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■■■ RESEARCH ARTICLE

Evaluation of osteopontin as a potential biomarker in hepatocellular carcinoma in a sample of Egyptian patients

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■■■ ABSTRACT

Background: In Egypt, hepatocellular carcinoma (HCC) is a disease with increased prevalence rate and a dismal prognosis and survival rate. For an early diagnosis of HCC, biomarkers are crucial. The glycoprotein osteopontin (OPN), which is released by T cells, osteoblasts, and macrophages, is also strongly expressed in a wide range of tumors, including stomach and colon. **Aim:** Our aim was to evaluate the serum level of OPN as a potential marker for HCC patients with HCV, in comparison with other tumor markers. **Materials and Methods:** This study included 140 patients (aged 27-79 years) divided into four groups. Group I involved 20 healthy controls, Group II involved 40 patients with HCV, Group III involved 40 patients with HCC. Group IV involved 40 patients with HCV and HCC. Routine clinical tests such as ALT, AST, ALP, ALBUMIN and BILIRUBIN were assayed for all patients. In addition, serum level of OPN was identified using Western blotting and its levels were quantified using sandwich ELISA. The absolute values of the investigated markers were statistically analyzed using SPSS program. **Results:** The mean OPN level was 11.89±7.5, 47.34 ±2. 71, 201 ±5.85, and 251.52± 19.63 pg/ml for G1, G2, G3 and G4, respectively. Of interest, Group IV (patients with HCC and HCV) showed much higher serum OPN levels than the normal group (p=0.001). Using Western blotting assay, OPN was identified at 68-kDa molecular mass in the sera from all patients. **Conclusion:** OPN has a greater sensitivity and specificity than CA.CA19.9 and AFP. It useful for early detection of HCC.

Keywords: Osteopontin, HCC, HCV, liver profile, AFP

■■■ ARTICLE INFO

INTRODUCTION

One of the leading causes of death worldwide is hepatocellular carcinoma (HCC), and its incidence is steadily rising (Fallah & Kharazmi, 2009; Tschiya et al., 2015). Hepatitis B (HBV) or C virus (HCV) are more likely to develop this condition than those with non-alcoholic fatty liver disease or a history of chronic alcohol misuse (Altekruse et al., 2009). In addition, the presence of cirrhosis and inflammation frequently makes an early diagnosis of the condition challenging. Hepatic carcinogenesis is a multiphase process characterized by strong cell proliferation and profound changes to the cell genome. Specifically, HCV-associated HCC appears to be associated with increased frequency following hepatic resection, indicating a major role for this viral infection in both metastasis and cell proliferation (Huang et al., 2004). HCV is the leading cause of death for individuals with HCC, even though the exact mechanism causing HCV-induced HCC is yet unknown. Furthermore, hepatic carcinogenesis can occur in conjunction with hepatic metabolic disturbance and even in the absence of cirrhosis. Numerous cytokines have been investigated as potential players in the formation of HCC and the progression of liver disease, as well as early circulating biomarkers that can identify individuals who are more vulnerable.

Liver cancer ranks as the fifth most prevalent cancer in both genders; it ranks sixth in women, accounting for 3.4% of cases with cancer, and second in men, following urinary bladder cancer, which accounts for 11.5% of all cancer

cases. Being the second most common cause of cancer-related deaths globally, primary liver cancer poses a serious threat to public health.

Ninety percent of all occurrences of primary liver cancer are caused by HCC alone, accounting for about 800,000 new cases each year (Liovet et al., 2016). Liver cancer is asymptomatic in the early stages, but develops symptoms in the advanced stages, which results in an inadequate curative outcome (Zhao et al., 2013). Liver cancer patients' lives are extended when they receive early diagnosis and efficient treatment.

Osteoblasts, T lymphocytes, and macrophages release phosphorylated glycoproteins called osteopontin (OPN). On Chromosome 4 region 22 (4q22.1) is where the OPN gene is found (Si et al., 2020). OPN is a secreted, pleiotropic, multi-phosphorylated glycoprotein, first recognized as secreted phosphoprotein 1 (SPP1) in 1979 (Yan et al., 2023). It was initially found in bone, but later research revealed that it is expressed in several tissues.

