Osteopontin as A Tumor Marker for Hepatocellular Carcinoma

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AIM: Hepatocellular carcinoma (HCC) is a global health problem because of its increasing prevalence worldwide and its poor prognosis. In Egypt, HCC incidence has increased sharply and nearly doubled over the last decade. The outcome of HCC depends mainly on its early diagnosis; therefore, new and specific markers for HCC are critically needed. Osteopontin (OPN) is a glycoprotein that overexpressed in HCC, and known to be an independent predictor of poor prognosis. The aim was to assess the value of OPN in Egyptian patients with HCC.

METHODS: This study included 40 patients with HCC, 20 patients with liver cirrhosis and 20 healthy controls. For all groups, clinical data and image findings were studied; serum alpha-fetoprotein & OPN levels were detected by enzyme immunoassay (EIA) kit. Tumor characteristics were assessed including size, number and site. Tumor staging was done using Okuda, CLIP, VISUM and Tokyo staging systems.

RESULTS: Serum OPN was significantly higher in HCC patients compared to cirrhotic patients and controls. The sensitivity and specificity in diagnosis of HCC were 92.5% and 85% respectively at cutoff of 239 ng/mL with 91.1% accuracy. OPN has a positive significant correlation with tumor number (p = 0.036), CLIP (p = 0.02), Tokyo (P = 0.03), and VISUM (P = 0.01) staging systems.

CONCLUSION: OPN could be a useful diagnostic & prognostic marker for detection of HCC.

Key words: Hepatocellular carcinoma; Osteopontin; Alpha-fetoprotein

INTRODUCTION

Worldwide, Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, with more than 748,000 new cases being diagnosed each year, accounting for 9.2% of all new global cancer cases (7.9% in men; 3.7% in women) [1,2]. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and one of the leading causes of death among patients with cirrhosis [3]. In Egypt, HCC is the second most common cancer in men and the sixth most common cancer in women [4]. 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 [5] and the increased survival rate among patients with cirrhosis allowing time for some of them to develop HCC [6]. Hepatic resection and transplantation remain the standard curative therapies for HCC. These treatments are limited to either patients with early-stage tumors in the case of transplantation or patients with preserved
liver function in the case of resection[10]. Early detection is critically important because the most effective treatment for HCC is surgical resection or ablation therapy when the tumor is small[7].

Prognosis for patients with HCC depends on tumor stage, with curative therapies only available for patients detected at an early stage. Patients detected at an early stage can achieve 5-year survival rates of 70% with transplant or resection, whereas those with advanced HCC are only eligible for palliative treatments and have a median survival of less than one year[20]. Up to 20% of HCC do not produce alpha-fetoprotein (AFP) even when very large[9]. The fibrolamellar variant most commonly occurs in young Caucasian females often with lymph node metastasis and without elevations of AFP[9] and slight increases are usual in acute hepatitis, chronic hepatitis and cirrhosis and over laps can cause diagnostic difficulties[11]. Osteopontin (OPN) is known to be a secreted adhesive glycoprotein, associated with tumorigenesis and metastasis in several cancers[12]. Plasma OPN level is a potential diagnostic marker for HCC, especially among high-risk group of patients[13]. Osteopontin (OPN) has been reported as one of the most promising markers for HCC[14]. Overexpression of OPN was associated significantly with the metastatic potential of primary HCC[15]. Moreover, subsequent study suggested OPN plasma level was elevated in HCC patients, and it has superior diagnostic accuracy as a tumor marker compared with a-fetoprotein[16]. In this study we aimed at evaluating the diagnostic and prognostic value of osteopontin in comparison to AFP levels in patients with HCC.

