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STUDY OF OSTEOPONTIN AS A NOVELTUMOR MARKER FOR DETECTION OF HEPATOCELLULAR CARCINOMA

By

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most common malignancies and the third most common cause of death from cancer worldwide. Approximately, 80 to 90% of HCC occur in a cirrhotic liver. HCC is now a rather common malignancy in Egypt which usually develops on top of liver cirrhosis secondary to viral infection. Hepatitis C virus increases the risk of HCC in the Egyptian patients. Osteopontin (OPN) is a glycoprotein secreted by cells as osteoblasts, osteoclasts, activated macrophages and T cells. It is over expressed in a variety of human tumors, including carcinomas of stomach, breast**,** prostate, lung, colon**,** and liver. Plasma level of osteopontin may be a biomarker for HCC. **Objective:** This study aimed to evaluate plasma osteopontin (OPN) level and determine its sensitivity and specificity as a screening tool for detection of HCC patients. **Subjects and Methods**: Plasma OPN level was measured in 64 patients(Twenty two patients with non-cirrhotic HCV, twenty two with cirrhotic HCV and twenty HCC patients on top of chronic cirrhotic HCV)in addition to twenty healthy controls. Plasma OPN level using ELISA technique was done to all participants. Also, liver enzymes (AST) and (ALT), serum bilirubin, albumin, alpha-fetoprotein and blood hemoglobin (Hb) were done to all participants. **Results:** The mean plasma osteopontin (OPN) level significantly elevated in patients with HCC group than all groups. The sensitivity and specificity in detection of HCC were 100% and 97% respectively at cut off level of 270 ng/ml with accuracy 95%. **Conclusion:** Plasma OPN level can be used as a routine biomarker for clinical prediction of the recurrence, metastasis, and prognosis in patients with HCC.

Keyword: Osteopontin, hepatitis C virus infection and hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem in Egypt and its incidence is increasing. The majority of cases were in rural areas, and the most were related to HCV infection (91%). The high prevalence of HCV infection makes screening programs and surveillance of those patients a very important tool to early detect cases of small HCCs ***(Abu El-Makaremet al., 2011).***

OPN is a negatively‑charged acidic hydrophilic protein of approximately 300 amino acid residues, and is secreted into

all body fluids. OPN has an arginine - glycine - aspartic acid (RGD) cell binding sequence, a calcium binding site and two heparin binding domains. Synthesis of osteopontin is stimulated by calcitriol ***(Kalmar, 2012)***. In the liver, hepatic Kupffer cells secrete OPN facilitating macrophage infiltration into necrotic areas following carbon tetrachloride toxicity ***(Hillas, 2013).***

The role of ostoepontin (OPN) in HCC has generated significant interest, especially with regards to its role as a prognostic factor. OPN level also increases in a range of inflammatory syndromes ***(Chabas, 2010).*** Recent work has highlighted the role of OPN in inflammatory liver diseases such as alcoholic and nonalcoholic disease and T-cell mediated hepatitis and can be used as a diagnostic biomarker. OPN is an attractive 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 ***(Zhang et al., 2012)***.

The objectives of this study were to study plasma osteopontin level in HCV infected patients without cirrhosis, chronic HCV infected patients with liver cirrhosis, and HCC patients on top of chronic cirrhotic HCV, and to compare all groups with each other and with the normal control, and to verify the possibility of using plasma OPN level as a potential biomarker for HCC.

SUBJECTS AND METHODS

Subjects: The current study included 64 patients aged between35 to 76 years old. Patients were attending the outpatient clinic of Internal Medicine Department of Al-Zaharaa University Hospital, Cairo during the period from 1st of November2013 to 31thof May 2014. Patients were divided into three groups; twenty two HCV patients without cirrhosis, twenty two chronic HCV patients with cirrhosis and twenty patients HCC on top of chronic HCV induced cirrhosis. Twenty healthy subjects with matched sex and age to the patients were included. A verbal consent from the all subjects was taken after explaining the study's purpose, procedures, and possible benefits to them. Any other tumor than HCC and with metastatic HCC, patients with chronic liver diseases other than chronic HCV and patients with inflammatory, autoimmune diseases, diabetes mellitus, and hypertension, renal or cardiopulmonary diseases were excluded from this study.

