Alfa-Fetoprotein L3 Subfraction and Osteopontin: Novel Markers for the Diagnosis of Hepatocellular Carcinoma

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Abstract: Objective: This study was designed to evaluate the role of serum alpha-fetoprotein-L3 subfraction (AFP-L3) and osteopontin (OPN) in the diagnosis of hepatocellular (HCC) and to consider their potential role as a novel prognostic marker. **Patients and Methods:** 120 patients with different stages of HCC were included in addition to 140 subjects with chronic liver diseases and 140 healthy control. Following clinical and radiological investigations, serum assay of AFP, AFP-L3 and OPN were performed.**Results:** AFP, AFP-L3 and OPN were significantly higher in HCC patients compared to chronic liver disease patients and normal control. A significant correlation was found between AFP and AFP-L3/AFP ratio among HCC patients. OPN varied significantly among different HCC stages. The best cutoff points revealed 100% sensitivity and 85% specificity for AFP-L3 and 100% for both sensitivity and specificity of OPN. **Conclusion:** Higher levels of AFP-L3 and OPN in HCC patients than controls propose them as potential markers for diagnosis of this disease. OPN has additional prognostic value through its significant difference among different HCC grades.

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in males and the eighth in females worldwide. Liver cancer accounts for 662,000 annual deaths and is the third leading cause of cancer-related deaths exceeded only by those of the lung and stomach [(1)]. It is strongly linked to hepatitis C (HCV) and B (HBV) viral infections. Egypt has the highest prevalence (13.8%) of HCV worldwide and has rising rates of HCC. Hospital-based studies in Egypt have reported an increase in the relative frequency of all liver-related cancers, from 4% in 1993 to 7.3% in 2003 [(2)].

The most common used laboratory marker for diagnosis is alpha-fetoprotein (AFP). However, it has a high rate of false-negative and false positive results. Its sensitivity and specificity depend on the cut-off value chosen. In cirrhotic patients, using a cut-off level of 20 ng/mL, sensitivity is only around 60% and specificity ranges from 80% to 94% [(3)]. This prompted the need of other reliable markers for this disease**.**

Total AFP is a collection of heterogeneous glycoproteins with different affinities to a lectin, the lens culinaris agglutinin (LCA), in affinity electrophoresis. AFP-L1 does not react with LCA and is present in chronic hepatitis and liver cirrhosis, constituting the major fraction of total AFP in non-malignant liver diseases. AFP-L3 has LCA-binding activity and is produced only by cancer cells. AFP-L2, which shows an intermediate affinity to LCA, is mostly derived from yolk sac tumors and could also be detected in maternal serum during pregnancy. Further clinical research has suggested AFP-L3 as a marker of HCC [(4)].

Osteopontin (OPN) is a phosphorylated acidic glycoprotein, which has been originally identified as a transformation-associated protein in culture of malignant cells. It is also believed to be involved in bone formation and resorption. Expression of OPN is highest in Kupffer cells, macrophages, and hepatic stellate cells [(5)]. OPN has a high level of expression in tumor tissues of HCC patients. It was suggested to be a potential tumor marker, because it exists not only as an immobilized extracellular matrix molecule, but also in a secreted form in body fluids including plasma and urine[(6)].

Our study aimed at assessment of the serum levels of both AFP-L3 and OPN in patients with different grades of HCC and evaluation of their diagnostic and prognostic utilities in comparison to total AFP.

2. Subjects and Methods:

Subjects:

This study was conducted on 120 HCC patients; 96 males and 24 females, with a median and interquartile range (IQR) of 49.5 (47-53) years, recruited from Tropical Medicine Department at Ain Shams University Hospital; in addition to 140 subjects with benign liver diseases as chronic hepatitis and liver cirrhosis, serving as pathological controls, 82 males and 58 females, with median (IQR) age of 53 (49.5-56) years; and 140 age-matched control subjects, 74 males and 66 females, with median (IQR) age of 51.5 (43.5-57.3) years.

According to the Barcelona Clinic Liver Cancer (BCLC) staging system, which uses variables related to tumor stage, liver functional and physical status, and cancer-related symptoms [7], HCC patients were subdivided into: stage A subgroup (n = 44), stage B subgroup (n = 48) and stage C subgroup (n = 28).

All studied individuals were submitted to full history taking, thorough clinical examination, radiological investigations and assessment of serum total AFP assay by chemiluminescent immunometric technique, serum AFP-L3 and OPN assay by enzyme-linked immunosorbent (ELISA) assay.

