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ROLE OF OSTEOPONTIN AND DIKKOPF RELATED PROTEIN - 1(DKK-1) AS DIAGNOSTIC MARKERS OF HCV RELATED HEPATOCELLULAR CARCINOMA

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ABSTRACT

Background: Osteopontin (OPN) is an important tumor marker, since it presents as an immobilized extracellular matrix molecule in addition to present as a secreted form in body fluids involving plasma. Osteopontin levels in the plasma were found to be significantly higher in hepatocellular Carcinoma (HCC) patients than in healthy control individuals and also higher than in patients with chronic liver diseases. Dikkopf related protein-1 (DKK-1) is a diagnostic and prognostic serologic marker for early HCC. The DKK-1 mRNA and protein levels were found to be up regulated in early HCC. **Objective:** The aim of the present study is to evaluate the role of plasma OPN level and Dickkopf-1 (DKK1) as potential markers of HCC among HCV infected patients, compared to AFP. Also, its relationship with clinicopathological features of HCC patients. **Study design:** This is a retrospective case control study. **Subjects and Methods**: The study included 90 adult subjects; they were classified in to 3 groups.Group1**:** It included 30 patients with HCC metastases. Group 2**:** It included 30 patients with chronic liver disease (CLD) (chronic hepatitis C without HCC). Group 3**:** It included 30 apparently healthy individuals as a control group. Plasma Osteopontin and Dikkopf related protein -1(DKK-1) were measured by Enzyme-linked immunosorbent assay ELISA. **Results**: The mean of DKK1 in control, CLD, HCC was 1.28± 0.383, 1.37± 0.414, and 2.58± 0.510 respectively, and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$). The

mean of OPN in control, CLD, HCC was 31.15± 15.031, 153.60± 93.931, 349.83± 183.912 respectively, P value = 0.000 and there were highly statistical significant differences between the three groups (p < 0.001). The mean of AFP in control, CLD, HCC was 1.110± 0.224, 34.06± 43.702, 4666.01± 3938.67 respectively, P value = 0.000 and there were highly statistical significant differences between the three groups (p < 0.001). DKK1 and OPN levels were significantly higher in metastasis cases P= 0.000 than non metastatic cases while AFP level was non-significant P= 0.424. Patients with large tumor size have significantly higher OPN levels p=0.025 while non significantly different as regard AFP and DKK1 levels. **Conclusion:** OPN and DKK1 can be used for diagnosis of HCC and differentiation between HCC and CLD. OPN and DKK1have higher sensitivity and specificity than AFP and can be used for early diagnosis. Combination between OPN and DKK1has increased both sensitivity and specificity for detection of of HCC.

KEYWORDS: Hepatocellular carcinoma, chronic liver disease, Osteopontin, Dickopf related protein- 1.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the major common cause of primary liver cancers and the fourth most frequent type of cancer all over the world following lung, breast and bowel cancers with a growing occurrence, causing one million deaths per year.[1]

Infection with Hepatitis C Virus (HCV) is the major factor associated with HCC mainly through indirect chronic inflammation, cell death and proliferation. The markers of HCV infection are present in the serum of 80% of patients with HCC.[2]

Hepatocellular carcinoma is a major health problem in Egypt and its incidence is increasing. 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.[3]

Owing to the lack of reliable clinical HCC markers, fewer than 20% of patients are diagnosed at a stage where curative treatment can be performed. The poor outcome of patients with HCC is related to the late detection with more than two-thirds of patients diagnosed at advanced stages of disease. Because the poor outcomes of HCC patients are often related to late detection, recent practice guidelines recommend continued surveillance for patients at high risk.[4]

Screening programs of HCC at earlier stages, involving alpha-fetoprotein (AFP) and ultrasound every 6 months in patients with liver cirrhosis for detection of HCC at earlier stages are important for achievement of effective treatment. Even though AFP's performance in early stage HCC is deficient, ~30% of HCC patients are negative for AFP and screening for this biomarker may not be satisfactory due to low sensitivity and specificity.[5]

Consequently, additional biomarkers have been proposed for screening HCC.

