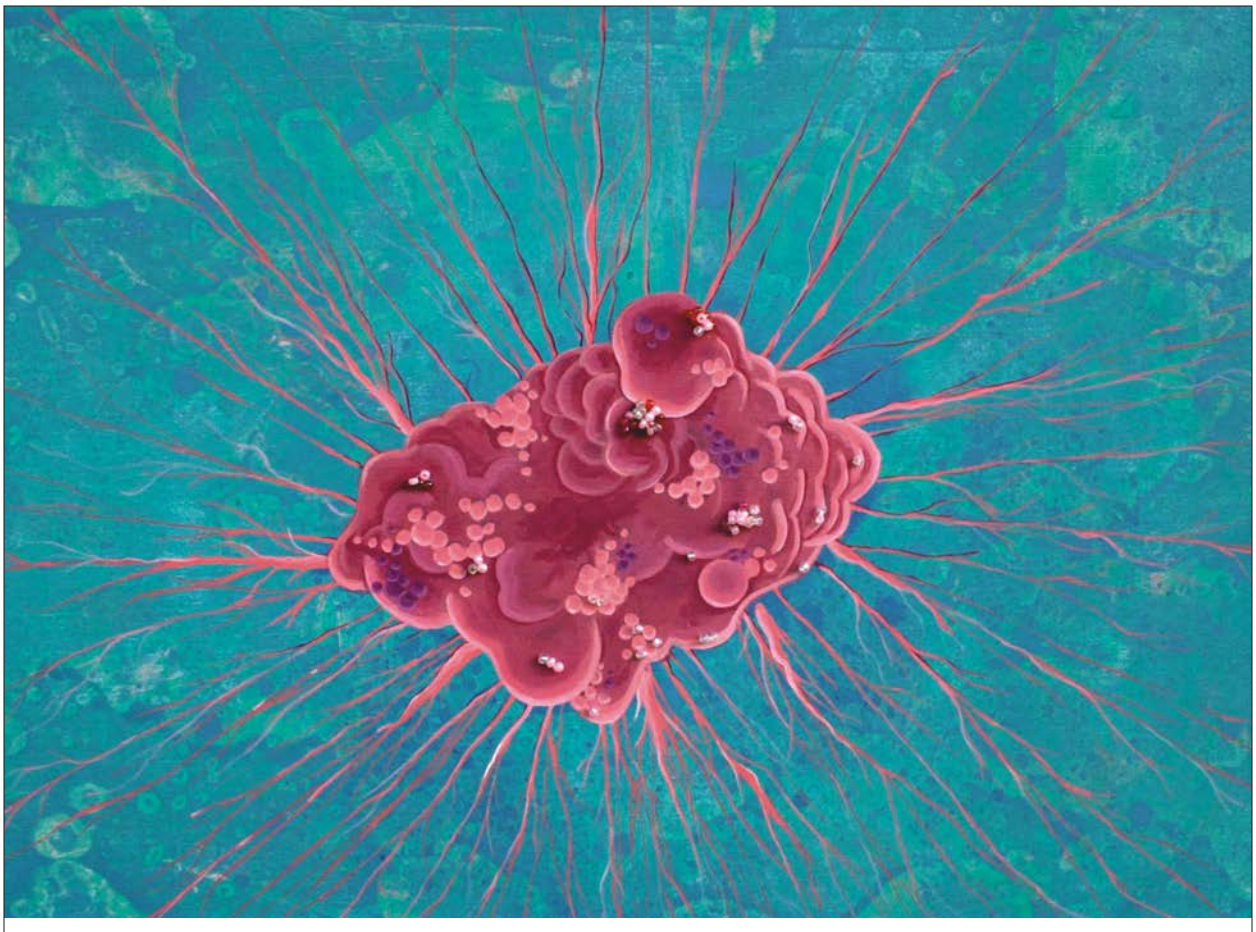

International Journal of Cancer Research

ISSN 1811-9727

Academic Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 10 (1): 37-45, 2014
ISSN 1811-9727 / DOI: 10.3923/ijcr.2014.37.45

