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RESEARCH ARTICLE

EVALUATION OF OSTEOPONTIN AND ALFA L-FUCOSIDASE AS DIAGNOSTIC MARKERS OF HEPATOCELLULAR CARCINOMA.

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Abstract

Introduction: Alpha fetoprotein (AFP) is not secreted in all cases of hepatocellular carcinoma (HCC), it may be normal in 40% of patients. Alpha-L-fucosidase (AFU) and osteopontin (OPN) have been suggested as tumor markers of HCC. This study aimed to evaluate the diagnostic values of AFU & OPN as tumor markers of HCC in hepatitis C virus patients. **Methods:** This case-control study was conducted on 60 patients (25 patients with HCC, 20 patients with chronic hepatitis and 20 patients with cirrhosis) in addition to 20 apparently healthy individuals who served as a healthy control group.

Results: AFP, OPN and AFU levels were significantly higher in the HCC group compared to chronic hepatitis, liver cirrhosis and healthy control groups ($p < 0.001$ in each) and in liver cirrhosis compared to the control group ($p < 0.01, p < 0.001, p < 0.001$) respectively. There was a statistically significant increase in AFU level in the liver cirrhosis compared to the chronic hepatitis group ($p < 0.001$), while there was no significant difference between both groups as regards AFP and OPN ($p > 0.05$). In patients with HCC, AFP level was significantly higher in TNM stage III than TNM stage II ($P < 0.001$). The AFP sensitivity was 75%, OPN sensitivity was 75% and AFU sensitivity was 70%. Combined use of OPN, AFP produced 80% sensitivity while combined use of AFU, AFP produced 90% sensitivity. Combined use of OPN, AFP and AFU produced 100% sensitivity.

Conclusion: Combined detection of OPN and AFU activity may be used as diagnostic markers for the diagnosis of HCC. Combined detection of AFU, OPN and AFP could improve early diagnosis of HCC and differentiate it from chronic liver disease.

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Introduction:-

Hepatocellular carcinoma (HCC) is considered the fifth commonest cancer in the world, and the third oncological cause of death. HCC is the major cause of mortality in patients with chronic liver diseases⁽³¹⁾. In Egypt, HCC represents 14.8% of all

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cancer mortality, with a higher incidence in males (17.3%) than in females (11.5%). Chronic hepatitis usually leads to the sequential occurrence of liver fibrosis and cirrhosis with a high risk of development of HCC^(3,9). It has been estimated that 20% of HCV-infected patients develop liver cirrhosis and approximately 40% of these develop HCC within 10-15 years⁽⁸⁾. Alpha-fetoprotein (AFP) is widely used as a screening test for HCC among patients with cirrhosis, despite its limited performance, particularly in early-stage HCC⁽¹⁰⁾. Other markers (e.g., lectinbound AFP [AFP-L3], des-gamma carboxyprothrombin (DCP) and glypican-3 have been proposed for HCC detection^(32,36). However, neither DCP nor AFP-L3 presented better performance characteristics than AFP for the diagnosis of early-stage HCC. American hepatopathologists treat every tumor larger than one cm in diameter in cirrhotic liver as HCC, consequently ignoring AFP serology (very frequently false negative)⁽²⁴⁾. Alpha-L-fucosidase (AFU) is a sort of enzyme to hydrolyze fucoseglycosidic linkages of glycoprotein and glycolipids. Its activity increases obviously in the serum of HCC patients compared with that in the serum of healthy adults, patients with cirrhosis and patients with chronic hepatitis⁽²³⁾. Osteopontin (OPN) is an integrin-binding glycoposphoprotein that is expressed by several cell types especially by transformed malignant epithelial cells and believed to be involved in many physiological cellular functions especially in regulation of migration, invasion and thus metastasis as well as survival of tumor cells⁽⁴⁾. OPN overexpression indicates a poor prognosis for patients with HCC, it may also have predictive role for HCC invasion and metastasis⁽⁴⁾. In our study we aimed to evaluate AFP, AFU and OPN together in order to improve sensitivity of HCC detection.