Osteopontin is a 314 amino acid phosphoglycoprotein that undergoes extensive post-translational modifications including phosphorylation, glycosylation and cleavage resulting in molecular mass variants ranging from 25 to 75kDa, it is a component of the non-collagenous bone matrix. It was firstly isolated from bone and a variety of calcified tissues.[6]

Osteopontin (OPN) is a glycophosphoprotein with cytokine and chemokine properties that was found to be circulating in the biological fluids of healthy individuals, but elevated in cancer patients as well as in individuals with systemic inflammatory response syndrome.[7]

Osteopontin is synthesized by many tissues as leucocytes, preosteoblasts, osteoblasts, osteocytes, fibroblasts, osteoclasts, bone marrow cells, hypertrophic chondrocytes, dendritic cells, macrophages, smooth muscle cells, skeletal muscle myoblasts, endothelial cells lining blood vessels and extra-osseous (non-bony) cells in the inner ear and brain.[8]

Many studies have provided the role exerted by osteopontin in many biological events including bone remodeling, immune regulations, tissue repair and wound healing, cell survival and protection from apoptosis and protection from urinary stones.[9]

Osteopontin was fond to be highly expressed in many malignancies and the expression level of OPN in tumor tissues or in blood of cancer patients has been positively correlated with tumor grade, tumor stage and early recurrence in many cancer types.[10]

Osteopontin has been shown to play an important role in tumorigenesis, tumor invasion and determining the oncogenic potential of various cancers. It is recognized as a key prognostic marker during the progression of cancer as it has been strongly correlated with poor prognosis in human cancers.[11]

Tumor cells with high metastatic potential had been found to show increased osteopontin expression. Osteopontin plays important roles in tumor metastasis through binding to OPN receptors which are αvβ integrins and CD44.[12]

Osteopontin is an important tumor marker, since it presents as an immobilized extracellular matrix molecule in addition to present as a secreted form in body fluids involving plasma. Osteopontin levels in the plasma were found to be significantly higher in HCC patients than in healthy control individuals and also higher than in patients with chronic liver diseases.[13]

Osteopontin gene has been proved to be correlated with the metastasis of HCC. The investigations were proved that: osteopontin has a significant role in hepatocellular carcinoma metastasis and tumor growth and is an attractive therapeutic target against hepatocellular carcinoma metastasis.[14]

The levels of OPN increase in HCC tissues when the carcinomas show invasion of bile duct or vascular tissues and intra hepatic spread. In additions patients with high OPN expression have significantly poor overall survival and shorter time to tumor returning (TTR) than the patients with low OPN expression.[15]

The DKK family consists of four members (DKK1 toDKK4). They are secreted glycoproteins of 225-350 amino acids with molecular weights between 25 and 29 kDa for DKK1, DKK2 and DKK4 and 38 kDa for DKK3. DKKs contain two conserved cysteine-rich domains, each of which is separated by a linker region of various lengths.[16]

Dickkopf related protein-1(DKK1), a secreted protein, is a known negative regulator of the Wingless integrin (Wnt) signaling pathway, which plays an important role in a variety of cellular processes, including proliferation, differentiation, survival, apoptosis and cell motility.[17]

The Dickkopf (DKK) genes are Wnt antagonists that were originally identified as inducers of head formation in Xenopus. The DKK gene family consists of DKK-1, -2, -3, -4, and a unique DKK-3-related gene. The expression of these genes is temporally and spatially regulated, and all DKK proteins show distinct and elevated expression patterns in tissues that mediate the epithelial-mesenchymal transformation. This suggests that they may participate in the epithelial to mesenchymal transition that is important not only in embryogenesis, but also in cancer progression.[18]