Methods: All patients and controls were subjected to complete history taking, full clinical examination, laboratory investigations as liver biochemical profile including; serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, albumin, bilirubin, international normalized ratio (INR), alpha fetoprotein (AFP), blood hemoglobin (Hb), and hepatitis viral markers including HCV antibody and HBV surface antigen. Radiological investigations included ultrasonograghy (US) and triphasic computed tomography (CT) scan of the underlying liver disease.

Determination of plasma osteopontin: In all patients and controls, venous blood samples were collected under complete aseptic conditions in a plastic tube containing ethylene diaminetetra acetic acid (EDTA) as an anticoagulant, centri-

fuged at 1000 rpm for 15 minutes within 30 minutes of collection, and isolated plasma sample were stored at -20?Cuntil measurements of osteopontin levels. Plasma osteopontin levels were measured by enzyme-linked immune sorbentassay (ELISA) using recombinant human OPN ELISA kit that was supplied by R & D Systems, Inc. 614 McKinley Place NE Minneapolis, MN 55413 United States of America, Catalog number: DOST00, according to the manufacturer's instructions.

Statistical analysis: Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 20. The quantitative data were presented as means, standard deviations and ranges. The comparison between the studied groups were done by using One Way Analysis of Variance (ANOVA) followed by post hoc analysis-least significant difference (LSD) test. The receiver operating characteristic curve (ROC) was used to assess the best cut off point with its sensitivity, specificity and accuracy. The confidence interval was set to 95% and the margin of error accepted was adjusted to 5%. So, the p-value was considered significant at the level of < 0.05.

RESULTS

The current study was conducted on64 patients ranged from 35 to 76 years old with a mean age of 45.55±16.1 years; forty one patients were males. Twenty healthy control subjects of comparable ages and sex were included.

Table (1): Comparison of laboratory parameters among all studied groups(Mean ± SD).

Groups / Parameters	Controls (n=20)	Non cirrhotic (n=22)	Cirrhotic (n=22)	HCC (n=20)	P-value
ALT(IU/L)	19.1±3.2	40.2±4.3	33.3±11.4	29.7±6.3	**0.001**
AST(IU/L)	14.1±2.9	42.5±19.1	39.4±15.3	25±13.2	**0.001**
T. Bilirubin (mg/dl)	0.90±0.06	0.89±0.29	2.5±0.7	3.5±0.7	**0.001**
Albumin(g/dl)	3.5±0.19	4.1±2.7	3.7±1.4	2.8±0.9	**0.001**
Hb (g/dl)	12±1.8	14±2.5	10±2.6	9±0.9	**0.001**
INR	1.03±0.5	1.06±0.3	1.9±0.8	2.5±0.5	**0.001**
AFP(ng/ml)	8.1±6.3	5.4±1.4	3.2±1.4	7.53±1.7	**0.001**

Post hoc test: LSD (P-values)						
Parameters	**Control vs Non cirrhotic**	**Controls vs Cirrhotic**	**Controls vs HCC**	**Non cirrhotic vs Cirrhotic**	**Non cirrhotic vs HCC**	**Cirrhotic vs HCC**
ALT(IU/L)	0.001	0.001	0.001	0.011	0.001	0.219
AST(IU/L)	0.001	0.001	0.001	0.555	0.001	0.002
T. Bilirubin (mg/dl)	0.880	0.001	0.001	0.001	0.001	0.001
Albumin(gm/dl)	0.327	0.530	0.001	0.540	0.046	0.018
Hb (gm/dl)	0.005	0.006	0.001	0.001	0.001	0.110
INR	0.812	0.001	0.001	0.001	0.001	0.006
AFP (ng/ml)	0.057	0.001	0.698	0.001	0.001	0.001

HCC group had the highest mean level of plasma osteopontin with statistically significant difference in comparison to other groups (Table 2).