METHODS

This study was approved by the Ethics and Research Committee of the Benha faculty of Medicine, Benha University, Egypt. Serum samples were obtained from sixty patients with chronic liver disease, divided into two groups: Group (I) included forty patients with HCC, Patients with cancers other than HCC or metastatic liver cancer were excluded. Group (II) included twenty patients with liver cirrhosis and without any evidence of HCC and Twenty healthy adults were recruited as controls (Group III). All patients included in this study had the procedure thoroughly explained to them. HCC was diagnosed by abdominal US and confirmed by triphasic CT scan. AFP was assayed by an enzyme immunoassay (EIA) Kit (Roche Mannheim, Germany). The clinical/pathological data of the patients were recorded, including age, sex, viral infections [Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV)], alcohol intakes, biochemical liver function test results, and AFP levels. Severity of liver disease was assessed by modified Child-Pugh score[17] and MELD (model for end stage liver disease) score[18] and the updated MELD (uMELD) score[19]. Tumor characteristics were detected by abdominal US with or without CT scan (including tumor size, number, site, halo sign and neovascularization). Tumor staging was done using Okuda[20], CLIP (The Cancer of the Liver Italian Program)[21], VISUM (Vienna survival model for HCC)[22] and Tokyo[23] staging systems.

Blood sampling and biochemical assays

Fasting venous blood samples (5 mL) were collected by trained laboratory technicians. A portion of blood was allowed to clot and then centrifuged at 3,500 g for 5 min to separate the serum used for assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and g-glutamyl transpeptidase (GGT), total bilirubin, direct bilirubin, albumin, creatinine and glucose concentrations were assayed using Beckman CX4 chemistry analyzer (NY, USA, supplied by the Eastern Co. For Eng. & Trade-Giza, Egypt). Viral infection status (HCV Ab and HBsAg) were measured using Abbott, Axyam (USA, Supplied by Al kanal company). Serum aliquots were stored at -80°C until assayed and thawed immediately before the measurements levels. Serum AFP and Osteopontin levels was determined using an enzyme-linked binding protein assay kit. All assays were performed in duplicate according to the manufacturer’s instructions.

Tissue Samples

For cases with HCC, liver samples were collected intraoperative after the patients were surgically operated for hepatic lobectomy. Specimens were obtained from the tumour tissue and fixed in formalin then embedded in paraffin blocks for histopathological examination. Using a microtome, sections 5 μm thick were cut from formalin-fixed paraffin embedded tissue blocks and subjected for H&E staining and pathological diagnosis, grading.

Statistical Analysis

Statistical package (SPSS, version 20.0) was used for data management. Descriptive statistics was presented as mean ± standard deviations for continuous variables, number and percentage for categorical variables (frequency distribution). Unpaired student t-test (two sided) was used to test the significance of difference between the mean value of studied groups and chi-square test was used for comparison of categorical variables. The diagnostic value for each marker was assessed using sensitivity, specificity, positive (PPV) and negative (NPV) predictive values. Receiver operating characteristic curves (ROC) were constructed to assess the validity of the markers in predicting HCC by calculating the area under the curve (AUC). Pearson correlation test was used to identify the correlation between Osteopontin and different clinico-pathological variables. The significance level was set at P < 0.05.