Subjects and Methods:-

This study has been conducted on 60 patients who attended clinics and admitted to the Internal Medicine and Oncology departments of Zagazig University Hospitals between February 2013 and May 2014. The plan for secondary data analysis was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Zagazig University, and all the patients gave their informed consent prior to their inclusion in the study. Inclusion criteria, patients with HCC, liver cirrhosis and chronic hepatitis were included in the study; all patients were HCV positive. Exclusion criteria were patients with HBV infection, cardiovascular, chest or renal diseases or patients receiving chemotherapy or radiotherapy.

Subjects:-

Studied subjects were divided into three groups; Control group: included 20 healthy volunteers (14 males and 6 females) with age range from 39 to 58 years with mean value \pm SD of 50.56 \pm 7.91 years. Chronic hepatitis group: included 20 patients (13 males and 7 females) with age range from 39 to 57 years with mean value \pm SD of 48.25 \pm 9.07 years. Chronic cirrhosis group: included 20 patients (14 males and 6 females) with age range from 39 to 59 years with mean value \pm SD 49.5 \pm 10.5 years. Hepatocellular carcinoma group: included 25 patients (16 males and 9 females) with age range from 43 to 57 years with mean value \pm SD of 54.0 \pm 8.56 years. According to American Joint Committee on Cancer (AJCC) Staging System this group was subdivided to TNM stage II and TNM stage III (tumor, node, and metastasis)⁽²²⁾. Abdominal ultrasonography, computerized tomography (CT) and laboratory investigations (liver functions, CBC and AFP) were done for all patients to prove the presence of chronic liver diseases or cirrhosis. Clinical assessment of liver disease severity was done by Child Pugh score based on serum albumin, bilirubin, prothrombin time, the presence of ascites and encephalopathy⁽²⁸⁾. HCC diagnosis was based on a clinical examination, triphasic abdominal computed tomography and the serum AFP level. HCV infection was diagnosed by real time PCR on Cobas[®] AmpliPrep/ Cobas[®] TaqMan[®] (Roche diagnostics- Switzerland). Peripheral blood was collected from each participant. Sera and plasma were stored at -80 °C until measurements of alpha L. fucosidase and OPN. The following laboratory tests were performed, liver function tests were done on Cobas 6000 Roche diagnostics- Switzerland and while AFP level was measured on Cobas E411 immunoassay Roche Diagnostics, USA. Complete blood count was performed on Sysmex-Kx-21 (Sysmex Corporation- Japan) and INR was performed on Sysmex CA-1500 instruments (Sysmex Corporation - Japan). The AFU assay was done on serum samples by a kit from Bio Supply UK, based on the enzymatic cleavage of the synthetic substrate 2-chloro-4-nitrophenyl- α -L-fucopyranoside to α -L-fucoside and 2-chloro-4-nitrophenol, which was quantified by measuring the absorbance at 405 nm in a kinetic fashion. One unit of AFU is defined as the amount of AFU that cleaves one μ mole of 2-chloro-4-nitrophenyl- α -L-fucoside per min at 37 °C with Intra assay CV% < 5.1%, Inter assay CV% < 6.2%⁽⁴⁴⁾. Plasma concentrations of OPN were measured by quantitative sandwich enzyme immunoassay technique (ELISA) according to the protocol provided by the manufacturer (R&D Systems, Minneapolis, USA). The optical density was measured at 450 nm using a micro plate reader. With an intra-assay CV 2.9% and inter-assay CV 5.4%.

Statistical analysis:-

Mean and standard deviation were used for descriptive statistics. Analysis of variance (ANOVA) and Least Significant Difference (LSD) were conducted for a comparison of parameters concentration levels among the different groups of subjects included. A comparison between two groups was performed using the student t-test. Receiver operating

characteristics (ROC) analysis was used to evaluate the diagnostic value of OPN, AFU and AFP to identify the cutoff values, sensitivity, specificity, positive and negative predictive values of OPN, AFU and AFP. Calculations were done with the Statistical Package for the Social Sciences version 19 (SPSS, Inc., Chicago, IL, USA).