Methods:

Assay of AFP by immunochemiluminescence:

The assay was done on Immulite 2000 system (Siemens Medical Solutions Diagnostics, Los Angeles, USA) using manufacturer's reagents. This is a solid-phase, two-site sequential chemiluminescent immunometric assay. Patients' samples were introduced into the reaction tubes and incubated with agitation to bind to the monoclonal anti-AFP antibody coated on a polystyrene bead solid phase. Unbound samples were removed by centrifugal wash. An alkaline phosphatase-labeled polyclonal anti-AFP antibody was then introduced, followed by incubation and wash steps to remove unbound antibodies. The substrate was then added, incubated, followed by the chemiluminescent substrate, a phosphate ester of adamantyl dioxetane. Substrate hydrolysis in the presence of alkaline phosphatase yielded an emission of light. The bound complex and the photon output, measured by a luminometer, were proportional to the concentration of AFP in the sample.

Assay of AFP-L3 and OPN by ELISA:

Both assay were based on a solid phase sandwich ELISA using reagents supplied by GenBio (Genbio, 4 ABO, Switzerland) and Ray Biotech (3607, Parkway Lane, Georgia, USA), respectively. In each assay, samples or standards were pipetted into wells containing capture monoclonal antibodies, respectively against AFP-L3 and OPN. Following incubation, unbound antibodies were removed by wash, followed by addition of the respective biotinylated anti-AFP-L3 and OPN antibodies. Another incubation and wash followed, with a subsequent addition of horse-radish peroxidase-conjugated streptavidin. After a final washing step, enzyme substrate solution was added to induce a colored reaction product, the intensity of which was directly proportional to the concentration of AFP-L3 or OPN present in the sample. Concentrations were deduced from calibration curves drawn from the standards assayed in the same run.

Statistical Analysis:

Statistical analysis was done through Statistical Package for Social Sciences (version 15.0, 2007, Echosoft Corporation, USA). Description was through median and IQR for numerical non-parametric data, and number and percentage for categorical data. Comparison of categorical data was performed using Chi-squared test, and that of 2 independent parametric data through Mann Whitney U test. On comparing more than 2 independent non-parametric variables, Kurskal Wallis test was used, followed by post-hoc multiple comparisons using the Mann Whitney U test. Spearman's correlation was used for correlating non-parametric variables. p value more than 0.05 was considered non-significant, less than 0.05 was considered significant, and less than 0.01 was considered highly significant. Diagnostic validity was estimated using sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, and efficacy, and represented on a receiver operator characteristics (ROC) curve.

3. Results:

Descriptive and comparative statistics of the studied HCC patients (group I), pathological controls (group II) and healthy controls (group III) are presented in Tables (1 and 2) and Figure (1). A highly significant difference of HCV infection was detected between each of groups I and II versus III. Such different was non-significant between groups I and II. None of age, sex or HBV infection showed any significant difference between groups. On comparing laboratory HCC markers, both AFP and OPN were highly significantly different between any 2 groups comparison, while AFP-L3 and its ratio to AFP showed high significant difference only between groups I versus II or III, but not between II versus III. As regards HCC stages (Tables 3 and 4, Figure 2), only OPN levels were highly significantly different among different stages, while none of AFP, AFP-L3 or AFP-L3/AFP ratio showed such significance.

On correlating different laboratory parameters (Table 5), highly significant correlation was revealed between AFP and each of AFP-L3 and AFP-L3/AFP ratio. All other correlations were non-significant.

At a cutoff of 20 ng/mL, AFP gave a sensitivity, specificity, PPV, NPV and efficacy of 96.7%, 85%,

90.6%, 94.4% and 92%, while a best cutoff of 12 ng/mL revealed 100%, 85%, 90.9%, 100% and 94%, respectively. The same diagnostic parameters for AFP-L3 at a cutoff of 2 ng/mL were 100%, 85%, 90.9%, 100% and 94%, and, for its ratio to AFP at 0.4, the parameters were 93.3%, 60%, 77.8%, 85.7% and 80%. OPN reached a 100% for all diagnostic performance parameters at a cutoff of 2000 pg/mL. The same applies for the combination of AFP at 12 ng/mL and AFP-L3 at 25 ng/mL (Table 6, Figures 3, 4, and 5).

Table (1): Comparison of the Demographic and Laboratory Data Between the Different Studied Groups Using Chi-squared Test for Qualitative and Mann Whitney U test for Quantitative Non-Parametric Data.