RESULTS

The demographic features and characteristics of the (two patients’ groups) were summarized in Table 1. A total of 80 adults, which comprised 40 patients with HCC, 20 patients with liver cirrhosis in addition to 20 apparently normal control subjects were studied for the osteopontin and AFP. The mean age of HCC patients was 60.9 ± 9 years with a range between 48 and 89 years. In liver cirrhosis patients the mean age was 56.5 ± 9.3 years with a range between 34 and 80 years, with no significant difference in the mean ages of patient groups, however HCC tends to occur at higher age. There was male predominance among the patients with HCC, 31 men (77.5%) versus 9 women (22.5%), with a male-to- female ratio of 3.4:1. There was a significant difference in the sex ratios of HCC patients, liver cirrhosis patients (P = 0.01). Concerning the residence, either rural or urban, 62.5% of HCC cases had urban residence. In addition, a positive history for farming was found in 35% of HCC cases. Regarding HCC etiology, the results showed that the frequency of HBV positivity had no statistically significant difference between the studied patients. It was detected only in two HCC cases and in 0% in cirrhotic cases. HCV was present in 92.5% of HCC cases and 90% of cirrhotic cases with no statistically significant difference between the two groups. The liver functions in cirrhosis assessed by Child–Pugh classification among the studied patients showed that 15% of HCC cases were Child A, while 20% of HCC cases were Child B and 65% were Child C with no statistical significant difference between the two groups. There was no statistically significant difference among the two groups as regards the severity of liver disease assessed by MELD scores (Table 1). Tumor imaging characteristics of HCC patients were illustrated in (Table 2). Abdominal CT showed the dominant
occurrence of HCC on top of cirrhosis (100%) and a percentage of portal vein thrombosis (PVT) of (12.5%) and the higher incidence of the focal lesion to be single (57.5%), large and affecting the right lobe (60%), and halo sign in 95% of the patients.

Regarding Okuda, CLIP, VISUM and Tokyo staging systems, most of HCC patients were relatively advanced stage at the disease. As regards AFP levels, the data revealed that as regards AFP values there was no significant difference between HCC cases and cirrhotics (P1=0.051) and between cirrhotics and control groups (P3=0.995), but AFP values were higher in HCC cases than control group (P2=0.000) (Table 3), Figure 1. As regards osteopontin values were higher in HCC cases than cirrhotics (P1=0.000), higher in HCC than control (P2=0.000), but no significant difference between cirrhotics & control (P3=0.227). (Table 3), Figure 2. In the receiver operating curve (ROC) (Table 4), Figure 3, the area under curve (AUC) for AFP was 78.7% when we use 12.9 ng/mL as a cutoff point, with a sensitivity of 70% and a specificity of 77.5%, PPV of 95.2% and NPV of 62.5%. For Osteopontin the cutoff point that gives an AUC of 91.1% was 239 ng/mL with a sensitivity of 92.5% and a specificity of 85%, PPV of 95.2% and NPV of 86.95%. There was significant positive correlation between AFP and CLIP (P = 0.005), Tokyo (p = 0.03) and VISUM (P = 0.02) staging system among HCC cases.

There was significant positive correlation between Osteopontin and Tumor number (P = 0.036), CLIP (p = 0.02), Tokyo (p = 0.03), and VISUM (P = 0.01) staging system among HCC cases, Figures (4, 5, 6, 7) respectively.

### Table 1 Demographic Features of the studied patient groups:

| Characteristics          | HCC Patients (n = 40) | Liver Cirrhosis (n = 20) | P value |
|--------------------------|----------------------|--------------------------|---------|
| Age (years)              | 48-89                | 34-80                    | 0.08    |
| Gender                   | 60.9 ± 9             | 56.5 ± 9.3               |         |
| Male                     | 31 (77.5%)           |                          | 0.01*   |
| Female                   | 9 (22.5%)            |                          |         |
| Residence                | 11 (55%)             |                          |         |
| Urban                    | 25 (62.5%)           |                          | 0.54    |
| Rural                    | 15 (37.5%)           |                          |         |
| Occupation               | 7 (35%)              |                          |         |
| Farmer                   | 14 (35%)             |                          |         |
| Non-farmer               | 26 (65%)             |                          | 0.186   |
| Etiology                 | 16 (80%)             |                          |         |
| Smoking                  | 22 (55%)             |                          | 0.2     |
| Alcohol                  | 1 (2.5%)             |                          | 0.44    |
| HBV                      | 2 (5%)               |                          |         |
| Severity of Liver disease| 0 (%)                |                          |         |
| Child A                  | 6 (15%)              |                          | 0.54    |
| Child B                  | 8 (20%)              |                          |         |
| Child C                  | 26 (65%)             |                           | 0.65    |
| MELD Score               | 4 - 44               |                          | 0.79    |
| Mean ± SD                | 19.9 ± 9.1           |                           |         |
| uMELD Score              | 2.6 ± 1.1            |                           | 0.58    |
| Mean ± SD                | 4.08 ± 1.1           |                           |         |

*: Significant; SD: Standard deviation; HCC: hepatocellular carcinoma; HCV: Hepatitis c virus; HBV: Hepatitis B virus; MELD: Model for end stage liver disease; uMELD: updated MELD.