Results:-

Table 1: Showed that, there were significant differences between all studied groups as regard liver function tests. Liver function tests were significantly difference in the HCC group compared to chronic hepatitis, liver cirrhosis and healthy control groups ($p < 0.001$ in each) except ALP & bilirubin there was no difference between HCC and cirrhotic groups ($p > 0.05$) while there was no significant difference between HCC and control groups as regard INR ($P > 0.05$). There was significantly differences between chronic hepatitis and cirrhotic groups ($p < 0.001$) except AST and ANR ($p > 0.05$).

Table 2: Presents the comparison of AFP, AFU, and OPN among the studied groups. There were statistically high significant difference in the mean values of AFP (ng/ml) ($p < 0.0001$), AFU (U/l) ($p < 0.001$) and OPN. (ng/dl) ($p < 0.001$). AFP, AFU and OPN levels were significantly higher in the HCC group compared to chronic hepatitis, liver cirrhosis and healthy control groups ($p < 0.001$ in each) and in liver cirrhosis group compared to the control group ($p < 0.01$, $p < 0.001$, $p < 0.001$) respectively. There was significantly increased in AFU level in the liver cirrhosis compared to chronic hepatitis group ($p < 0.001$), while there was no significant difference between both groups as regard to AFP and OPN ($p > 0.05$ in each). AFP level was significantly higher in the chronic hepatitis group compared to control group ($p < 0.01$) while there was no significant difference between both groups as regard to AFU and OPN ($p > 0.05$ in each).

Table 3: AFP was significantly elevated in TNM stage III than TNM stage II of HCC ($t = 4.6$, $p < 0.001$). while there was no significant difference between stage III and stage II as regard AFU and OPN ($t = 0.69$, $p > 0.05$), ($t = 0.91$, $p > 0.05$) respectively.

Table 4: Presents the diagnostic performance of AFP, APO and AFU in diagnosis of HCC, At cutoff value 200 ng/ml, AFP had 65% sensitivity, 80% specificity, this mean 35% of HCC patients gave false negative, However 100% sensitivity and 93.8% specificity were obtained in combination between AFU, OPN and AFP. Receiver operator characteristic (ROC) curves showed that the area under the curve (AUC) for OPN and AFU was 0.85 with 95% confidence interval (CI) (0.75 - 0.95) and 0.87: 95% CI (0.56 - 1), respectively. (Figure 1, 2).

Table 1: Comparison between studied groups as regards biochemical parameters

Parameters	Control n=20 X±SD	Chronic hepatitis n=20 X±SD	Liver Cirrhosis n=20 X±SD	HCC n=25 X±SD	P. Value
Bilirubin (mg/dL)	0.56±0.17	1.18±0.72	2.52±0.85	2.57±1.4	<0.001
Albumin(g/dL)	4.43±0.49	3.91±0.39	2.8±0.54	3.11±0.54	<0.001
AST (IU/L)	19.1±7.09	90.1±19.9	79.7±14.2	105.1±33.5	<0.001
ALT (IU/L)	20.03±5.88	108.5±19.9	62.4±17.9	164.6±17.9	<0.001
ALP (IU/L)	196.4±31.1	199.8±45.8	265.3±55.4	255.1±79	<0.001
INR	1.05±0.1	1.77±0.36	1.79±0.4	1.14±0.14	>0.05

LSD test was carried out between two groups, HCC group versus control group: Bilirubin ($P < 0.001$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P < 0.001$), ALP ($P < 0.001$), INR ($P > 0.05$). HCC vs. chronic hepatitis group: Bil. ($P < 0.001$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P < 0.01$), ALP ($P < 0.001$), INR ($P < 0.01$). HCC vs. cirrhotic group: Bil. ($P > 0.05$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P < 0.01$), ALP ($P > 0.05$), INR ($P < 0.01$). Chronic hepatitis vs. control: Bil. ($P > 0.05$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P < 0.01$), ALP ($P > 0.05$), INR ($P < 0.01$). Cirrhosis vs. Control: Bil. ($P < 0.01$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P < 0.01$), ALP ($P < 0.01$), INR ($P < 0.01$). Cirrhosis vs. chronic hepatitis: Bil. ($P < 0.01$), albumin ($P < 0.01$), ALT ($P < 0.001$), AST ($P > 0.05$), ALP ($P < 0.001$), INR ($P > 0.05$).