Role of Serum Osteopontin Level as a Diagnostic Biomarker for Early Hepatocellular Carcinoma

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ABSTRACT

Osteopontin (OPN) was shown to play an important role in the pathogenesis of various inflammatory and fibrotic processes. Recently osteopontin has attracted attention as a promising biomarker for hepatocellular carcinoma (HCC) particularly in patients with low alpha-fetoprotein (AFP) levels. However, the significance of OPN in the early stage hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) remains unclear and is therefore evaluated in this study. Serum AFP and OPN levels were detected by ELISA in 29 patients with hepatocellular carcinoma HCC, in 29 patients with chronic hepatitis C virus (HCV) and in 30 healthy subjects. The diagnostic accuracy of each candidate marker was evaluated using Receiver-Operating Characteristic (ROC) curve analysis, reporting the Area Under the Curve (AUC) and its 95% Confidence Interval (CI). The mean serum OPN level in HCC patients (67.8 ng mL^{-1}) wasn't significantly different from HCV patients (71.7 ng mL^{-1}) while both were significantly higher than control group (11.6 ng mL^{-1}) ($p<0.001$). When HCC patients were compared to HCV patients, AUC for OPN and AFP were 0.46 and 0.68, respectively. Based on the ROC analysis, there were no satisfactory cut-off values for OPN that would best distinguish HCC from the non HCC patients. Osteopontin isn't a useful diagnostic marker for HCC. The current study recommended further search to find more specific biomarker for diagnosing HCC patients with low AFP.

Key words: Osteopontin, hepatitis C, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The 5-year survival rate for this malignancy is depressingly low, ranging from 4-6% in different countries (El-Garem *et al*., 2013). Major etiologic factors associated with HCC include infection with Hepatitis C Virus (HCV) mainly through indirect chronic inflammation, cell death and proliferation. Owing to the lack of reliable clinical HCC markers, fewer than 20% of patients are diagnosed at a stage where curative treatment can be performed (Szalay, 2010).Traditionally, the most commonly used serum marker for HCC is AFP. However, its use has been questioned due to a reported sensitivity of 39-65% and specificity of 65-94%. Moreover, multiple limitations exists when applied to chronic HCV patients who might express high levels of AFP which have been related to the hepatic fibrosis and necroinflammation resulting from the natural process of disease progression in HCV and unrelated to HCC (Di Bisceglie, 2004). Recently, the American and the European

Associations for the Study of Liver Diseases guidelines for HCC screening have recognized the overall poor performance of AFP and have excluded it from the screening recommendations (Bruix and Sherman, 2011). Therefore, it is necessary to identify new HCC biomarkers that have a sufficient sensitivity and specificity for the diagnosis of HCC patients. Osteopontin (OPN) is a glycophosphoprotein with cytokine and chemokine properties that was found to be circulating in the biological fluids of healthy individuals, but elevated in cancer patients as well as in individuals with systemic inflammatory response syndrome (Yang *et al*., 2014). OPN seems to drive hepatic inflammation; it attracts hepatic NKT cell and neutrophil infiltration, regulates interleukin-17 production in hepatitis and it's a chemotactic factor in the recruitment of macrophages to the liver which themselves produce abundant OPN (Diao *et al*., 2012). With respect to HCC, OPN was found to bind to cancer cells and endothelial cells which increases cell survival, cell migration and angiogenesis. OPN was shown to up regulate hyaluronic acid synthase which may contribute to survival of cells in the absence of adhesion, another key feature of metastatic cells (Tajima *et al*., 2010).

This study aimed at investigating the potential utility of serum OPN as a complement diagnostic biomarker for AFP in early HCV related HCC patients.

