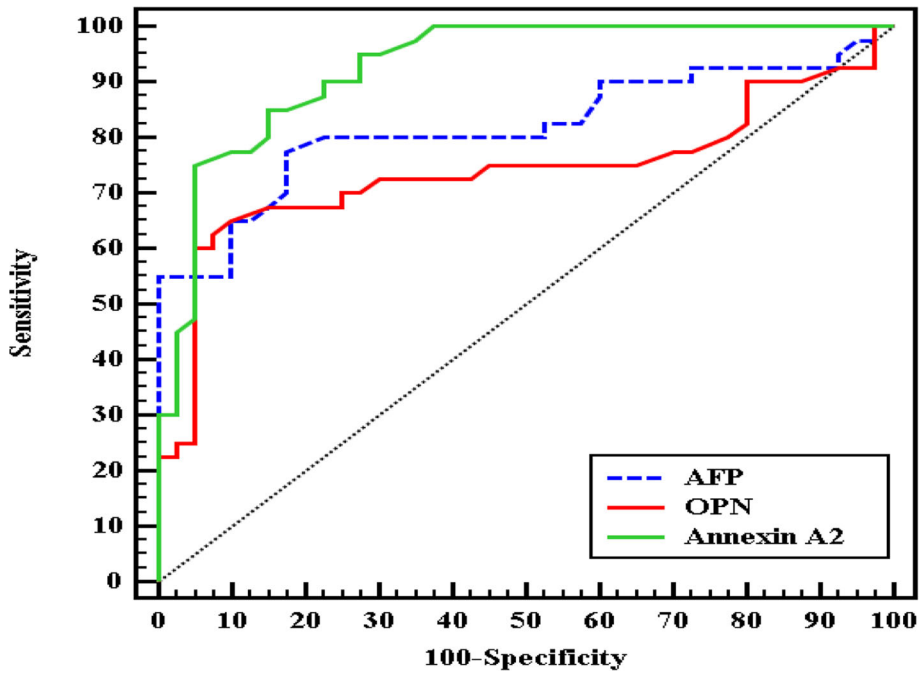

Fig. 2 ROC curve for AFP, OPN, and ANXA2 to diagnose HCC

Table 4 Agreement (sensitivity, specificity) for combination of different tumor markers to diagnose HCC

	AUC	*P*	95% C.I.	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP & OPN	0.853*	< 0.001*	0.763 – 0.943	70.0	85.0	82.35	73.91	77.50
AFP & ANXA2	0.958*	< 0.001*	0.920 – 0.996	82.50	92.50	91.67	84.09	87.50
OPN & ANXA2	0.954*	< 0.001*	0.904 – 1.004	92.50	92.50	92.50	92.50	92.50
AFP & OPN & ANXA2	0.964*	< 0.001*	0.925 – 1.003	87.50	92.50	92.11	88.10	90.0

AUC area under a curve, *CI* confidence intervals, *NPV* negative predictive value, *PPV* positive predictive value
*Statistically significant at $p \leq 0.05$

correlated with AFP levels but no significant correlation between OPN level and other parameters in HCC patients [20].

The ROC curve for OPN for detection of HCC was significant, the cutoff point was 13.2 (ng/ml) with sensitivity of 65.0% and specificity of 90.0%. These results are compatible with the study performed by Lee et al. who reported that the accuracy achieved by using plasma OPN levels for diagnosis of HCC was inferior to the accuracy achieved using AFP. At a cutoff value of 6 (ng/ml), plasma AFP showed high sensitivity (63.9%) and specificity (95%). Plasma OPN at a cutoff value of 557 (ng/ml) showed a high specificity (92.5%) but a lower sensitivity (26.1%) [21].

These results are incompatible with the study performed by Shang et al. who reported that OPN at higher threshold of 91 (ng/ml), its diagnostic performance higher than AFP (0.739, 0.680, respectively) for discriminating between early HCC and cirrhosis. OPN demonstrated 75% sensitivity and 62% specificity for early stage HCC, compared to 46% sensitivity and 93% specificity for AFP [19]. The exact reason for these differences as regards cutoffs is not clear, but these discrepancies may be in consequence of the different assay systems and conditions of sample collection used in different studies.