OPN mediates a multitude of biological processes and plays significant roles in bio mineralization, and several physiological processes involved in cellular homeostasis, as well as in pathologies such as chronic inflammation and tumor biology (Tang Z et al., 2023). OPN was also known as early T-lymphocyte activation 1 protein, and bone sialoprotein 1 and was reported to activate immune cells, including T-cells, B-cells, macrophages, natural killer and Kupffer cells. Currently, OPN is classified as one of the members of the small integrin-binding ligand N-linked glycoprotein family and modulates cell signaling and connections with the matrix through interacting with integrin's and CD44 receptors (Sinha et al., 2023).

AIM OF THE WORK

Our aim was to evaluate the level of OPN as a potential marker of HCC between patients with HCV, in comparison with other tumor markers representing a new strategy for liver cancer diagnosis with high degrees of sensitivity and specificity.

MATERIAL AND METHODS
Patients, controls, and sample collection

The recent study involved 140 patients. Ethical committee approval was taken from Faculty of Pharmacy in Minia University (MPEC 230112) and informed consent was approved for all patients from Minia Oncology. Samples were collected in the period from April 2023 to January 2024. The following patients were excluded: (1) those with any other type of tumor other than HCC; (2) those with autoimmune, B, or Acute hepatitis; (3) those with localized hepatic lesions other than HCC; (4) those with inflammatory illnesses or bone lesions; and (5) those who had previously had treatment for HCC.

The samples were classified into four groups: Group I (control) involved twenty patients who appeared to be healthy; they had normal liver function tests and were seronegative for HCV antibodies and hepatitis B surface markers. Group II (HCV without HCC) consisted of forty patients without HCC based on ultrasonography results, biochemical evidence of parenchymal damage, and a biopsy of the liver. Hepatitis C by PCR was approved. Group III (HCC without HCV) included forty patients. Radiological investigations, such as abdominal ultrasonography and triphasic CT, as well as laboratory tests, were used to diagnose HCC in this group. Group IV (HCV-positive chronic hepatitis with HCC) involved forty patients. Ultrasonography findings, biochemical evidence of parenchymal damage, and a liver biopsy were used to diagnose the patients in this group. Hepatitis C by PCR was approved by the patient. 10 ml of venous blood from each patient was taken, and the serum was separated by centrifugation for 15 minutes at $1,000 \times g$ to measure liver function tests, serum OPN, and serum alpha-fetoprotein. Serum was subsequently refrigerated at -80˚C. The following procedures were applied to all patients and controls: complete clinical assessment, taking a full history, and abdominal ultrasonography.

Biochemical Examination

Standard laboratory testing was measured, including total and direct bilirubin, serum albumin, ALT, AST, and liver function tests.

Hepatitis markers included PCR for HCV, tumor markers such CA19.9, and an ELISA kit to evaluate CEA levels.

Assessment of Serum OPN by ELISA: Using a human osteopontin assay kit and a human alpha-fetoprotein assay kit, all serum samples were analyzed for OPN and AFP using ELISA technique as directed by the manufacturer. In summary, serum samples and prepared standards were added to the relevant ELISA plate wells in 100 µL increments, and the assay was conducted. A micro test plate spectrophotometer (Abcamm CA, USA) was used to detect the absorbance at 450 nm. Human OPN was used as a standard to quantify OPN using a calibration curve. Every standard or sample underwent duplicate analysis.

SDS-PAGE and Western blot: To determine the molecular weight of OP, after optimization of reaction condition, resolved proteins on SDS-PAGE were electro transferred onto nitrocellulose (NC) filter in protein transfer unit. The NC filter was blocked using 5% (w/v) non-fat dry milk for 1-h at room temperature then rinsed in TBS and incubated overnight with specific mouse antibody to ESM-1 with constant shaking. The blots were washed 3 times (30 min each) in TBS, followed by 2-hours incubation with goat anti-mouse IgG alkaline phosphate conjugate. After washing 3 times with TBS (15 min), the blots soaked in alkaline phosphatase BCIP/NBT substrate, pH 9.5. The color reaction was observed within 20 min, and the developed color then stopped by dipping the blots in distilled water and then left to dry.

Statistical analysis

The data were analyzed using the statistical software SPSS 18. Result was displayed as the mean (M ± SD) plus standard deviation. The quantitative variables between the two groups were compared using chi-square analysis. P value <0.05 was considered statistically significant, while a p value <0.001 considered highly significant. The ROC curve was applied to determine the test's sensitivity and specificity, as well as the optimal cut-off value for the diagnostic biomarker under consideration. Furthermore, the area under the curve (AUC) was calculated to assess accuracy.