MATERIALS AND METHODS

Materials: Serum samples for the assessment of biomarkers were obtained from 88 individuals. Samples were obtained from patients presented before treatment to the viral hepatitis research lab at The National Hepatology and Tropical Medicine Research Institute (NHTMRI) in Cairo during the period of January to December 2012. Informed consent was obtained from each patient and the study protocols conformed to the ethics guidelines of the Institutional Review Board. Subjects were divided into three groups. Group 1 included patients with histological proven early HCV-related HCC (HCC; n = 29). Early stage HCC was defined as a single tumor nodule <3 cm in diameter with no evidence of vascular invasion or metastasis.

Group 2 included patients with chronic hepatitis C (HCV; n = 29), with or without cirrhosis. All patients were HCV positive as detected by hepatitis C antibody and HCV RT-PCR and HBV negative as detected by hepatitis B antigen and HBV DNA/PCR. Child-Pugh class was used to evaluate status of liver function. Group 3 included normal healthy age-matched subjects (Control; n = 30) that were negative for HCV and HBV with no history of liver disease.

Sera collected from 10 mL of coagulated blood by centrifugation were immediately separated and frozen at -80°C until assayed.

Methods: Serum levels of human OPN was measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R and D systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Each sample was examined in duplicate and the average value (mean) was used for data analysis.

The quantitative measurement of serum AFP was performed using AxSYM AFP (Abbott Laboratories, USA and Diagnostics Division) AxSYM AFP is based on the Microparticle Enzyme Immunoassay (MEIA).

Statistical analysis: Results are expressed as the Mean±SD. Kolmogorov-Smirnov test was done to evaluate the distribution of variables. Data were log transformed if they did not adhere to normal distribution. Differences between means were analyzed by the Student's t-test and *post hoc*

Bonferroni was applied to compare individual groups. Receiver-Operating Characteristics curves (ROC) were constructed to evaluate the diagnostic performance of the serum markers in discriminating HCC from other groups. Sensitivity, specificity, positive and negative predictor values and diagnostic accuracy were calculated in accordance with standard methods. $p<0.05$ for a two-tailed test was considered statistically significant. All statistical analyses were performed using the SPSS software version 15.0 (SPSS, Chicago, IL). This study calculated the optimal cutoffs using the maximum sum of sensitivity and specificity as well as using the minimum distance to the top-left corner of the ROC curve.

RESULTS

The clinical characteristics of the studied groups which included gender, age, liver function tests, cirrhosis stage, cancer stage, mean serum level of alph-afetoprotein and osteopontin are presented in Table 1.

The mean, median, minimum, maximum and standard deviation of OPN and AFP in all studied groups were shown in Table 2.

The mean serum OPN level in HCC patients (67.8 ng mL^{-1}) wasn't significantly different from HCV patients (71.7 ng mL^{-1}) while both were significantly higher than control group (11.6 ng mL^{-1}) at $p<0.001$ (Fig. 1).

The mean serum AFP level in HCC patients (1172.4 ng mL^{-1}) was significantly higher than both HCV patients and control group (22.5, 7.01 ng mL^{-1} respectively) while there were no significant difference between AFP serum level in HCV patients and control group (Fig. 2).

Table 1: Clinical and laboratory characteristics of the studied groups

Factor	HCC	HCV	Control	P1	P2	P3
n	29	29	30	-	-	-
Age (year)	63.16±2	62.4±1.9	64.8±1.73	NS	NS	NS
Sex (M/F)	23 M/6 F	20 M/9 F	30 M/0 F	-	-	-
ALT (IU L^{-1})	45.00±2.73	36.03±2.14	31.89±2.84	<0.01	NS	<0.001
AST (IU L^{-1})	58.5±3.9	38±2.5	33.2±4	<0.001	NS	<0.001
Bilirubin (mg dL^{-1})	2.2±0.5	1.1±0.42	0.6±0.2	<0.001	<0.01	<0.01
Albumin (g dL^{-1})	2.1±0.2	3.4±0.34	3.2±0.3	<0.05	NS	<0.05
Child-pugh class A,B,C (%)	18 (62%)	9 (31%)	-	-	-	-
	11 (38%)	17 (58.6%)				
	0 (0%)	3 (10.3%)	-	-	-	-
Okuda stage	I	-	-	-	-	-
BCLC	A	-	-	-	-	-
AFP (ng mL^{-1})	1172.4	22.5	7.01	<0.001	NS	<0.001
OPN (ng mL^{-1})	67.8	71.7	11.6	<0.001	<0.001	NS