### Table 2 Tumour related findings and characteristics of the HCC Patient group:

| Characteristics | HCC Patients (n = 40) | Percentage (%) |
|-----------------|-----------------------|----------------|
| Computed Tomographic Features |                      |                |
| Tumor size      | 3 / 3.5 / > 5 cm      | 22.5/ 42.5/ 35 |
| No. of nodules   | 1-3 / 3 or more       | 57.5 / 42.5    |
| Site of Tumor    | Right liver / left liver / both | 10 / 55 / 35 |
| Shape            | Irregular / round / defined | 11 / 40 / 35 |
| Echogenicity     | Hyperechoic / hypoechoic / isoechoic | 10 / 55 / 35 |
| Halo sign        | 38                    | 95             |
| Metastasis       | 1                     | 2.5            |
| Portal vein invasion | 5                    | 12.5           |
| Tumor staging    |                        |                |
| Okuda stage      | I / II / III          | 15 / 40 / 45   |
| CLIP stage       | I / II / III          | 10 / 55 / 35   |
| Tokyo stage      | Early (0-4)            | 42.5           |
| Advanced (5 or more) | 23                    | 57.5           |
| VISUM stage      | I / II / III          | 47.5/ 32.5/ 20 |
| AFP (ng/mL) level| 2.5 - 30.00           | 10/ 8.2        |
| Mean              | 2687.5                |                |
| Osteopontin (ng/mL) level | 40-913             |                |
| Mean              | 357.2                 |                |

### Table 3 AFP and Osteopontin as markers for HCC AFP and Osteopontin values in the studied groups.

| Marker | Group I HCC (N = 40) | Group II cirrhosis (N = 20) | Group III control (N = 20) | P value |
|--------|----------------------|-----------------------------|---------------------------|---------|
| Range  | Mean ± SD            | Range                       | Mean ± SD                 |         |
| Osteopontin (ng/mL) | 40-913 | 357.2 ± 211.3 | 40-321                      | 202.2 ± 62.49 | 42-722 | 134.1 ± 176.09 | P1=0.000* | P2=0.000* | P3=0.0227 |
| AFP (ng/mL) | 2.5-30.000 | 2687.5 ± 687.4 | 0.5-187                       | 20.5 ± 40.9   | 4.2-35 | 10.8 ± 8.2   | P1=0.057 | P2=0.05* | P3=0.095 |

*: significant.

### Table 4 AFP and Osteopontin as markers for HCC.

| Test            | Cut off | Sensitivity % | Specificity % | PPV % | NPV % | AUC % | P-value |
|-----------------|---------|---------------|---------------|-------|-------|-------|---------|
| Osteopontin/ml  | 239     | 92.5%         | 85%           | 95.2% | 86.95%| 91.1% | 0.000*  |
| AFP ng/ml       | 12.5    | 70%           | 77.5%         | 95.2% | 62.5% | 78.7% | 0.000*  |

*: significant; PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve; AFP=alphafetoprotein.
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Figure 1 Alpha fetoprotein levels among the studied groups.

Figure 2 Osteopontin levels among the studied groups.

Figure 3 ROC curve of AFP and osteopontin as a marker for HCC.