DKK-1 mRNA is expressed at low levels in most normal human tissues with the exception of the placenta. A wide range of DKK-1 gene expression levels has been reported at various phases of tumorigenesis in multiple cancer phenotypes including prostate, breast, colorectal, esophageal, lung and multiple myeloma.[19]

DKK1 was frequently found to be overexpressed in patients with Wilms tumor, hepatoblastoma, multiple myeloma and breast cancer, indicating a potential oncogenic role of DKK1 in carcinogenesis.[20]

DKK-1 has been postulated to be a tumor suppressor or tumor promoter depending on the tumor type. Evidence for the potential involvement of DKK-1 inactivation in human cancers is accumulating.[21]

DKK-1 is a diagnostic and prognostic serologic marker for early HCC. The DKK-1 mRNA and protein levels were found to be up regulated in early HCC. Serum levels of DKK-1 in patients with early HCC were significantly elevated. DKK-1 had a better sensitivity and accuracy than AFP. More importantly, 73.1% of the patients negative for AFP could be diagnosed with early HCC using DKK-1. A combination of DKK-1 and AFP further improved the diagnostic efficacy.[22]

It was found that the increased DKK1 expression was correlated with poor overall and disease-free survival of HCC, indicating that DKK1 could serve as a novel prognostic marker and therapeutic target for HCC. The prognostic value of DKK1 has been confirmed in some cancers.[23]

The increased expression of DKK1was correlated with beta-catenin cytoplasmic/nuclear accumulation in clinical HCC samples and high DKK1 expression predicted unfavorable prognoses in HCC patients. These findings suggest that DKK1 is a novel prognostic predictor for HCC patients.[24]

On the other hand, serum DKK1 level is a predictive marker of HCC invasiveness. Elevated expression of DKK1 in serum or transcript indicates poor clinical outcome, especially in early stage HCC and AFP-normal HCC. Furthermore, HCC patients with high DKK1 expression and high cytoplasmic/nuclear β-catenin accumulation had very poor prognosis.[25]

Subjects and Methods

This study was carried out in the Medical Biochemistry and Tropical Medicine Departments between October 2016 and August 2017, AL-Azhar University. Approval for the study was obtained from the research Ethics Committee, Faculty of Medicine-AL-Azhar University and patients were recruited amongst those attending the Tropical Medicine Department in Al-hussein University Hospital.

Subjects**:** The study included 90 adult subjects; they were 57 males and 33 females. Informed consent was obtained from all of them to use their samples for research. They were classified in to 3 groups:

Group 1: It included 30 patients with HCC; 18 males (60%) and 12 females (40%). In 15(50%) of patients the primary HCC lesion was less than 5cm and in the remaining 15 patients (50%) was more than 5cm. 21 (70%) of patients showed HCC metastases and 9 patients (30%)showed no HCC metastases.

Group 2: It included 30 patients with CLD (chronic hepatitis C without HCC); 21 males (70%) and 9 females (30%).

Group 3: It included 30 apparently healthy individuals (control group); 18 males (60%) and 12 females (40%).

Exclusion criteria

The following patients will be excluded from the study:

1. Patients having extra hepatic malignancy.
2. Patients having any bony lesions or inflammatory diseases.
3. Patients with any chronic liver disease other than HCV.

All individuals included in this study will be subjected to the following:

1- Full history taking focusing on previous hepatic disorders, predisposing factors preceding liver disease, age, sex, alcohol intake andblood transfusion.

2- Thorough clinical examination, with special emphasis on abdominal examination, jaundice, edema and ascites.

3- laboratory investigations

4- Imaging studies

- Abdominal ultrasonography for all patients (liver, spleen, portal vein, ascites).

- Triphasic computed tomography for HCC group (HCC size, number, site, portal vein thrombosis).

Sample Preparation

10 ml of venous blood were collected aseptically from each subject and each sample was distributed as follows:

1.8 ml blood were collected into tube containing 0.2 ml of trisodium citrate solution (32g/l) to perform PT& concentration.