P1: HCC and control, P2: HCV and control, P3: HCC and HCV

Table 2: Descriptive statistics of AFP and OPN in the studied groups

Health condition and tumor factor	HCC AFP OPN (n = 29)	HCV AFP OPN (n = 29)	Control AFP OPN (n = 30)
Mean	1172.4 (67.8)	22.5 (71.7)	7.01 (11.6)
Minimum	2.6 (9.6)	5.3 (0)	2.50 (0.0)
Maximum	2600.0 (267.9)	100.7 (246.6)	13.50 (60.9)
Median	223.6 (50.1)	11.5 (47.8)	6.50 (6.9)
Mode	1050.0 (9.6)	9.5 (28.8)	5.80 (0.0)
SD	1890.0 (64.2)	23.6 (60.4)	2.49 (13.95)

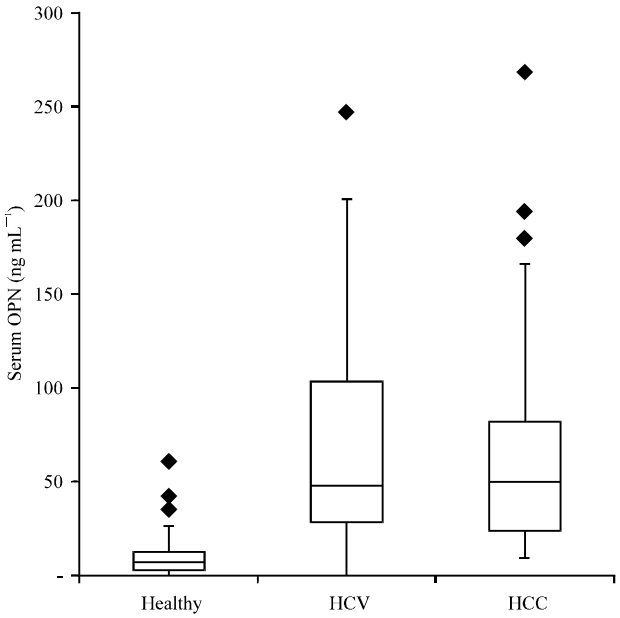

Fig. 1: Serum levels of OPN among healthy individuals and those with HCV and HCC. Box refers to the 25th and 75th percentile values, with a line indicating median levels, whereas the interquartile range extends outside the box. Points outside the interquartile range are outliers. HCC wasn't significantly different from HCV patients while both were significantly higher than control group at $p<0.001$

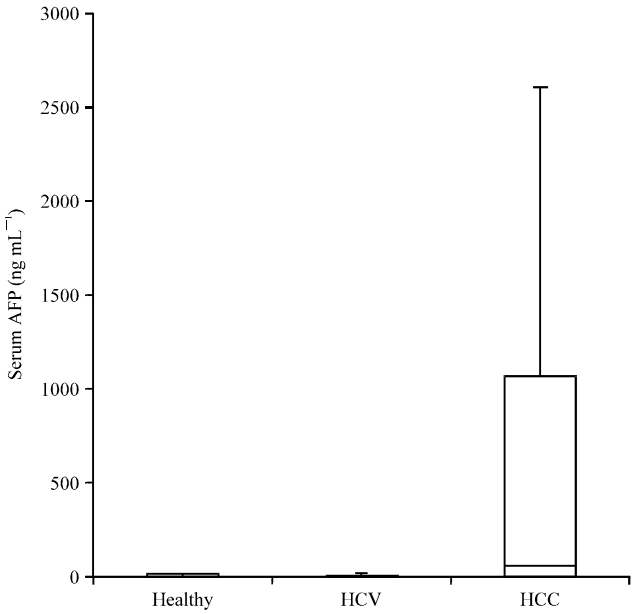

Fig. 2: Serum levels of AFP among healthy individuals and those with HCV and HCC. Box refers to the 25th and 75th percentile values, with a line indicating median levels, whereas the interquartile range extends outside the box. HCC had higher AFP serum levels, compared with HCV and healthy control groups ($p<0.001$)