The binding of secreted OPN from HCV-infected cells to integrin$\alpha_v\beta_3$ and CD44 leads to elevation of reactive oxygen species and activation of Ca^{2+} signaling and downstream cellular kinases; all of which promote epithelial-mesenchymal transition, cell migration, and invasion to enhance tumor progression and metastasis in HCC [22]. The role of OPN in metastasis is more prominent because OPN expression facilitates recurrence and reduces patient survival after liver transplantation for HCC. Thus, OPN may be a useful marker for detecting early recurrence of HCC after surgery [23].

In the present study, ANXA2 level was found to be significantly higher in early HCC patients than chronic hepatitis C patients. These results are compatible with El-Gezawy et al. who pastulated that ANXA2 was significantly higher in early HCC patients than cirrhotic patients [18]. Shaker et al. also reported that ANXA2 was significantly higher in early HCC patients than chronic liver disease (CLD) patients [24].

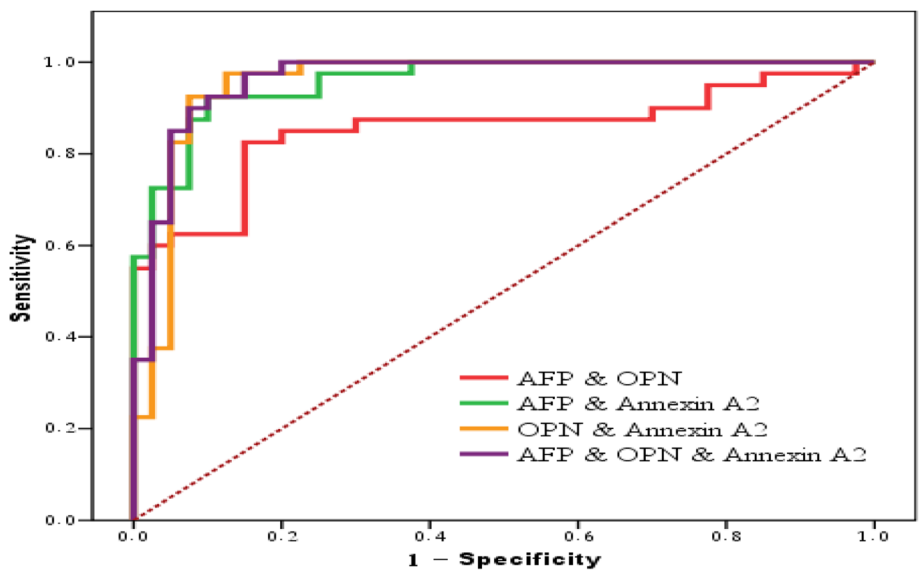

Fig. 3 ROC curve for combination of different tumor markers to diagnose HCC

Interestingly, in the HCC group, there was significant positive correlation between ANXA2 level and alkaline phosphatase and there was significant relation as regards fatigue, abdominal pain, and vomiting. However, no significant correlation was noted between annexin level and other parameters. There was no significant correlation between ANXA2 level and AFP; this agrees with the studies done by El-Gezawy et al. [18], Shaker MK et al. [24], and Sun et al. [13]. The correlation coefficient between serum ANXA2 and AFP values was not significant, indicating that measuring both markers (AFP and ANXA2) in serum can improve the diagnostic value.

The ROC curve for ANXA2 for detection of HCC was significant, the cutoff point was 10.1 (ng/ml) with sensitivity of 85.0% and specificity of 85.0%. These results are compatible with El-Gezawy et al. who reported for early stage HCC, ANXA2 (optimal cutoff of 24.99 IU/ml), higher sensitivity, and specificity (79.34% and 85.56% respectively) than those of AFP (optimal cutoff of 5.96 IU/ml) (67.78% and 59.85% respectively) [18]. The ANXA2 mRNA expression level was obviously, significantly higher and over expressed in HCC tissues rather than in the other patient groups. One explanation showed that ANXA2 synthesis is induced in transformed hepatocytes [25].