Table 2: Comparison of AFP, OPN, and AFU among studied groups

Parameters	Control (n = 20)	chronic hepatitis (n = 20)	Cirrhosis (n = 20)	HCC (n = 25)	P. value
AFP (ng/ml) X±SD	4.48±0.29	49.05±9.3	68.95±18.6	456.3±153	<0.0001
OPN (ng/dl) X±SD	85.4±32	91.4±31.2	116.6±40.4	540.9±87.5	<0.001
AFU (U/L) X±SD	16.4±5.7	22.7±7.6	40.6±11.4	83.9±31	<0.001

AFP: Alpha fetoprotein, OPN: Osteopontin, AFU: Alpha-L-fucosidase. LSD test was carried out between two groups, HCC group versus control group: AFP (P = 0.000), AFU (P = 0.000), OPN (P = 0.000). HCC vs. cirrhosis: AFP (P = 0.000), AFU (P < 0.001), OPN (P = 0.000). HCC vs. chronic hepatitis group: AFP (P = 0.000), AFU (P < 0.001), OPN (P = 0.000). Cirrhosis vs. control group: AFP (P < 0.01), AFU (P < 0.001), OPN (P < 0.01). Cirrhosis vs. chronic hepatitis: AFP (P > 0.05), AFU (P < 0.001), OPN (P > 0.05). Chronic hepatitis vs. Control group: AFP (P < 0.01), AFU (P > 0.05), OPN (P > 0.05).

Table 3: Relation between TNM classification, AFP, AFU, and OPN within HCC group.

Parameters	TNM (II) n=15	TNM (III) n=10	t	P
AFP(ng/ml) X±SD	368.33 ±122.83	588.25 ±79.68	4.46	<0.001
OPN (ng/dl) X±SD	557.1±89.4	521.1±86.1	0.91	>0.05
AFU (U/L) X±SD	87.6±25.3	79.3±21.2	0.69	>0.05

TNM: (tumor, node, metastases), AFP: Alpha fetoprotein, OPN: Osteopontin, AFU: Alpha-L-fucosidase

Table 4: Diagnostic Performance of AFP, OPN and AFU in diagnosis of HCC.

Variables	Cut-Off	Sensitivity	Specificity	PPV	NPV	Accuracy
AFP(ng/ml)	200	65%	80%	100%	90.3%	91.8%
OPN(ng/dl)	170	75%	73.8%	47.9%	90.6%	95.3%
AFU (U/L)	64	70%	86.2%	60.9%	90.3%	82.4%
Combined AFP&AFU	AFP=200 AFU=64	90%	80%	58.6%	96.3%	82.4%
Combined AFP& OPN	AFP=200 OPN=170	80%	80%	55.2%	92.9%	80%
Combined OPN& AFU	AFU=64 OPN=170	100%	93.8%	83.3%	100%	95.3%
Combined AFP, OPN& AFU	AFU=64 OPN =170 AFP=200	100%	93.8%	83.3%	100%	95.3%

PPV: Positive predictive value, NPV: Negative predictive value, AFP: Alpha fetoprotein, OPN: Osteopontin, AFU: Alpha-L-fucosidase,