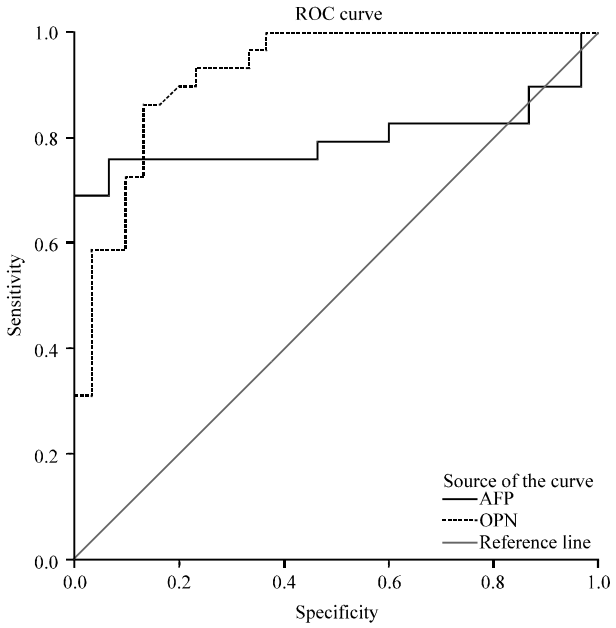

Fig. 3: Receiver Operating Characteristic (ROC) plots curve for Osteopontin (OPN) and alpha-fetoprotein in diagnosis of early stages of HCC versus Healthy control. Mean of area under ROC curve for osteopontin (OPN) marker was 0.92 while for AFP was 0.8

There were no correlation between OPN levels and the severity of chronic liver disease expressed as Child-Pugh classes.

When HCC patients were compared to healthy control, the AUC for OPN was [0.92; 95% Confidence Interval (CI) (0.852-0.988)] which was significantly higher than that yielded by AFP (0.8; with 95% CI (0.666-0.932) (Fig. 3).

The sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Likelihood Ratio (LR) and diagnostic accuracy (ACC) of serum OPN levels in HCC patients relative to control group were 86, 87, 86.2 % and 86.6, 6.4, 86.4%, respectively, at a cut-off value = 21.5 ng mL^{-1} (Table 3).

For AFP in HCC patients relative to control group at a cut-off value = 12 ng mL^{-1} (the optimal cutoff); the values of sensitivity, specificity, PPV, NPV, LR and ACC were 76, 93.3, 90.9% and 75.6, 9.8, 81.3% (Table 3).

Based on the ROC analysis, there were no satisfactory cut-off values for OPN that would best distinguish HCC from the non HCC patients (healthy control and HCV patients). As for AFP, it had a cut-off value ≥51.2 ng mL^{-1} (the optimal cutoff); the values of sensitivity, specificity, PPV, NPV, LR and accuracy were 55.1, 93.2, 80% and 80.8, 8.1, 80.6%. AUC for both OPN and AFP dropped to 0.69 and 0.74 respectively (Table 3).

When HCC patients were compared to HCV patients, AUC for OPN and AFP were 0.46 and 0.68 respectively (Fig. 4).

When HCV patients were compared to healthy control the AUC for OPN was [0.86; 95% Confidence Interval (CI) (0.76-0.96)] which wasn't significantly different from the AUC yielded by AFP [0.87; with 95% CI (0.773-0.961] (Fig. 5).