DISCUSSION

AFP is the most established tumour marker in HCC and is the gold standard by which other markers for the disease are judged[24]. In the current study AFP in HCC cases had a mean value of 2687.5 ng/mL which was higher than that of patients with cirrhosis (although not statistically significant) (20.5 ng/mL) and control (10.8 ng/mL) subjects, these results are in agreement with Abdel-Hamid et al[25] who reported that AFP in HCC cases had a mean value of 1172.4 ng/ml which was statistically higher than that of patients with cirrhosis (22.5 ng/mL) and control (7.01 ng/mL) subjects. Furthermore, similar

Figure 4 Correlations of osteopontin values and Tumor number among HCC cases.

Figure 5 Correlations of osteopontin values and CLIP stage among HCC cases.

Figure 6 Correlations of osteopontin values and Tokyo stage among HCC cases.

Figure 7 Correlations of osteopontin values and VISUM stage among HCC cases.
findings were observed by Salem et al\textsuperscript{[28]}, Lee et al\textsuperscript{[27]}, Abdel gawad et al\textsuperscript{[26]} and El-Shenawy et al\textsuperscript{[25]} who reported higher mean values of AFP for HCC cases. ROC analysis of AFP used as a diagnostic test suggested that a value of about 12.9 ng/mL provided the optimal balance between sensitivity and specificity, however at this level the sensitivity was only 70\% and the specificity was 77.5\%, which is inadequate sensitivity and specificity for early diagnosis of HCC. This goes in agreement with Bruix and Sherman\textsuperscript{[30]} and Abu El-Makarem et al\textsuperscript{[26]} who reported the same results. Accordingly, the role of AFP in the diagnosis of HCC is limited, these results were supported by El-Serag et al\textsuperscript{[28]} who mentioned that AFP is not elevated in all patients with HCC, its sensitivity for detecting HCC ranges between 25\%-60\%, and its specificity is also low because serum AFP can also be detected in patients with cirrhosis and chronic hepatitis. Therefore, the finding of a mass in the liver with an elevated AFP does not automatically indicate HCC; intrahepatic cholangiocarcinoma (ICC) is also more common in cirrhosis than in non-cirrhotics. The fact that both are more common in cirrhosis means that care must be taken to distinguish between them given the differences in treatment and outcomes. Since AFP can be elevated in either condition, it is recommended that it no longer be used\textsuperscript{[31]}. There is a debate in defining the AFP cut-off level for the diagnosis of HCC, an AFP value above 400-500 ng/mL has been considered to be diagnostic for HCC in patients with cirrhosis, however, such a cut-off value is problematic in absolute diagnostic terms, since high levels of this magnitude are not as common in the presence of smaller tumors (< 5 cm) and furthermore, only 30\% of HCC patients have levels higher than 100 ng/mL in this context\textsuperscript{[32]}. There was non-significant correlation between AFP and the severity of the liver disease (Child, MELD and uMELD scores), tumor number, size, OPN and Okuda stage, this finding is supported by Huo et al\textsuperscript{[33]} and Atta et al\textsuperscript{[34]} who reported that no correlation was observed between AFP and severity of the liver disease. This can be explained by that transient increases and fluctuations in serum AFP may occur in chronic liver disease and cirrhosis, especially during exacerbations of hepatitis\textsuperscript{[34]}, mild to moderate elevations (20-400 ng/mL) occur in cirrhosis\textsuperscript{[35]}. But the frequency of elevated AFP did not differ with regards to MELD score or cirrhotic disease\textsuperscript{[36]}. But there was significant positive correlation between AFP and CLIP, Tokyo, and VISUM staging system among HCC cases, these were in agreement with El-Shenawy et al\textsuperscript{[37]} and El-Zayadi et al\textsuperscript{[38]} who reported that there was significant positive correlation between AFP and CLIP staging systems.