Parameter	Group I (HCC Patients) [n=120]	Group II (Chronic Liver Disease Controls) [n=140]	Group III (Healthy Controls) [n=140]	Group I vs II		Group I vs III		Group II vs III	
				X^2 / Z^*	*p*	X^2 / Z^*	*p*	X^2 / Z^*	*p*
Age ǂ (years)	49.5 (47-53)	53 (49.5-56)	51.5 (43.5-57.3)	1.90*	> 0.05	0.72*	> 0.05	0.42*	> 0.05
Sex Male: Female:	 M: 96 (80%) F: 24 (20%)	 M: 82(58.6%) F: 58 (41.4%)	 M: 74 (52.9%) F: 66 (47.1%)	0	> 0.05	1.60	> 0.05	0.95	> 0.05
Positive HCV	112 (93.3%)	135 (96.4%)	0 (0%)	3.73	> 0.05	31.11	< 0.001	10.78	< 0.001
Positive HBV	8 (6.7%)	5 (3.6%)	0 (0%)	1.48	> 0.05	0.70	> 0.05	2.22	> 0.05

ǂ = median (interquartile range)

Table (2): Comparison of AFP, AFP-L3, AFP-L3/AFP ratio and OPN Between the Different Studied Groups Mann Whitney U test.

Parameter	Group I (HCC Patients) [n=120]	Group II (Chronic Liver Disease Controls) [n=140]	Group III (Healthy Controls) [n=140]	Group I vs II		Group I vs III		Group II vs III	
				Z	*p*	Z	*p*	Z	*p*
AFP ǂ (ng/mL)	93.5 (55.3-422)	10 (8-62.5)	3 (2-4)	3.41	< 0.001	4.69	< 0.001	3.77	< 0.001
AFP-L3 ǂ (ng/mL)	38.5 (13.3-113.2)	0 (0-10)	0 (0-0.08)	4.02	< 0.001	4.71	< 0.001	1.31	> 0.05
AFP-L3/AFP ratio ǂ	0.26 (0.12-0.50)	0 (0-0.18)	0 (0-0.03)	3.55	< 0.001	4.35	< 0.001	1.17	> 0.05
OPN ǂ (pg/mL)	9750 (9000-11000)	975 (750-1175)	575 (400-675)	4.73	< 0.001	4.73	< 0.001	3.59	< 0.001

ǂ = median (interquartile range)

Table (3): Statistical Comparison of Laboratory Parameters Among the Different HCC Stage Subgroups Using Kruskall Wallis Test.

Parameter	Stage A (n =44) Median (IQR)	Stage B (n = 48) Median (IQR)	Stage C (n = 28) Median (IQR)	*H*	*p*
AFP (ng/mL)	80 (40-422)	100 (51.8-345.8)	93 (90-789)	0.231	> 0.05
AFP-L3 (ng/mL)	43.4 (10-113.7)	25.8 (10-126)	40 (20-111)	0.171	> 0.05
AFP-L3/AFP ratio	0.27 (0.10-0.49)	0.31 (0.10-0.58)	0.24 (0.14-0.43)	0.001	> 0.05
OPN (pg/mL)	9000 (8000-9000)	10000 (9500-11000)	11000 (11000-12000)	19.79	< 0.001

Table (4): Post-hoc Multiple Comparisons of Laboratory Parameters Among the 3 Stages of HCC Cases Using Mann Whitney U test.

Parameter		Stage A vs B	Stage A vs C	Stage B vs C
AFP	Z	0.03	0.68	0.17
	p	> 0.05	> 0.05	> 0.05
AFP-L3	Z	0.34	0.18	0.34
	p	> 0.05	> 0.05	> 0.05
AFP-L3/AFP	Z	0.03	0.09	0.09
	p	> 0.05	> 0.05	> 0.05
OPN	Z	3.57	3.60	2.18
	p	< 0.001	< 0.001	< 0.05

Table (5): Correlation Between Different Laboratory Parameters Among HCC Patients (group I) using Ranked Spearman Correlation Test

		AFP-L3	AFP-L3/AFP Ratio	OPN
AFP	r_s	0.80	0.50	0.08
	P	< 0.001	< 0.01	> 0.05
AFP-L3	r_s		0.01	0.05
	P		> 0.05	> 0.05
AFP / AFP-L3 ratio	r_s			0.02
	p			> 0.05

Table (6): Diagnostic Performance of AFP, AFP-L3, AFP-L3/AFP ratio and OPN in Discrimination Between HCC Cases (group I) Versus Both Control Groups (II and III).