RESULTS
Demographic data of selected patients and controls
The study comprised 140 patients, aged between 27 and 79 years, with 83 men (59.3%) and 57 females (40.7%). Group I (control) comprised 20 subjects who appeared healthy, with ages ranging between 33 and 70 years and a mean value was 53.1±9.6. Group II (HCV only) comprised 40 patients with liver hepatitis without HCC; their ages ranged from 33 to 75 years, with a mean value of 58.3 ±12.2. Group III (HCC) comprised 40 patients with HCC, whose ages varied from 27 to 79 years, with a mean value of 60.4±12.1. Group IV (HCV positive chronic hepatitis with HCC) Comprised 40 patients, whose ages varied from 36 to 73 years with mean value 52.7±10. All examined groups' mean age and gender values did not differ statistically (P > 0.05). Table 1 and Figure 1 describe the laboratory results for the liver function tests for each of the 140 patients in the various groups. The mean albumin, ALP, AST, and ALT values in the liver function tests revealed a highly significant difference between the patients and controls (*P* value = 0.0001).

Immunochemical identification and quantification of Osteopontin in human serum

Using western blotting, A sharp band was found at 68-kDa in the sera of infected patients with significant HCC (G3-G4) as shown in Figure 3. The concentration of OP in sera was quantified using sandwich ELISA technique. Figure 2 and Table 2 show the ELISA-measured serum levels of OPN, CEA, and CA19.9 in the patient groups and the healthy control group.

The OP cutoff of ELISA was determined by calculating the mean ELISA OD±3SD of 60 sera samples from G1-G2 (non-HCC), to determine if a tested sample is positive or negative. The best cutoff level was set at concentrations equal = 197 pg/mL. By applying cut-off for all 140 sample were tested by ELISA, 71 out of 80 patients with significant HCC showing positive ELISA for OPN and 59 out of 60 patients with non-significant HCC displaying negative test result (Table 3) by using the area AUC, diagnostic value of the OPN evaluated. The AUC of OPN for discriminating significant HCC patient from non-

Table 1. Liver function tests in HCC patients in comparison with healthy control.

Biochemical Marker	G1 Control	G2 HCV Only	G3 HCC	G4 HCC+HCV	**P value
1) ALT	30.65 ± 3.70	56.08 ± 15.42	88.87 ± 22.99	95.45 ± 24.22	0.0001
2) AST	33.45 ± 4.89	61.40 ± 16.96	89.40 ± 17.93	93.3 ± 17.85	0.001
3) ALB	4.41 ± 0.43	3.90 ± 0.49	2.98 ± 0.30	2.93 ± 0.34	0.001
4) ALP	84.60 ± 18.97	102.58 ± 35.55	234.00 ± 65.73	256.13 ± 75.15	0.0001

Overall significance of differences among the groups was determined by t-test for ALT, AST, ALBUMIN and ALP. Significance differences ($P<0.0001$) were shown between significant HCC patients (G2-G3) and non-significant HCC patients (G1-G2). **$P > 0.05$ is considered not significant, $P < 0.05$ considered significant, $P < 0.001$ considered very significant, *P* < 0.0001 is considered extremely significant

Table 2. Levels of Tumor markers in HCC patients in comparison with healthy control.

TUMOR Marker	G1 Control	G2 HCV Only	G3 HCC	G4 HCC+HCV	**P value
1) Osteopontin	11.89± 7.28	47.34± 2.71	201.97 ± 5.85	251.52 ± 19.63	0.0001
2) AFP	4.80 ± 2.33	7.35 ±2.38	380.00 ± 12.8	399.08 ± 13.9	0.001
3) CA19.9	67.45 ± 37.92	81.83 ± 40.16	196.17 ± 15.4	275.48 ± 9.1	0.001
4) CEA	5.63 ±1.44	18.71± 12.66	330.85 ± 14.5	346.58 ± 10.11	0.001

Overall significance of differences among the groups was determined by t-test for CEA, CA19.9, AFP, and OP. Significance differences ($P<0.0001$) were shown between significant HCC patients (G2-G3) and non-significant HCC patients (G1-G2). **$P > 0.05$ is considered not significant, $P < 0.05$ considered significant, $P < 0.001$ considered very significant, $P < 0.0001$ is considered extremely significant.

Table 3. The diagnostic performances of OPN ELISA at the best cut off level.