Table (2): Comparison of plasma osteopontin level among all studied groups (Mean ± SD)

Groups / Parameter	Controls (n=20)	Non cirrhotic (n=22)	Cirrhotic (n=22)	HCC (n=20)	P-value
Osteopontin (ng/ml)	40.3±5.2	171.5±40.5	260.4±38.6	399.5±69.3	0.001

Post hoc test: LSD(P-values)						
Parameters	**Control vs Non cirrhotic**	**Controls vs Cirrhotic**	**Controls vs HCC**	**Non cirrhotic vs Cirrhotic**	**Non cirrhotic vs HCC**	**Cirrhotic vs HCC**
Osteopontin (ng/ml)	0.001	0.001	0.001	0.001	0.001	0.001

Regarding the diagnostic accuracy of plasma OPN as predictor for cirrhotic liver disease area under the curve was 0.71, at cut off value 170 ng/ml; Plasma OPN showed 91% sensitivity, 62% specificity with accuracy 70%(Table 3 & Figure 1). As regard the diagnostic accuracy of plasma OPN as predictor for HCC, we analyzed the receiver operator characteristic curve (ROC); area under the curve (AUC) was 0.98. At a cut off value 270ng/ml, plasma OPN showed 100% sensitivity, 97% with accuracy 95%. So this lead to that the validity of osteopontin was overall prediction of HCC with high validity and overall accuracy (Table 3 & Figure2).

Table (3): Diagnostic sensitivity, specificity and accuracy of osteopontin in prediction of HCC and cirrhosis

Parameters / Groups	Sensitivity%	Specificity%	Accuracy%	Cut off (ng/ml)
Cirrhosis	91%	62%	70%	170
HCC	100%	97%	95%	270

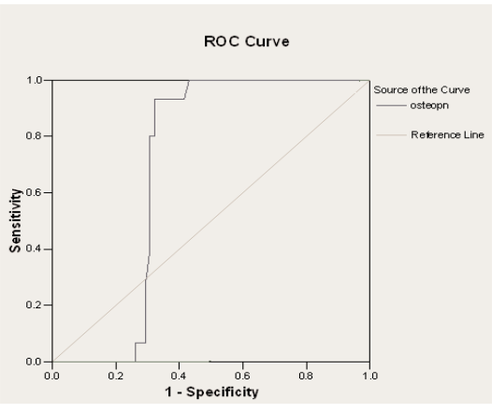

Figure (1): ROC curve analysis of OPN in diagnosis of liver cirrhosis.

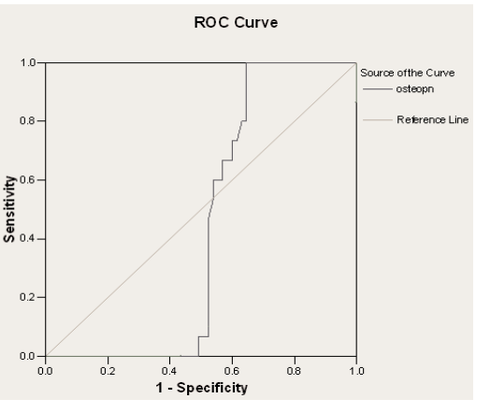

Figure (2): ROC curve analysis of OPN in diagnosis of HCC.

DISCUSSION

Hepatocellular carcinoma (HCC) represents one of the most common primary malignancies of the liver worldwide, with an incidence that varies in the different geographic areas as a consequence of the regional variations in exposure to risk factors for this tumour ***(Hsu et al., 2013 and Santambrogio et al., 2013).*** Major risk factors for development of HCC include chronic infections with HBV, HCV and liver cirrhosis ***(Sherman, 2011 and Sirivatanauksorn*** & ***Tovikkai, 2011).***Early detection of HCC opens doors for various effective treatments such as surgical resection, radiofrequency ablation, and transplantation, which can subsequently lead to long-term survivals in a great number of HCC patients ***(Choi et al., 2012and Shao et al., 2012)***.