4 ml blood were collected in tube containing k3-EDTA as an anticoagulant, for CBC, The remaining part was centrifuged for 15 minutes at 1000 x g within 30 minutes of collection. Samples stored at ≤-20°C. The plasma was used for determination of OPN and DKK1.

The remaining of the blood was collected in a plain vacutainertube and allowed to clot then the serum was separated by centrifugation at 3000 r.p.m for 15 minutes and was used forroutine laboratory investigations and AFP measurement.

Analytical Methods

A-Complete Blood Count: Complete blood count is performed on Sysmex XS- 500Ifrom sysmex corporation, Japan.

B- Liver and kidney function tests: The analysis was done on Dimension Rxl max auto analyzer provided by Siemens by using reagents supplied by Dimension® clinical chemistry system.

C- Prothrombin Time: The analysis was done on Sysmex CA- 1500. Auto analyzer using reagents supplied by Siemens.

D- Hepatitis markers: For HBsAg: The analysis of serum HBsAg was done by electrochemiluminescence immunoassay "ECLIA' on the cobas e 411 immunoassay analyzer from Roche diagnostics.

For anti HCV antibody: The analysis of serum anti HCV antibody was done by "ECLIA' on the cobas e 411 immunoassay analyzer.

E- Serum AFP: The analysis of serum AFP was done "ECLIA' on Cobas e 411 system from cobas.

F- Serum Osteopontin assay: Serum osteopontin levels were determined and measured by (ELISA) using Chromate ELISA reader Diagnostics (USA) using Sunred ELISA kit (China).

G-Serum Dickkopf assay: Serum Dickkopf1 levels were determined and measured by Chromate ELISA reader Diagnostics (USA) using Sunred ELISA kit (China).

Statistical Analysis

Data collected throughout history, basic clinical examination, laboratory investigations and outcome measures coded, entered and analyzed using Microsoft Excel software. Data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis. According to the type of data. Quantitative data were represented as number and percentage, mean ± SD, the following tests were used in parametric quantitative independent groups which were Student t- test in non-parametric normally distributed data while skewed data by Mann Whitney. Differences and association of qualitative variables between two groups by Chi square test (X^2). While between multiple groups by one way ANOVA for normally distributed data and Kruskal Wallis for skewed data, correlation by Pearson's or Spearman's correlation. P value was set at <0.05 for significant results and <0.001 for high significant result.

ROC curve

A receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot which illustrates the performance of a binary classifier system as its discrimination threshold is varied. It was used for determination of cut off values of AFP, OPN and DKK1for diagnosis of HCC.

RESULTS

Table (1): Statistical comparison of clinical findings among studied groups.

			GROUP			Total	P
			Control	CLD	HCC		
Encephalopathy	No	N	30	18	9	57	0.00
		%	100.0%	60.0%	30.0%	63.3%	
	Yes	N	0	12	21	33	
		%	0.0%	40.0%	70.0%	36.7%	
Ascites	No	N	30	9	6	45	0.00
		%	100.0%	30.0%	20.0%	50.0%	
	Yes	N	0	21	24	45	
		%	0.0%	70.0%	80.0%	50.0%	
Edema	No	N	30	9	3	42	0.00
		%	100.0%	30.0%	10.0%	46.7%	
	Yes	N	0	21	27	48	
		%	0.0%	70.0%	90.0%	53.3%	
Portal vein thrombosis	No	N	30	27	12	69	0.00
		%	100.0%	90.0%	40.0%	76.7%	
	Yes	N	0	3	18	21	
		%	0.0%	10.0%	60.0%	23.3%	
Jaundice	No	N	30	12	9	51	0.00
		%	100.0%	40.0%	30.0%	56.7%	
	Yes	N	0	18	21	39	
		%	0.0%	60.0%	70.0%	43.3%	
Total		N	30	30	30	90	
		%	100.0%	100.0%	100.0%	100.0%	

Table (1): shows that there were highly significant differences among studied groups as regard clinical characters (Encephalopathy, Ascites, Edema, Portal vein thrombosis and Jaundice)P value =0.00.