Pathology	OPN Elisa		Total
	+ve	-ve	
(HCC) +ve	71 (True +Ve)	9 (False -Ve)	80
(Non HCC) –ve	1 (False +Ve)	59 (True -Ve)	60
Total	72	68	140

Sensitivity = 71/80×100=88.75 %
Specificity = 59/60×100=96.3 %
Efficiency = 130/140×100=92.8 %
NPV= 59/68×100=86.76%
PPV= 71/72×100=96.6 %

HCC patients were 0.913 ($P < 0.0001$) in comparison with CA19.9 and CEA (Figure 4). By using OPN cut-off = 197 Pg/mL, significant HCC was expected, moreover the sensitivity, specificity and efficiency were evaluated as 88.75%, 96.3% and 92.8%; respectively by using the same cut off (Figure 5).

DISCUSSION

One of the most prevalent malignant tumors is HCC. Since HCC is typically detected at a later stage, the prognosis may be bad, with an overall survival rate after five years less than 5% (Calderon-Martinez et al., 2023).

Patient survival can be considerably increased by early HCC differential diagnosis and accurate identification. Diagnostic imaging methods and the identification of blood biomarkers, such as α-AFP, fucosylated AFP, and des-γ-carboxyprothrombin, are currently used in clinical practice to detect HCC. Nonetheless, these techniques have constraints in terms of sensitivity and specificity, particularly in the first phases of HCC. High-throughput technologies have recently led to the identification of numerous novels, non-invasive blood biomarkers as well as the elucidation of numerous new pathways implicated in hepatic carcinogenesis. Specifically, the utilization of AFP in conjunction with these novel candidate compounds has demonstrated encouraging outcomes (Reichl and Mikulits, 2016).