	Cut-off	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive value (%)	Efficacy (%)
AFP	20 ng/mL	96.7	85	90.6	94.4	92
AFP	12 ng/mL	100	85	90.9	100	94
AFP-L3	2 ng/mL	100	85	90.9	100	94
AFP-L3/AFP ratio	0.4	93.3	60	77.8	85.7	80
OPN	2000 pg/mL	100	100	100	100	100
AFP and AFP-L3	12 ng/mL and 25 ng/mL	100	100	100	100	100

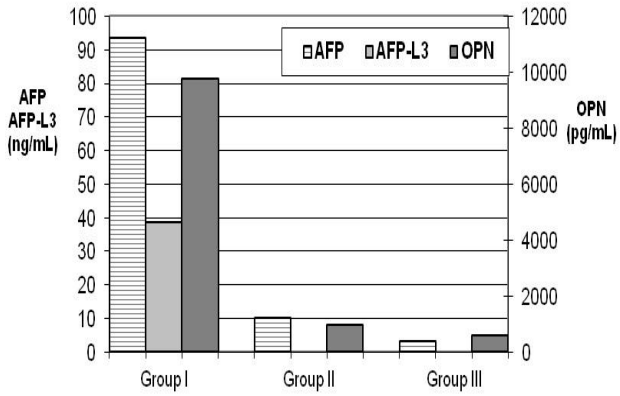


Figure (1): Comparison of AFP, AFP-L3 and OPN among all studied groups.

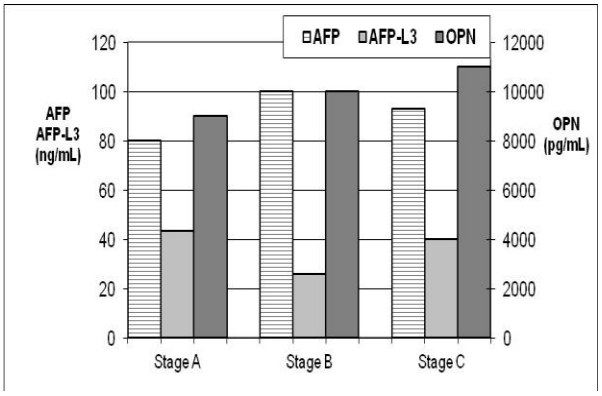


Figure (2): Comparison of AFP, AFP-L3 and OPN among different stages of HCC patients (group I).

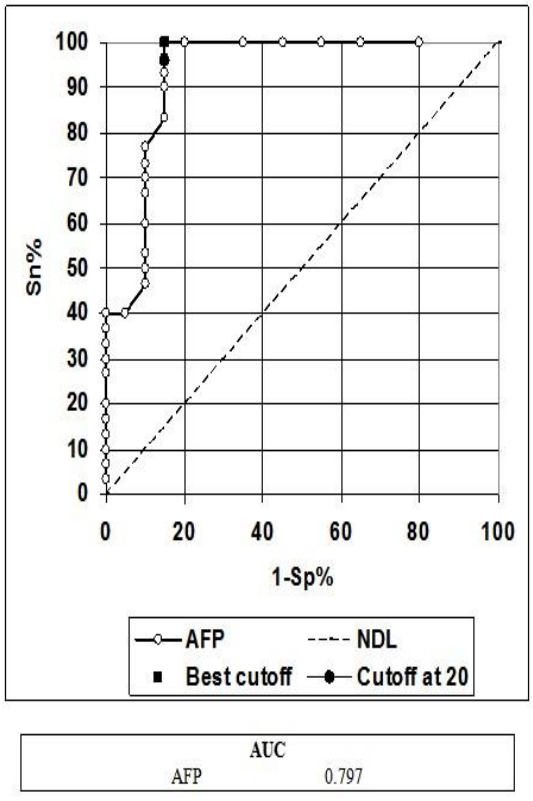
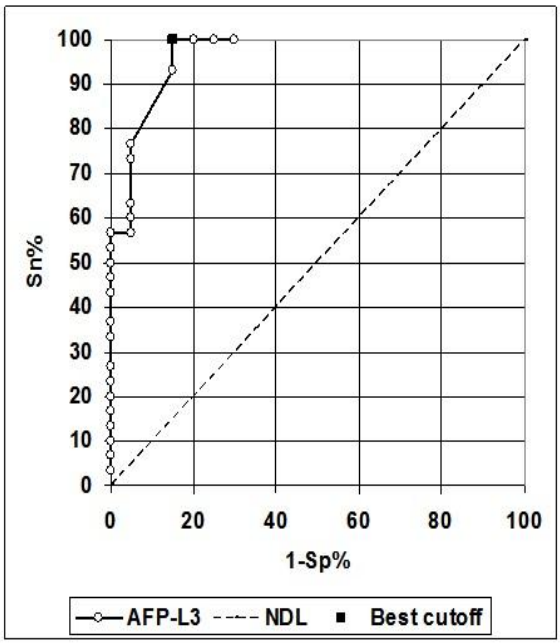
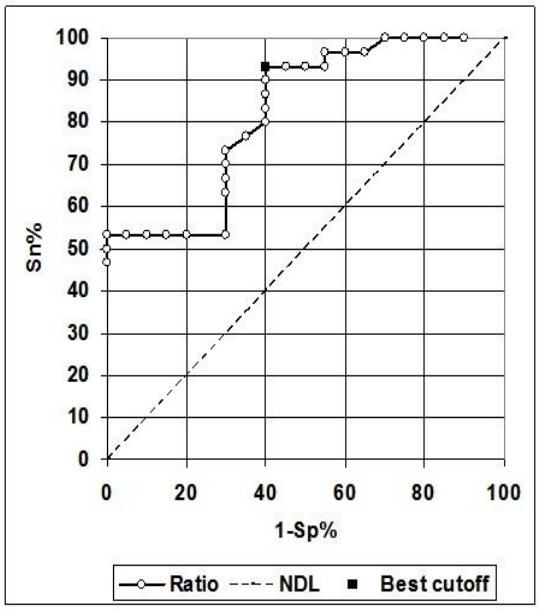