Table (2): Child classification between HCC and CLD.

			GROUP		Total	P
			CLD	HCC		
Child	B	N	9	6	15	0.37
		%	30.0%	20.0%	25.0%	
	C	N	21	24	45	
		%	70.0%	80.0%	75.0%	
Total		N	30	30	60	
		%	100.0%	100.0%	100.0%	

Table (2): shows that in HCC groups, 6 patients (20%) were classified as stage B and 24 patients (80%) were classified as stage C. In CLD groups, 9 patients (30%) were classified as stage B and 20 patients (70%) were classified as stage C.

Table (3): Statistical comparison of hematological findings among the studied groups.

		N	Mean	Std. Deviation	Minimum	Maximum	P
HB	Control	30	10.55	0.69	9.90	12.00	0.163
	CLD	30	10.29	0.442	9.80	11.20	
	HCC	30	10.55	0.64	9.80	12.00	
TLC	Control	30	6.21	0.55	5.50	7.00	0.000
	CLD	30	7.41	1.34	6.00	10.00	
	HCC	30	9.14	1.46	7.00	11.00	
PLT	Control	30	286.20	26.36	245.00	330.00	0.000
	CLD	30	84.40	10.06	70.00	105.00	
	HCC	30	101.90	9.18	90.00	120.00	

Table (3): shows that the mean of HB in control, CLD, HCC was 10.55± 0.69, 10.29± 0.442, 10.55± 0.64 respectively, P value = 0.163 and there was no statistical significant difference between the three groups.

The mean of TLC in control, CLD, HCC was 6.21± 0.55, 7.41± 1.34, and 9.14± 1. 46 respectively, P value = 0.000 and there was a highly statistical significant difference between the three groups ($p < 0.001$).

The mean of PLT in control, CLD, HCC was 286.20± 26.36, 84.40± 10.06, and 101.90± 9. 18 respectively, P value = 0.000 and there was a highly statistical significant difference between the three groups ($p < 0.001$).

Table (4): Comparisons between laboratory data among studied groups.

		N	Mean	Std. Deviation	Minimum	Maximum	P
PT	Control	30	12.10	0.874	11.00	14.00	0.000
	CLD	30	20.80	1.494	18.00	23.00	
	HCC	30	27.80	1.689	25.00	31.00	
Creatinine	Control	30	1.0540	0.065	1.00	1.20	0.000
	CLD	30	2.280	0.721	1.00	3.30	
	HCC	30	3.40	1.12	1.10	5.10	
Urea	Control	30	45.40	4.319	38.00	52.00	0.000
	CLD	30	66.60	7.68	58.00	82.00	
	HCC	30	73.50	7.735	58.00	84.00	
AST	Control	30	19.60	2.49	15.00	24.00	0.000
	CLD	30	50.10	26.34	28.00	110.00	
	HCC	30	70.10	36.25	29.00	132.00	
ALT	Control	30	19.00	1.43	17.00	21.00	0.000
	CLD	30	45.10	21.65	29.00	97.00	
	HCC	30	61.00	26.26	30.00	100.00	
Albumin	Control	30	4.40	0.32	4.00	5.20	0.000
	CLD	30	3.11	0.36	2.70	4.00	
	HCC	30	2.22	0.29	1.90	3.00	
Total Bilirubin	Control	30	1.08	0.127	.90	1.30	0.000
	CLD	30	3.51	0.731	1.90	4.30	
	HCC	30	5.44	1.337	2.80	7.10	

Table (4) shows that

The mean of Prothrombin time in control, CLD, HCC was 12.10± 0.874, 20.80± 1.494, and 27.80± 1.689 respectively and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of Creatinine in control, CLD, HCC was 1.0540± 0.065, 2.280± 0.721, and 3.40± 1.12respectively, and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of Urea in control, CLD, HCC was 45.40± 4.319, 66.60± 7.68, and 73.50± 7.735respectively and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of AST in control, CLD, HCC was 19.60± 2.49, 50.10± 26.34, 70.10± 36.25 respectively, and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of ALT in control, CLD, HCC was 19.00± 1.43, 45.10± 21.65, 61.00± 26.26 respectively, and P value = 0.000 and there was a highly statistical significant difference between the three groups ($p < 0.001$).