It is critically necessary to determine novel, sensitive, and specific biomarkers for early diagnosis (Tang et al., 2017). The diagnostic usefulness of several different new biomarkers for early hepatocellular carcinomas has been studied, however it is still up for debate.
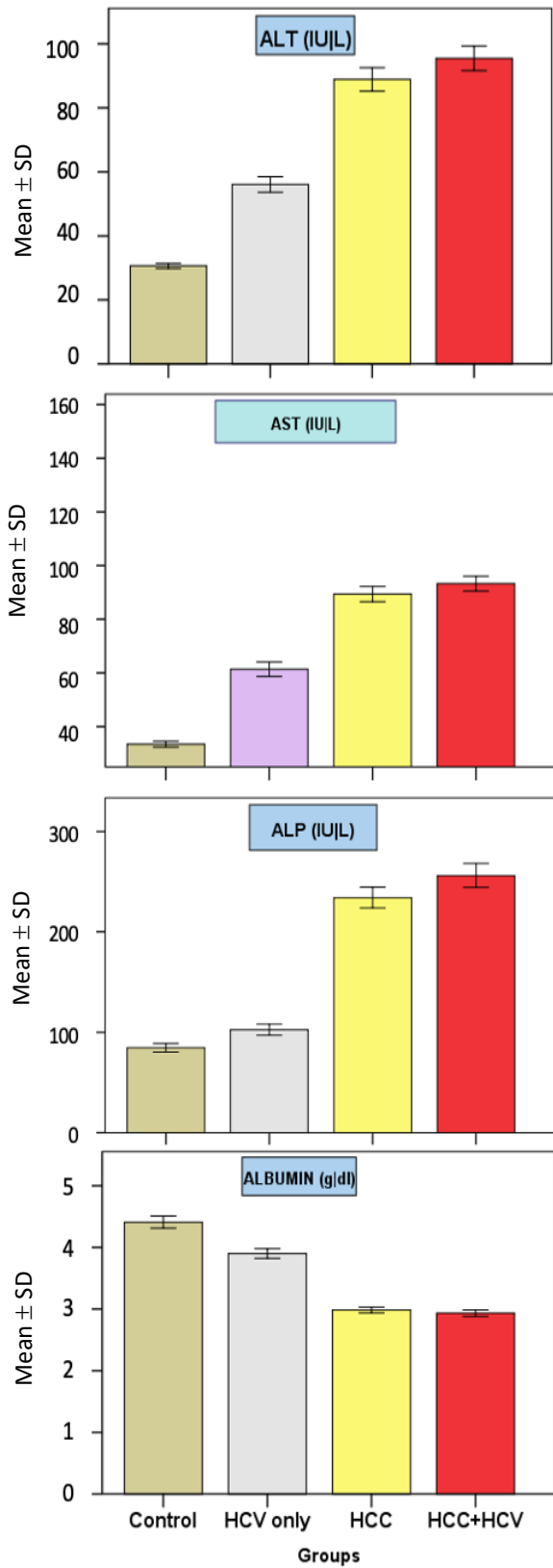
Figure 1. Liver function tests in healthy control in comparison with patients. Serum levels of ALT (A), AST (B), ALP (C) and albumin (D). Bars represent mean ± SD
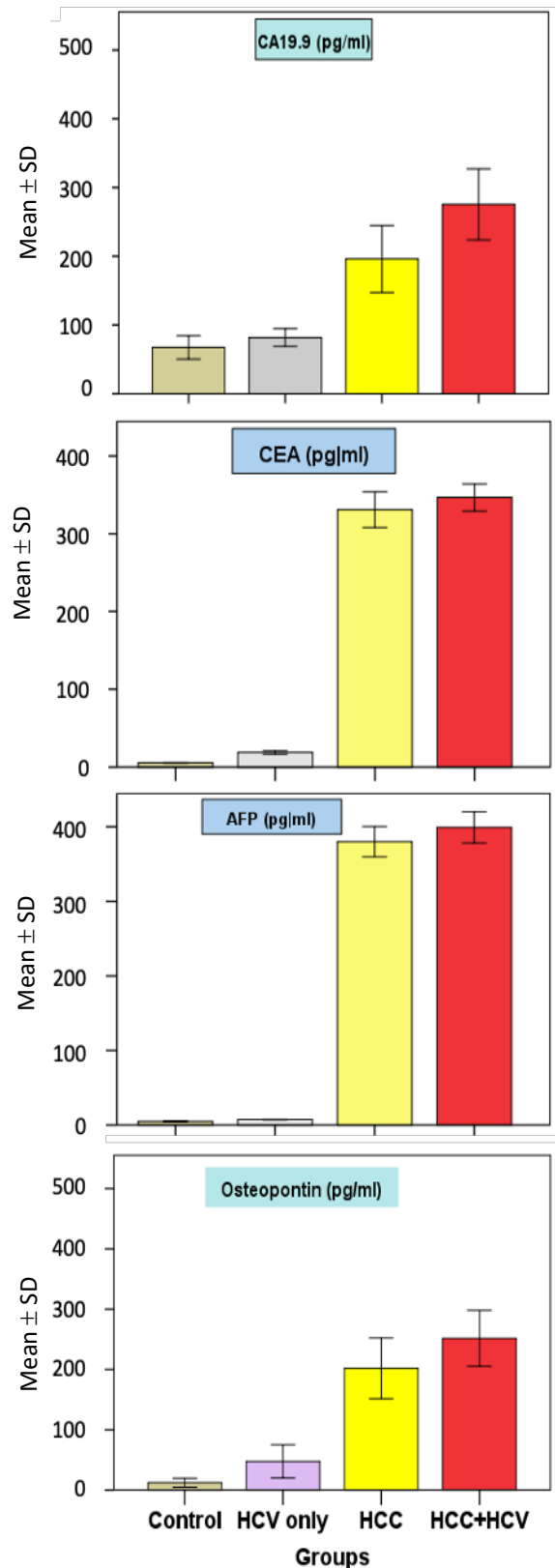
Figure 2. Levels of tumor markers in healthy control in comparison with patients. Serum levels of CA-19.9 (A), CEA (B), AFP (C) and OP (D). Bars represent mean ± SD
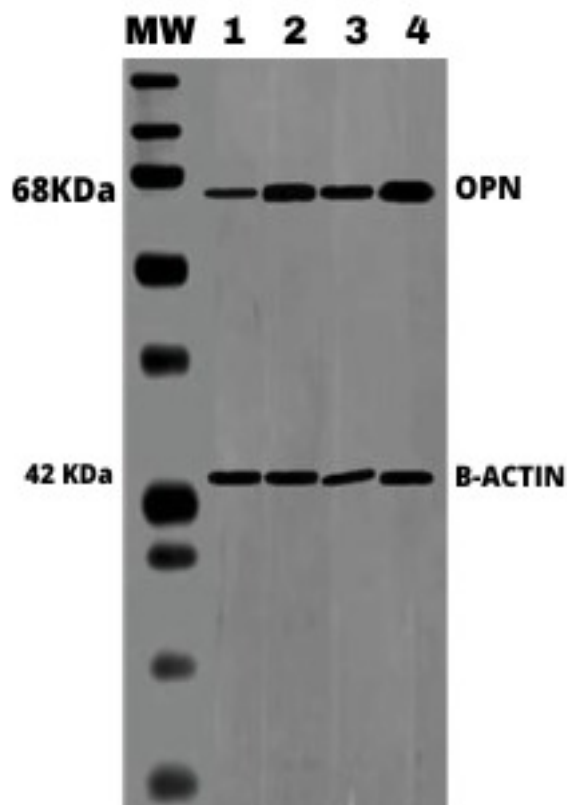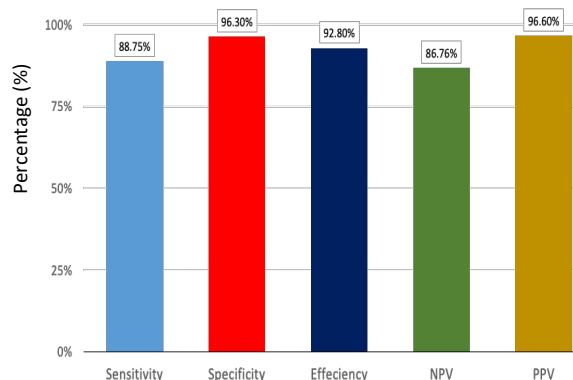
Figure 3. Detection of OPN in sera from infected patients with HCC using western blot.
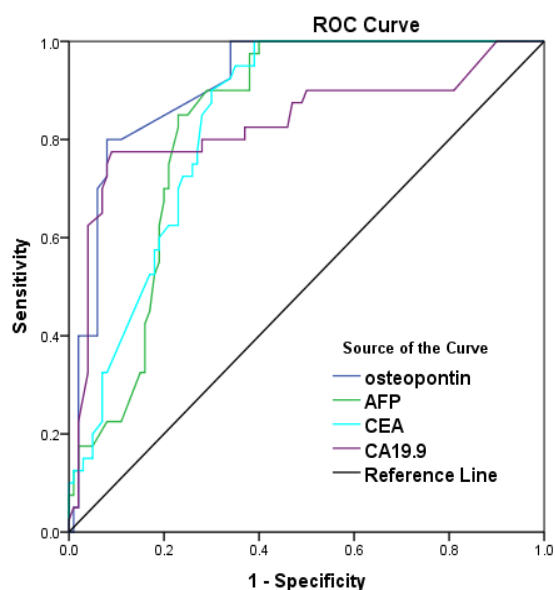
MARKER	Area Under the Curve
Osteopontin	0.913
CEA	0.836
CA19.9	0.833
AFP	0.831