Figure (3): ROC curve analysis showing the diagnostic performance of AFP for discriminating HCC patients (group I) versus all controls (groups II and III).



AUC	
AFP-L3	0.887

Figure (4): ROC curve analysis showing the diagnostic performance of AFP-L3 for discriminating HCC patients (group I) versus all controls (groups II and III).



AUC	
Ratio	0.683

Figure (5): ROC curve analysis showing the diagnostic performance of AFP-L3/AFP ratio for discriminating HCC patients (group I) versus all controls (groups II and III).

4. Discussion:

Hepatocellular carcinoma is usually asymptomatic in the early stages and tends to be invasive. Therefore, most patients are presented with an incurable disease at the time of detection which makes its early diagnosis critical for a good prognosis. Surgical resection remains the treatment of choice for these tumors, but unfortunately only 10-20 % of primary HCCs are resectable at the time of diagnosis. Continuous researches are ongoing worldwide to find and evaluate an early sensitive and specific marker for HCC [8].

In our study, 93.3% of the patients with HCC were positive for HCV infection which is an impact of increased prevalence of HCV infection among the Egyptians in 14% of the general population [9]. HCV infected persons represent 78.5% of all HCC patients in Egypt [10]. Usage of glass syringes in the early campaigns of Schistosoma treatment appeared to be responsible for wide spread transmission of HCV [11].

We found that the median value of both serum AFP-L3 and AFP-L3/AFP ratio were significantly higher in HCC patients group when compared to pathological and normal control individuals. That is matching with the study of ***Davis et al.*** [12] revealing that AFP-L3 is produced only from malignant liver cells. ***Li et al.*** [4] studied the presence of AFP-L3 in chronic liver disease patients suspected to have HCC. They found that 57% of AFP-L3 positive cases were diagnosed as HCC in the following 6 months and 6 of them were diagnosed to be the single small HCC at the early stage through ultrasonic diagnosis or CT.

In accordance with ***Kim et al.*** [13] ***and Bessa et al.*** [14]***,*** serum OPN level was significantly higher in HCC patients group than the pathological and healthy controls. The same was demonstrated by ***Gotoh et al.***[15] and ***Sun et al.*** [16] who revealed, through immunohistochemistry, that OPN protein was expressed mainly in cancer cells. OPN regulates the transformation of normal cells to malignant cells by induction of phosphorylation and activation of phosphoinositide 3-kinase. This induces DNA binding and activation of various transcription factors, including nuclear factor kappa-beta. The latter helps "switching on" of genes expressing anti-apoptotic proteins. The end-result is anti-apoptosis, tumor cell growth, motility and invasion. A role of OPN in tumor development through enhancement of angiogenesis had been recorded *in vitro* [17].