These results are also, more or less, compatible with the study performed by Shaker et al. who reported that ANXA2 was significantly higher in HCC patients than chronic liver disease patients, the cutoff point for predicting the probability for early HCC was 18 ng/mL, the diagnostic sensitivity was 74%, the specificity was 88%, the PPV was 92.5%, the NPV was 62.9%, and the efficacy was 78.7% which is higher than AFP (cutoff value was 19.8 ng/ml) as regards diagnostic sensitivity (70%), but similar to AFP as regards specificity, positive and negative predictive values, and efficacy [24].

Shaker et al. stated that there was a significant difference observed between patients with CLD and healthy people with respect to AFP, who declared that one of the limitations in the use of AFP for the diagnosis of HCC is its increase in patients who have hepatitis and CLD but who do not have HCC, but found that ANXA2 levels were highly and significantly increased in patients with HCC compared with the levels in patients with CLD and in controls; however, no statistical significance was found between patients with CLD and the healthy people with respect to ANXA2 expression [24].

This was explained by Zhang et al. who stated that the ANXA2 gene is upregulated in HCV-associated HCC [26]. In addition, Mohammad et al. stated that ANXA2 is rarely detected in either normal or chronic hepatic tissues but is over expressed at both the mRNA and protein levels HCC [27].

Wang et al. stated that, one of the possible mechanisms explaining the relationship between ANXA2 and HCC is promotion of HCC cell migration and invasion by ANXA2 pseudogene [28]. A recent study done by Lou et al. who reported that ANXA2 binds with Lung cancer associated transcript 1 (LUCAT1), which plays a key role in tumorigenesis, progression of HCC and a better therapeutic target for HCC patients. LUCAT1 inhibits the phosphorylation of ANXA2 and increase the secretion of plasminogen into plasmin [29].

Conclusions

OPN had better specificity but lower sensitivity, diagnostic performance, and accuracy than serum AFP for detection of early HCC in patients with CHC. ANXA2 was found to have better diagnostic performance, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy than AFP so it could be developed as an effective diagnostic and predictive marker for early HCC in patients with CHC. Using a combination of OPN and ANXA2 is associated with high sensitivity and specificity that could be a potential method for surveillance.

Abbreviations

AFP: Alpha-fetoprotein; ALP: Alkaline phosphatase; ALT: Alanine transaminase; ANXA2: Annexin A2; AST: Aspartate transaminase; BCLC: Barcelona Clinic Liver Cancer; CHC: Chronic hepatitis C; CLD: Chronic liver disease; ELISA: Enzyme-linked immunosorbent assay; GGT: Gamma glutamyl transferase; HCC: Hepatocellular carcinoma; HCV: Hepatitis C Virus; LUCAT1: Lung cancer associated transcript 1; OPN: Osteopontin; SVR: Sustained virological response

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Authors' contributions

AH is a Professor of Tropical Medicine at the Faculty of Medicine, Alexandria University, (the first author) and designed the work. FA is a Professor of Tropical Medicine at the Faculty of Medicine, Alexandria University, and revised the work. AD is a Professor of Clinical and Chemical Pathology at the Faculty of Medicine, Alexandria University, and interpreted the data of the work. EHE is an Assistant professor of Tropical Medicine at the Faculty of Medicine, Alexandria University, and revised the work. AIA is from the Tropical Medicine Department, Faculty of Medicine, Alexandria University, and is the corresponding author of the work. All authors have read and approved the manuscript.

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Availability of data and materials

Available upon request.

Ethics approval and consent to participate

The study was approved by the Research Ethical Committee of Faculty of Medicine, Alexandria University which is constituted and operates according to ICH GCP guidelines and applicable local and institutional regulations and guidelines which govern EC operation. IRB no. 00007555, FWA no. 00015712, EC serial protocol number 020912.
A written informed consent was obtained from all patients and human data not applicable.

Consent for publication

Not applicable

Competing interests
The authors declare that they have no competing interests.

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