Osteopontin (OPN) is a phosphorylated glycoprotein secreted by activated macrophages, leukocytes, and activated T lymphocytes, and is present in extra cellular fluids, at sites of inflammation, and in the extra cellular matrix of mineralized tissues. OPN interacts with a variety of cell surface receptors including several integrin and CD44. Binding of OPN to these cell surface receptors stimulates cell adhesion, migration, and specific signaling functions. It is present in elevated levels in the blood and plasma of some patients with metastatic cancers ***(Barros, 2013).*** OPN is an attractive potential tumor marker, because it exists not only as an immobilized extra cellular matrix molecule but also in a secreted form in body fluids including plasma ***(Zhang et al., 2012)***.

As regard the results of the laboratory parameters in different studied groups, we found a statistically significant difference in ALT and AST levels with the least significant level difference between the control group versus other groups. Also, there was a statistically significant difference in serum bilirubin with the least significant difference between the control

group versus other groups except non cirrhotic and between non cirrhotic versus cirrhotic and HCC. There was a statistically significant difference in albumin level with the least significant difference between control and non-cirrhotic versus cirrhotic and HCC. There was a statistically significant difference in INR between groups with the least significant difference between cirrhotic versus other groups.

Regarding the results of mean level of AFP in the studied groups, it showed that serum level of AFP was significantly higher in HCC patients than all other groups including the cirrhotic group. These findings were in agreement with ***Gad et al.(2005*)** who found a significantly higher sensitivity of AFP in Egyptian patients in comparison with Japanese patients for HCC diagnosis.

By studying plasma OPN level in different groups, we found significant elevation of plasma osteopontin levels in HCC patients than cirrhotic HCV patients' levels, and lower levels in normal control group. Plasma osteopontin level was higher in cirrhotic HCV patients than non-cirrhotic group. ***El-Din Bessa et al.(2010), Abu El Makarem et al.(2011), and Zhang et al.(2012)*** found that the median plasma OPN level was significantly higher in the HCC group than in the cirrhotic patients or in the normal control group. Also, ***Kim et al.*(*2006)*** showed that median plasma OPN level in the chronic liver disease group was significantly higher than that of healthy controls. A possible proinflammatory role of OPN in chronic hepatitis and cirrhosis is suggested.

As regard the diagnostic accuracy of plasma OPN as predictor for cirrhotic liver disease, the cut off value of 170ng/ml, plasma OPN showed 91% sensitivity, 62% specificity, with accuracy 70% .In our study sensitivity, specificity of OPN for selective detection of HCC group were 100% and 97% respectively at cut off value of 270ng/ml with accuracy 95%. So, osteopontinhas good validity and overall accuracy in prediction of both liver cirrhosis and HCC. The previous results were in agreement with a study done by ***Kim et al. (2006)*** who reported diagnostic sensitivity and specificity of OPN for HCC group over non-HCC group (CLD group and healthy control) to be 93.5% and 84.2%, respectively, at a cut-off level of 552.9ng/ml. Results of our study were in accordance also with the study done by ***Abu El-Makarem et al. (2011).*** They found that the diagnostic efficacy of OPN was superior to AFP in terms of sensitivity and specificity. The sensitivity, 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 300ng/ml.

In agreement with our results, ***Keddeas and Abo-Shady (2011)*** found that plasma OPN level was significantly higher in patients with HCC compared to CLD and control participants. OPN at the best cutoff value (325.5 ng/ml) had sensitivity of 87.5% and specificity of 80% for detection of HCC cases (area under the

curve = 0.876). Also, ***Sufen et al. (2012)***compared HCV patients associated HCCs with HCV patients associated cirrhosis, the AUC for AFP was 0.64, whereas OPN had an even higher AUC (0.80).The sensitivity and specificity of plasma OPN levels in HCV associated HCCs relative to HCV-associated cirrhosis were 82% and 65% respectively, at a cut-off value of 91ng/ml. For AFP at a cut-off value 20ng/mL, the values of sensitivity and specificity were 46% and 88% respectively.