The present study demonstrated that OPN levels significantly differed among advancing tumor stages, where median serum OPN levels was 9000 pg/mL in stage A, 10000 pg/mL in stage B and 11000 pg/mL in stage C. These results were similar to ***Pan et al.*** [18], who measured OPN mRNA levels in 240 surgically removed primary HCCs using the reverse transcription PCR. HCC tumors showed OPN mRNA

over-expression in TNM stage III and IV more than I and II. They also found that elevated OPN mRNA levels in HCC tissues were significantly associated with higher grade and early recurrence of cancer, resulting in poorer prognosis. Similarly, ***Bessa and her coworkers*** [14] found a statistically significant increase of the mean OPN levels from stage A (6800 pg/mL) to stage B (11400 pg/mL) to stage C (15330 pg/mL). This suggested that OPN could induce increased invasiveness and promote progression.

Assessment of the diagnostic performance of AFP for distinguishing HCC from benign liver diseases and healthy candidates revealed, at a cutoff of 12 ng/mL, a diagnostic sensitivity of 100%, specificity of 85%, PPV and NPV of 90.9% and 100%, and efficacy of 94%, which was superior than the diagnostic performance at the common cut-off of 20 ng/mL that yielded sensitivity, specificity, PPV, NPV and efficacy of 96.7%, 85%, 94.4%, 90.6% and 92%, respectively. ***Kim et al.*** [13] reached 100% specificity with a raised cutoff to 70.4 ng/mL, while ***Motawa et al.*** [19], using a cutoff of 19.8 ng/mL demonstrated 68.2% sensitivity and 75% specificity. AFP -L3 showed sensitivity of 100%, specificity of 85%, PPV of 100% and NPV 90.9%. That is close to study by ***Debruyne and Delanghe*** [20], (n=334), in which AFP- L3 showed sensitivity of 90% and 95% specificity. Using much larger number of patients (n = 2000), the study of ***Shiraki et al.*** [21] revealed a sensitivity of 75% and specificity of 90%. In a meta-analysis performed by ***Li and his coworkers*** [4], the sensitivity of AFP-L3 was found to be stage-related in HCC. In small HCC (HCC <2 cm in diameter), AFP-L3 had a sensitivity of only 35-45%. The sensitivity increased with increase in size of the HCC, and reached 80-90% when HCC was 5 cm in diameter or greater. When we tested the diagnostic performance of AFP and AFP-L3 in combination, diagnostic specificity improved from 85% to 100%, as revealed by ***Leerapun et al.*** [22] (n=272) who stated that determination of AFP-L3, in combination with AFP, increases the specificity of diagnosis of HCC in individuals with HCC.

In our study, AFP-L3/AFP ratio of 0.4 had a sensitivity of 93.3%, specificity of 60%, PPV of 85.7% and NPV of 77.8%.This was close to the study by ***Tanwandee et al.*** [23] (n =61) where a cut-off 0.15 yielded a sensitivity of 82% specificity of 71%, PPV of 83% and NPV of 69%. ***Leerapun et al.*** [22] studied 166 cases of HCC and 106 cases of benign liver conditions. When a cut-off of 0.1 was used, it gave a sensitivity of 71% and specificity of 63% while raising the cut-off to 0.35 led to a specificity of 100% at the expense of a sensitivity of 33%. A study by ***Sangiovanni et al.*** [24] assessed the diagnostic performance of AFP-L3/AFP ratio in 86 HCC patients and 38 patients with other liver conditions. By a cut-off of 0.07, a sensitivity of 60% and specificity of 80% were reached. A probable cause for the discrepancy in the cut-offs of the above studies lies in the different numbers of subjects included in each.

Assessment of serum OPN in our study revealed that a best cut-off of 2500 pg/mL yielded 100% in all aspects of diagnostic performance. According to these results, serum OPN can be considered an efficient marker for screening of HCC. These results were comparable to those of ***Abu El Makarem et al.*** [25], who studied the diagnostic performance of OPN level for discrimination of the HCC patients (n = 113) from chronic liver disease subjects (n = 120) and healthy subjects (n = 120). The sensitivity, specificity, PPV and NPV were 97.7%, 100%, 100%, 97.6%.

The results of the current study indicate clearly that serum AFP-L3 and OPN are promising tumor markers that could be added to the current standard tests for diagnosis of HCC in order to detect the disease at an early stage and hence improving the prognosis and survival rate of the patient.

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