The mean of Albumin in control, CLD, HCC was 4.40± 0.32, 3.11± 0.36, 2.22± 0.29 respectively and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of Total Bilirubin in control, CLD, HCC was 1.08± 0.127, 3.51± 0.731, and 5.44± 1.337 respectively and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

Table (5): Comparison between DKK1, OPN, and AFP as regard control, CLD and HCC groups.

		N	Mean	Std. Deviation	Minimum	Maximum	P
DKK1	Control	30	1.28	0.383	.88	2.20	0.000
	CLD	30	1.37	0.414	.90	2.30	
	HCC	30	2.58	0.510	1.80	3.30	
OPN	Control	30	31.15	15.031	14.50	65.00	0.000
	CLD	30	153.60	93.931	59.00	389.00	
	HCC	30	349.83	183.912	129.00	622.00	
AFP	Control	30	1.110	0.224	.80	1.50	0.000
	CLD	30	34.06	43.702	1.10	128.00	
	HCC	30	4666.01	3938.67	1.80	9875.00	

Table (5) shows that

The mean of DKK1 in control, CLD, HCC was 1.28± 0.383, 1.37± 0.414, and 2.58± 0.510 respectively, and P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of OPN in control, CLD, HCC was 31.15± 15.031, 153.60± 93.931, 349.83± 183.912 respectively, P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

The mean of AFP in control, CLD, HCC was 1.110± 0.224, 34.06± 43.702, 4666.01± 3938.67 respectively, P value = 0.000 and there were highly statistical significant differences between the three groups ($p < 0.001$).

Table (6): Spearman's Correlation between DKK, OPN, AFP and other parameters in HCC group.

		DKK1	OPN	AFP
OPN	R	.764	1	.474
	P	.000		.000
AFP	R	.533	.474	1
	P	.000	.000	
Age	R	.326	.196	.223
	P	.002	.064	.034
TLC	R	.564	.490	.453
	P	.000	.000	.000
PLT	R	-.366-	-.548	-.312-
	P	.000	.000	.003
PT	R	.714	.718	.615
	P	.000	.000	.000
Creatinine	R	.620	.630	.216
	P	.000	.000	.041
Urea	R	.540	.756	.352
	P	.000	.000	.001
AST	R	.609	.652	.183
	P	.000	.000	.085
ALT	R	.620	.637	.231
	P	.000	.000	.029
Albumin	R	-.648-	-.686-	-.485-
	P	.000	.000	.000
Bilirubin	R	.675	.708	.554
	P	.000	.000	.000
Size	R	.470	.741	-.045-
	P	.009	.000	.813

Table (6): Shows that there was a statistical significant positive correlation between the levels of OPN with AFP (r=0.474& P= 0.000), age(r=0.196& P= 0.64), DKK1(r=0.764& P< 0.001), total bilirubin (r=0.708& P= 0.001), AST(r=0.652& P=0.001), ALT(r=0.637&P=0.000), urea(r=0.756&P=0.001), creatinine(r=0.630&P=0.000), TLC (r=0.490&P=0.001), PT(r=0.718& P= 0.000) and size (r=0.741& P=0.001), except for albumin which showed negative significant correlation (r=- 0.686& P=0.000) and platelets which showed negative significant correlation (r=- 0.548& P=0.000).