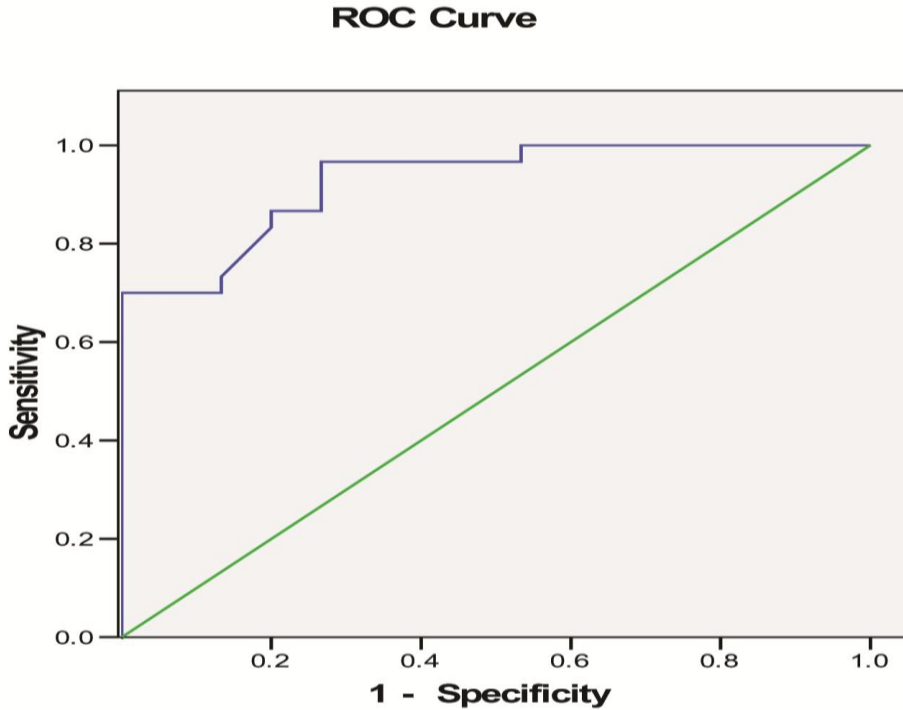


Figure 1: ROC curve of OPN. The curves show cut-off value for OPN of 170 ng/dl, AUC was 0.8: 95%CI(0.75 - 0.95)

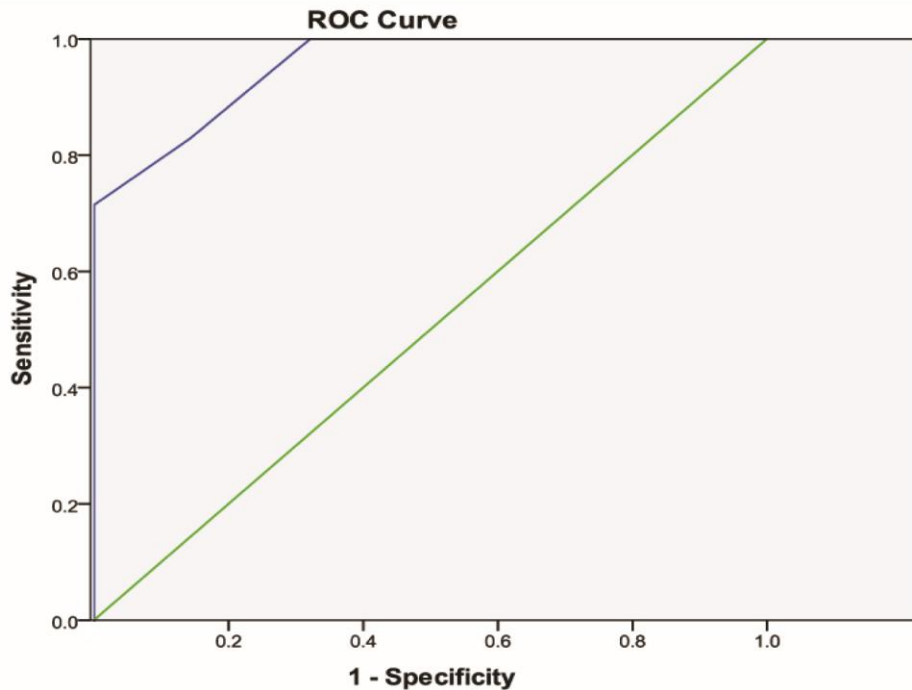


Figure 2: ROC curve of AFU. The curves show cut-off value for AFU of 64 U/L, AUC was 0.87: 95% CI (0.56 -1)