Figure 4. AUROC for OP in comparison with CA19.9, CEA and AFP for predicting Hepatocellular carcinoma.

Figure 5. Overall performance characteristics of OP detection using ELISA. By applying OPN cut-off = 198 pg/mL, significant HCC patients were predicted, with high degree of sensitivity, specificity, NPV and PPV and efficiency (>85%).

Therefore, it's imperative to find novel serologic biomarkers that exhibit enough sensitivity and specificity for early detection of hepatocellular carcinomas and/or negative AFP (Tsuchiya et al., 2015). In clinical research, OP is regarded as a crucial molecule that can be targeted to improve understanding of the pathophysiology, prognosis, and rational design of many diseases, including HCC (Kariya and Kariya, 2022).The current study's objective is to assess the plasma OPN level's potential as an HCC marker. In our study, biochemical laboratory tests (AST, ALT, albumin, and ALP) were analyzed using standard procedures. All evaluated biochemical markers differed significantly. There was an extremely significant difference between AST, ALT, ALB, and ALP level in patients with HCC patients (G3-G4) and non-significant HCC (G1-G2). We used a polyclonal antibody (poAb) specific to OPN and a western blot analysis approach to identify OPN in serum samples of patients with HCC.

The level of OPN as a potential marker of HCC between patients with HCV, in comparison with other tumor markers. Osteoblasts, T lymphocytes, and macrophages release phosphorylated glycoproteins called OP. On Chromosome 4 region 22 (4q22.1) is where the OPN gene is found. Angiogenesis, fibrosis, carcinogenesis, inflammation, and other pathological diseases have all been linked to OPN expression, which has been found in a wide variety of organs.

In conclusion, we found that serum OPN levels were significantly raised in severe HCC patients and could be clearly distinguished from non-HCC. According to our research, OPN may be a potential biomarker for HCC, which could make it a useful diagnosis.

CONFLICT OF INTEREST

All authors state that they have no conflicts of interest.

FUNDING

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