Table 3: Area under the ROC Curves (AUC), cut off value, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio (LR) and diagnostic accuracy (ACC) of osteopontin and alpha fetoprotein in diagnosis of HCC from each of control, non HCC, HCV and also diagnosis of HCV from control

Tumor marker	AUC	Cut off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR	ACC (%)
OPN								
HCC to control	0.92	≥21.5	86.0	87.0	86.2	86.6	6.4	86.4
HCC to non HCC	0.69	None	-	-	-	-	-	-
HCC to HCV	0.46	None	-	-	-	-	-	-
HCV to control	0.86	≥21.6	90.0	86.6	86.6	89.6	6.9	88.1
AFP								
HCC to control	0.8	≥12	76.0	93.3	90.9	75.6	9.8	81.3
HCC to non HCC	0.74	≥51.2	55.1	93.2	80	80.8	8.1	80.6
HCC to HCV	0.68	≥70	51.7	79.3	71.4	62.1	2.5	65.5
HCV to control	0.867	≥9						
	75.8	73.3	72.8	74.6	2.9	74.5		

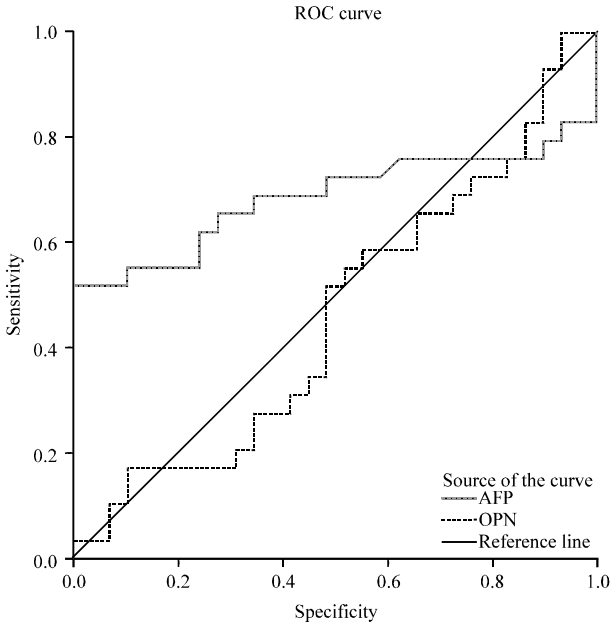

Fig. 4: Receiver operating characteristic (ROC) plots curve for OPN and AFP in diagnosis of early stages of HCC versus HCV patients. Mean of area under ROC curve for OPN marker was 0.46 while for AFP was 0.68

DISCUSSION

HCC ranks almost the first among all malignancies in Egypt, where it has the highest prevalence of Hepatitis C Virus (HCV) infection worldwide, with 14% of the population infected and seven million with chronic liver hepatitis (El-Zayadi *et al*., 2010). The poor outcome of patients with HCC is related to the late detection with more than two-thirds of patients diagnosed at advanced stages of disease (Akahoshi *et al*., 2010). Several tumor markers have been proposed as complements or substitutes for AFP in HCC diagnosis. OPN has been suggested as a potential marker for cirrhosis and HCC. OPN was fond to be highly expressed in many malignancies and the expression level of OPN in tumor tissues or in blood of cancer patients has been positively