In the view of current data, the present study provided the evidence that the plasma OPN level could serve as a prognostic indicator for patients with HCC, and can be used in early detection and diagnosis of HCC. Also, OPN is superior to the traditional tumor biomarkers as AFP in diagnosing liver cirrhosis, a chronic liver disease which is risk factors for HCC.OPN level is a good prognostic value for HCC, especially among high-risk group of patients. The results obtained in this study will be valuable for the future application of plasma OPN level as a routine biomarker for clinical prediction of the recurrence, metastasis, and prognosis in patients with HCC. Greater number of patients is recommended to gain greater insight into potential usefulness of OPN in patients with HCC especially those with normal levels of AFP.

REFERENCES

1. **Abu El Makarem MA, Abdel-Aleem A, Ali A, Saber R, Shatat M, Rahem DA and Sayed D (2011):** Diagnostic significance of plasma osteopontin in hepatitis C virus-related hepatocellular carcinoma. Annals of Hepatol., 10(3):296-305.
2. **Barros NM (2013):** Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia. J. Bone Miner. Res., 28 (3): 688–99.
3. **ChabasD (2010):**The influence of the pro-inflammatory cytokine, osteopontin, on auto-immune demyelinating disease. Science, 294 (5547): 1731–5.
4. **Choi JG, Chung YH and Kim JA(2012):**High HBV-DNA titer in surrounding liver rather than in hepatocellular carcinoma tissue predisposes to recurrence after curative surgical resection. J. Clin. Gastroenterol.,46(5):413-9.
5. **El-Din Bessa SS, Elwan NM, Suliman GA and El-Shourbagy SH (2010):** Clinical significance of plasma osteopontin level in Egyptian patients with hepatitis C virus-related hepatocellular carcinoma. Arch Med Res., 41(7):541-547.
6. **Gad A, Tanaka E, Matsumoto A, Serwah A, Attia F and Hassan A (2005):** Ethnicity affects the diagnostic validity of alpha-fetoprotein in hepatocellular carcinoma. Asia-Pacific Journal of Clinical Oncology, 1: 64-70.
7. **Hillas G(2013):**Increased levels of osteopontin in sputum supernatant of smoking asthmatics. Cytokine, 61 (1): 251–5.
8. **Hsu CY, Lee YH, Hsia CY, Su CW and Lin HC(2013):**Performance status in patients with hepatocellular carcinoma: determinants, prognostic impact, and ability to improve the Barcelona. Clinic Liver Cancer system. Hepatology, 57(1):112-9.
9. **Kalmar L(2012):**Structural disorder in proteins brings order to crystal growth in biomineralization. Bone, 51 (3): 528–34.
10. **Keddeas MW and Abo-Shady RA (2011):** Evaluation of plasma osteopontin level as a biomarker for hepatocellular carcinoma in

Egyptian patients. Egyptian Liver Journal, 1:38–42.

11. Kim J, Ki SS, Lee SD, Han CJ, Kim YC, Park SH and Cho SY (2006): Elevated plasma osteopontin levels in patients with hepatocellular carcinoma. Am. J Gastroenterol., 101(9):2051-2059.

12. Santambrogio R, Salceda J, Costa M, Kluger MD, Barabino M and Laurent A(2013): External validation of a simplified BCLC staging system for early hepatocellular carcinoma. Eur J SurgOncol.,39(8):850-7.

13. Shao YY, Lu LC, Lin ZZ, Hsu C, Shen YC and Hsu CH(2012): Prognosis of advanced hepatocellular carcinoma patients enrolled in clinical trials can be classified by current staging systems. Br J. Cancer, 107(10):1672-7.

14. Sherman M(2011): Hepatocellular carcinoma: screening and staging. Clin. Liver Dis., 15(2):323-34, vii-x.

15. Sirivatanauksorn Y and Tovikkai C (2011): Comparison of staging systems of hepatocellular carcinoma. HPB Surg., 818(21)2165-70.