Discussion:-

Patients with chronic liver disease and cirrhosis should be carefully monitored for the early detection of HCC. AFP is the main tumor marker of HCC, Even in patients with advanced HCC, the AFP levels may remain normal in 15–30% of the patients. Therefore, it is of maximum importance to identify sensitive biomarkers that allow the prediction of HCC

development at an early stage and at the same time are easily measurable and minimally invasive^(7,32). Our study investigated the concept of a combined detection using several markers in order to support the detection of HCC using AFP. In this study serum levels of ALT, AST and Bilirubin were higher in HCC group than other groups, that was in agreement with Bruix and Sherman⁽³⁰⁾ who showed that the serum level of ALT and AST were elevated in HCC especially the advanced cases; and the difference becomes greater as the disease progresses. Hepatocellular carcinoma can cause either obstructive jaundice or hepatocellular jaundice (30). In current study there were elevation of AFP levels in HCC patients compared to cirrhotic and chronic hepatitis patients, these results were supported by Nagwa et al.⁽²⁷⁾ who found that the mean concentration of AFP was significantly high in untreated patients with HCC as compared to patients with chronic liver disease. Marrero et al.,⁽²⁴⁾ found that elevation of AFP levels in patients with cirrhosis or exacerbations of chronic hepatitis while it may be normal in up to 40% of patients with HCC, particularly during the early stages, this disagreement may be due to discrepancy between cases number and stages of liver disease. In our study there were elevation of OPN levels in cirrhotic patients compared to chronic hepatitis patients and healthy subjects. Urtaşun et al.⁽³⁵⁾ reported that the OPN levels were significantly higher in HCV-cirrhotic patients compared to healthy individuals. OPN expression increases in tumor genesis, angiogenesis and in response to inflammation, cellular stress and injury⁽¹²⁾. However OPN were statistically elevated in patients with HCC compared to patients with cirrhosis or chronic hepatitis, these results were in accordance with Bessa et al.⁽⁵⁾ who found that OPN level was significantly higher in HCC patients group than the pathological and healthy controls. The mean value of AFU in patients of HCC in this study was significantly higher than those found in patients of cirrhosis, chronic hepatitis and control, this was in agreement with^(34,38). One possible explanation for the increase of AFU is an increased synthesis of proteins by tumor with a consequent increase in fucose turnover. Grizzi et al.⁽¹⁵⁾ and Ishizuka et al.⁽¹⁸⁾ demonstrated the activity of AFU was increased in the sera of HCC patients compared with chronic liver disease and healthy individuals. In this study we showed that HCC stage III had significantly high AFP more than HCC stage II these results supported by Shian-Yang et al.⁽³¹⁾ they reported that HCCs with high AFP had more frequent large size (> 5 cm) and high-grade tumors as compared to those with lower AFP. In our study there were no significant relation between AFU levels and tumor stages these may be due to most of the HCC patients had an early stage tumor (60%, TNM stage II). In agreement with our results, El-Houseiniet al.⁽¹¹⁾ showed no significant relation between AFU and tumor size. The liver contains various cell types that produce cytokines and chemokines⁽²¹⁾, which up regulate AFU in the later stages of inflammation, this process is consistent with activation of a natural regulatory loop, resulting in a gradual reduction of the potential of blood-borne leukocytes to enter the endothelium at the sites of current inflammation⁽²⁾. This anti-inflammatory immune-suppressive response may stimulate HCC metastases. In the present study there were no significant difference between tumor stages and plasma OPN levels this may be due to small number of cases specially advanced HCC, this was not in accordance with Lee et al.⁽²⁰⁾ who found that plasma OPN levels increased significantly in patients with large and multiple tumors and distant metastasis. In addition, plasma OPN levels were increased significantly with advanced tumor stages and with tumor recurrence these studies suggested that plasma OPN levels could be used as a prognostic and predictive marker for HCC^(19, 39, 41). 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⁽²⁵⁾. In the current study for HCC diagnosis, AFP had sensitivity 65% at cut off value 200ng/ml this means that 35% of the studied patients with HCC are negative for AFP. Our findings are in concordance with reports by Bruix J⁽⁸⁾ and Spangenberg et al.⁽³²⁾ who showed AFP levels do not discriminate between benign liver disease and HCC. Additionally, they have poor sensitivity and specificity and vary with the etiology of liver disease, treatment and tumor stage. We found that the sensitivity, specificity and diagnostic accuracy of AFU at the cut-off value of 64 U/L were 70, 86.2 and 82.4%, respectively. Montaser et al.⁽²⁵⁾ reported that 87.5 % sensitivity and 98 % specificity of AFU at 25 U/L cutoff value, at a cut-off value of 2.3005 $\mu\text{mol l}^{-1} \text{min}^{-1}$, AFU yielded a sensitivity and specificity of 90% and 97.5%, respectively⁽²⁶⁾. In our study a relatively differ cut off values of AFU compared to other studies mostly due to the difference in cases number. AFU has been recommended as a serum biomarker for HCC in some studies. The present study shows that the concentration of AFU is significantly higher in patients with HCC than patients with benign liver diseases and control subjects; In addition, AFU recorded 70% sensitivity and 86.2% specificity showing its validity as a diagnostic biomarker of HCC. Therefore, AFU activity may be regarded as a biomarker for the diagnosis of HCC. Zhao et al.⁽⁴²⁾ showed that, AFU may be a valuable supplementary marker in HCC detection. However, the specificity of AFU is relatively poor, and is also overexpressed in diabetes, pancreatitis and hypothyroidism patients. The activity of AFU is also susceptible to ethnicity. Therefore, the clinical value of AFU requires additional investigation.