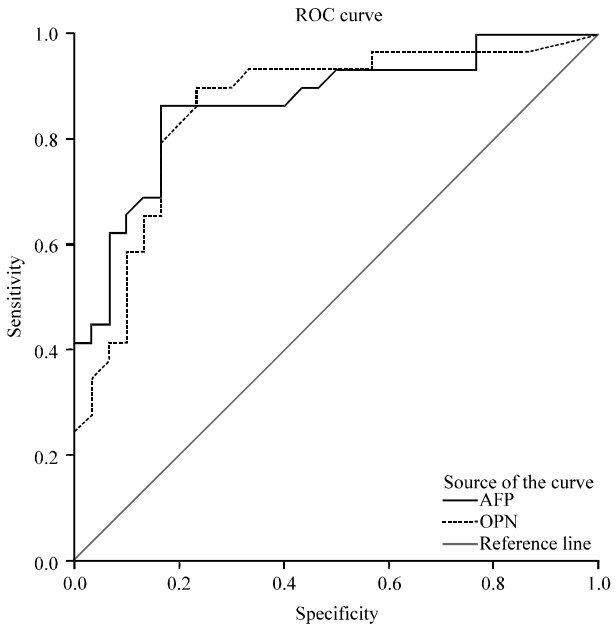

Fig. 5: Receiver operating characteristic (ROC) plots curve for OPN and AFP in diagnosis of HCV versus healthy control subjects. Mean of area under ROC curve for OPN marker was 0.86 while for AFP was 0.867

correlated with tumor grade, tumor stage and early recurrence in many cancer types (Sun *et al.*, 2011). Similarly, some studies found that OPN level in HCC patients was significantly higher than in patients with chronic liver disease or in healthy individuals (Kim *et al.*, 2006; Abu El Makarem *et al.*, 2011; Shang *et al.*, 2012), suggesting the use of circulatory OPN as a complement diagnostic biomarker for AFP. In this study, the mean OPN level in HCC patients wasn't significantly different from HCV patients while both were significantly higher than control group. Based on the ROC analysis, OPN in HCC patients relative to healthy control had good AUC, sensitivity, specificity, positive predictive value and negative predictive value (0.92 and 86, 87, 86.2 and 86.6% respectively at cut off ≥21.5) suggesting that OPN could performs as a rule-in as well as a rule-out test. Compared to healthy control; 25/29 (86.2%) HCC patients had OPN level of ≥21.6 ng mL^{-1} (optimal cut-off value). In contrast, 20/29 (69%) of HCC patients had AFP levels ≥12 ng mL^{-1} (optimal cut-off value). However when control group was extended to include other health conditions such as chronic hepatitis C, there were no satisfactory cut-off values for OPN that would best distinguish HCC from the non HCC patients and from HCV patients with AUC of 0.69 and 0.46 respectively suggesting that the use of OPN in this case is worthless.

As for AFP in HCC patients relative to healthy control, it had excellent specificity and positive predictive value (93.3%, 90.9) suggesting that AFP performs better as a rule-in test. In HCC patients relative to HCV group; the AUC of serum AFP was 0.74 which was higher than that for OPN but still was not good enough. The sensitivity, specificity, PPV and NPV were 51.7, 79.3, 71.4 and 62.1.6% respectively, at a cut-off value ≥70 ng mL^{-1}. The low sensitivity, specificity PPV and NPV of AFP found in this study group led us to recommend further search to find new complementary biomarker. OPN performance in patients with chronic hepatitis C was superior to AFP with a sensitivity, specificity, PPV, NPV, LR and ACC of 90, 86.6, 86.6% and 89.6, 6.9 and

88.1% at cut off value of 21.6 ng mL^{-1} compared to 75.8 and 73.3, 72.8% and 74.6, 2.9 and 74.5 for AFP at cut off value of 9 ng mL^{-1}. Taken together, these results suggest that elevated OPN levels in HCC and HCV patients are strongly related to the role of OPN in mediating hepatic inflammatory environment rather than mediating carcinogenesis. Based on the fact that ideal tumor biomarkers should possess high specificity and sensitivity not to be detected in premalignant liver disease, the current study concluded that simultaneous use of OPN and AFP doesn't add to the surveillance for HCC patients among HCV ones.

In contrast to these result, Shang *et al.* (2012) concluded that combining OPN and AFP in detecting HCC resulted in enhanced sensitivity and specificity indicating that these two markers are complementary.

In agreement with the current study, Zekri *et al.* (2011) reported that OPN levels in HCC patients weren't significantly higher than in those with chronic liver diseases. This study had, however, some limitations, such as the small sample size. Further large scale studies are worthwhile to investigate the exact role of OPN in HCC and to find new biomarker for diagnosing HCC patients with low AFP.

ACKNOWLEDGMENTS

The authors want to express great appreciation to all the team workers in viral hepatitis research lab.

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