16. Sufen Shang, AmeliePlymoth, ShaokuiGe, Ziding Feng, Hugo R. Rosen, Suleeporn Sangrajrang, Pierre Hainaut, Jorge A. Marrero and Laura Berett (2012): Identification of Osteopontin as a Novel Marker for Early Hepatocellular Carcinoma Hepatology, 25(4):695-705.

17. Zhang CH, Xu GL, Jia WD, Ge YS, Li JS, Ma J Land Ren WH (2012): Prognostic significance of osteopontin in hepatocellular carcinoma: a meta-analysis. Int J Cancer, 130(11):2685-2692.

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خلفيه البحث: الإصابة بفيروس سى المزمن هو السبب الرئيسي لتليف وسرطان الكبد، والذى هو ثالث أسباب الوفاة فى العالم، وبسبب زيادة معدل حدوثه والتدهور السريع الذى يحدث للحالات فقد أصبح من أهم الأسباب التى تؤدى إلى الوفاة، كما يعتبر سرطان الكبد واحدا من الأورام القليلة التى يمكن التعرف فيها على مسبباته. وتعتبر مصر واحدة من البلدان النامية ذات الموارد المحدودة وبرامج الفحص بإستخدام وسائل منخفضة التكلفة، عالية الدقة للكشف المبكر عن سرطان الكبد،وقد أوضحت الحاجة إلى دلالات أورام جديدة لسرطان الكبد و التي ينبغي أن تكون حساسة ومحددة، ويمكن إستخدامها كأداة واحدة لتشخيص سرطان الكبد.ودلالات الأورام هى مواد تتكون وتفرز بواسطة الخلايا السرطانية وتعتبر غير نشطة حيويا ولا تتواجد بصورة طبيعية بالجسم، وإذا وجدت فهى دائما بكميات قليلة غير مؤثرة، وعدد قليل من هذه المواد يفرز بتركيزات عالية، ولذا يمكن إستخدامها والإستفادة منها فى تشخيص الحالات مبكرا والمتابعة المستمرة للحالات.

والأوستيوبونتين هو بروتين سكرى يفرز من خلايا البلعوم الكبرى النشطة،وخلايا الدم البيضاء،والخلايا الليمفاوية T، ويزداد فى أورام بشرية متنوعة تشمل سرطان المعدة، والثدى، والرئة، والبروستاتا ، والقولون، والكبد.

هدف البحث: تقييم مستوى مادة الأوستيوبونتين فى البلازما في المرضى الذين يعانون من سرطان الكبد وتحديد حساسيتها وخصوصيتها كأداة فحص للكشف عن سرطان الكبد.

مواد وطرق البحث:شملت الدراسة على 64 مريضا منقسمة إلى 3 مجموعات:مجموعة فيروس سي بدون تليف الكبد(22 مريضا) ومجموعة تليف الكبد نتيجة الإصابة بفيروس سى غير المرضى بسرطان الكبد(22 مريضا) والمجموعة الثالثة تشمل20 مريضا يعانون من سرطان الكبد. كما كانت هنا كمجموعة مقارنه (20 شخصا) من الأصحاء. وتم قياس الهيموجلوبين والبيليروبين ، والزلال، وسرعة التجلط، ووظائف كبد ، وألفا فيتو بروتين والأوستيوبونتين للمشاركين.

النتائج: أثبتت النتائج أن ألفا فيتو بروتين فى المصل أعلى في مجموعة سرطان الكبد عن مجموعة تليف الكبد بدلالة إحصائية بين مجموعة تليف الكبد وسرطان الكبد.وكان متوسط الأوستيوبونتين أعلى في مجموعة سرطان الكبد عن مجموعة تليف الكبد بدلالة إحصائية بين مجموعة تليف الكبد وسرطان الكبد، وأيضا كان أعلى في مجموعة تليف الكبد عن مجموعة فيروس سي بدون تليف وكان بدلالة إحصائية . وكانت الحساسية 100%، والنوعية97% للأوستيوبونتن لتشخيص سرطان الكبد.

الاستنتاج: يمكن إستخدام الأوستيوبونتين كعلامة جيدة جديدة للكشف المبكر عن سرطان الكبد.