We have been reported that the combined detection of AFP and AFU can improve the sensitivity of HCC detection to 80%, while specificity slightly not changed; this finding came in agreement with BO Tao et al.⁽⁶⁾. And Huang Xingang et

al.⁽¹⁷⁾ they demonstrated that combined use of AFU and AFP will improve the sensitivity and accuracy of diagnosis of primary hepatic carcinoma. The combined use of AFP and AFU can improve the sensitivity, specificity and diagnostic accuracy of AFP from 70, 85 and 79.7%, respectively, to 95, 100 and 99.1%, respectively⁽²⁶⁾. The combined detection of AFU with other tumor markers should be commonly used in clinical practice to improve the diagnostic sensitivity of AFU⁽⁴³⁾. In our study, plasma OPN sensitivity, specificity and diagnostic accuracy were 75, 73.8 and 95.3%, respectively at a cut off value 170 ng/dl. Abu El Makarem et al.⁽¹⁾ who studied the diagnostic performance of OPN level for discrimination of the HCC from chronic liver disease and healthy subjects found that the sensitivity, specificity, PPV and NPV were 97.7%, 100%, 100%, 97.6% respectively at a cut off value 300ng /ml. In the current study the combined detection of AFP and OPN can improve the sensitivity and specificity of HCC detection to 80 % and 80%, respectively Hafez et al.⁽¹⁶⁾ found that diagnostic performance of OPN for discrimination of the HCC patients was 100% in all aspects of diagnostic performance at a cut off value 2000pg/ml.

In this study OPN and AFU levels are elevated in HCC patients than other liver diseases, these results suggest that OPN and AFU together can use as diagnostic markers for HCC. In addition combined use of AFU and OPN can improve the sensitivity (100 %) and specificity (93.8%) of HCC detection, the AFP does not add any value to diagnosis when combined with them. Although has to be considered AFP the gold standard for HCC tumor markers and serve as an important tool in the monitoring of HCC patients, its performance in early stage of HCC is deficient^(34,36). OPN is expressed by several cell types especially by transformed malignant cells⁽⁴⁾, AFU activity is increased in another diseases rather than liver diseases as diabetes⁽⁴²⁾, Consequently, in order to improve the diagnostic performance for HCC, the combination of AFP with other serum tumor markers is recommended in the diagnosis of HCC^(12,41). Previous studies have proven that a combination of serum tumor markers had a better diagnostic performance for HCC^(12, 14, 41), to avoid misdiagnosis especially in early stage HCC could use the combination of AFP, PON and AFU if they available to decrease the false negative rate of AFP level when it used alone.

Conclusion:-

Combined detection of OPN and AFU activity may be used as diagnostic markers for the diagnosis of HCC. Combined detection of AFU, OPN and AFP could improve early diagnosis of HCC and differentiate it from chronic liver disease.

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