Regarding AFP as prognostic predictor in HCC, our results supported by Kim et al\textsuperscript{[39]} and Tandon and Garcia-Tsao\textsuperscript{[40]} who concluded that serum AFP may have a prognostic meaning in patients with HCC, and is included in prognostic classifications such as CLIP score and VISUM staging system, this may be explained by the issue that an increase in AFP concentration might reflect tumor aggressiveness including differentiation degree and vascular invasion\textsuperscript{[41]}. Concerning OPN mean value, HCC cases had a mean value of (357.2 ng/mL) which was higher than that of patients with cirrhosis (202.2 ng/mL) and control (134.1 ng/mL) subjects, these results were in agreement with Shang et al\textsuperscript{[42]} who reported that plasma levels of OPN were significantly higher in HCC patients (mean 271.4 ng/mL) than in cirrhosis (mean 86.2 ng/mL) and healthy controls (mean 32 ng/mL). Similar results by Kim et al\textsuperscript{[40]}, El-Din Bessa et al\textsuperscript{[41]} and Abu El Makarem et al\textsuperscript{[28]} were reported, who found that the median plasma OPN level was significantly higher in the HCC group than in the cirrhotic group or in the normal control group. ROC analysis of OPN used as a diagnostic test suggested that a value of 239 ng/mL provided the optimal balance between sensitivity and specificity, at this level the sensitivity was 92.5\% and specificity was 85\%, which is considered good sensitivity and specificity for diagnosis of HCC. This goes in agreement with Abu El Makarem et al\textsuperscript{[37]} who reported that OPN had high diagnostic efficiency for HCC (the sensitivity and specificity of plasma OPN levels in HCC patients relative to the CLD group were 97.67\% and 100\% respectively, at a cut-off value of 300 ng/mL).

Similar results by Kim et al\textsuperscript{[39]} and El-Din Bessa et al\textsuperscript{[41]} were observed, who reported that OPN had high diagnostic sensitivity and specificity for HCC group over non-HCC group (cirrhotic group and healthy control). There was no significant correlation between plasma OPN and severity of liver disease; this was in agreement with Abu El Makarem et al\textsuperscript{[37]} and Abdel-Hamid et al\textsuperscript{[30]} who reported the same results. There was significant correlation between plasma OPN and tumor number; this goes in agreement with Salem et al\textsuperscript{[28]} and Abu El Makarem et al\textsuperscript{[37]} who reported that there was a significant correlation between plasma OPN and number of nodules. There was no significant correlation between plasma OPN and the size of the tumor, this goes in agreement with Salem et al\textsuperscript{[28]}, but disagree with Abu El Makarem et al\textsuperscript{[37]} who reported that there was significant correlation between plasma OPN and size of tumor nodules.

There was significant positive correlation between plasma OPN and CLIP, Tokyo, and VISUM staging system among HCC cases, these results were in agreement with Abu El Makarem et al\textsuperscript{[37]} and Zhang et al\textsuperscript{[43]} who reported that plasma OPN was significantly correlated with advancing degree of tumor stage and tumor progression. There was no significant correlation between plasma OPN and AFP in the present study, this goes in agreement with Abu El Makarem et al\textsuperscript{[37]} and Kim et al\textsuperscript{[39]} who reported that exactly.

CONCLUSION

Regarding OPN as prognostic marker for HCC, these results suggested that plasma OPN level could be a potential prognostic marker for HCC, this suggestion was supported by Hua et al\textsuperscript{[44]} and Abu El-Makarem et al\textsuperscript{[37]} who documented that there is significant correlation between OPN overexpression and tumor progression (high grade, late stage, LNVascular invasion). Also Chen et al\textsuperscript{[45]} stated that OPN overexpression has been revealed as an independent prognostic factor for poor overall and disease free survival in HCC patients. According to this study, plasma OPN level was elevated in HCC patients than cirrhotic and apparently healthy patients, with better diagnostic sensitivity and specificity for HCC group over non-HCC group (cirrhotic group and healthy control) with slightly better prognosis than AFP, these results suggested that plasma OPN level could be a potential diagnostic and prognostic marker for HCC.

CONFLICT OF INTEREST

The authors declare that they do not have conflict of interests.

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