Also it shows that was a statistical significant positive correlation between the levels of DKK1with AFP (r=0.533& P= 0.001), age (r=0.326& P=0.002), total bilirubin (r=0.675& P= 0.001), AST(r=0.609& P= 0.001), ALT(r=0.620& P=0.001), urea (r=0.540&P=0.001), creatinine (r=0.620& P<0.001), TLC (r=0.564&P= 0.001), PT(r=0.714& P= 0.000) and size (r=0.470& P=0.009) except for albumin which showed negative significant correlation (r=-

0.648& P=0.001) and platelets which showed negative significant correlation (r=- 0.366& P<0.001).

Lastly it shows that there was a statistical significant positive correlation between the levels of AFP with age (r=0.223& P=0.034), total bilirubin (r=0.554& P= 0.001), ALT (r=0.231& P=0.029), urea (r=0.352& P= 0.001), creatinine (r=0.216& P=0.04), TLC(r=0.453&P= 0.001), PT(r=0.615& P= 0.001) except for albumin which showed negative significant correlation (r=- 0.485& P=0.001) and platelets which also showed negative significant correlation (r=- 0.312& P=0.003).

Table (7): Comparison between metastasis cases among HCC group as regard DKK1, OPN and AFP.

	Metastasis	N	Mean	Std. Deviation	P
DKK1	+VE	15	3.006	0.243	0.00
	-VE	15	2.16	0.311	
OPN	+VE	15	506.26	127.484	0.00
	-VE	15	193.40	37.047	
AFP	+VE	15	4079.13	3422.202	0.424
	-VE	15	5252.89	4436.772	

Table (7): Shows that DKK1 and OPN levels were significantly higher in metastasis cases than non metastatic cases P= 0.000, while AFP level was non-significant P= 0.424.

Table (8): Comparison between tumor size among HCC as regard DKK1, OPN and AFP.

	Size	N	Mean	Std. Deviation	P
DKK1	> 5	21	2.564	0.621	0.930
	< 5	9	2.54	0.364	
OPN	> 5	21	398.04	192.18	0.025
	< 5	9	237.33	99.65	
AFP	> 5	21	4627.17	4043.93	0.936
	< 5	9	4756.64	3916.46	

Table (8): Shows that patients with large tumor size have significantly higher OPN levels P=0.025 while non significantly differrent as regard AFP and DKK1 levels.

Table (9): Area under the curve and cut off value of DKK1, OPN and AFP.

Test Result Variable(s)	Area	Cutoff	95% Confidence Interval	
			Lower Bound	Upper Bound
DKK1	0.970	>1.75	.941	.999
OPN	0.930	>164.5	.879	.981
AFP	0.910	>7.15	.840	.980

Table (9): Shows that Area Under the Curve for DKK1, OPN and AFP was 0.970, 0.930 and 0.910 and cut off values were >1.75, >164.5 and >7.15 respectively.

Table (10): Sensitivity and specificity of DKK, OPN and AFP.

	Sensitivity	Specificity	+VE predictive	-VE predictive	Accuracy
DKK1	86.7%	88.3%	78.7%	92.9%	87.7%
OPN	90.0%	90.0%	81.8%	94.7%	90.0%
AFP	80.0%	55.0%	47.05%	84.6%	63.3%
AFP & DKK1	83.3%	93.3%	86.2%	91.8%	90.0%
AFP & OPN	76.7%	95.0%	88.4%	89.0%	88.8%
OPN & DKK1	83.3%	96.6%	87.5%	90.9%	93.3%

Table (10) shows that

The sensitivity, specificity, positive predictive value and negative predictive value of DKK1, OPN were higher than AFP.

The combination of AFP and DKK1has increased both sensitivity and specificity of AFP for detection of HCC.

The combination of AFP and OPN has increased specificity of AFP for detection of HCC to 95% but decreased sensitivity to76.7%.

The combination of OPN and DKK1has increased both sensitivity and specificity for detection of HCC to 83.3% and 96.6% respectively.

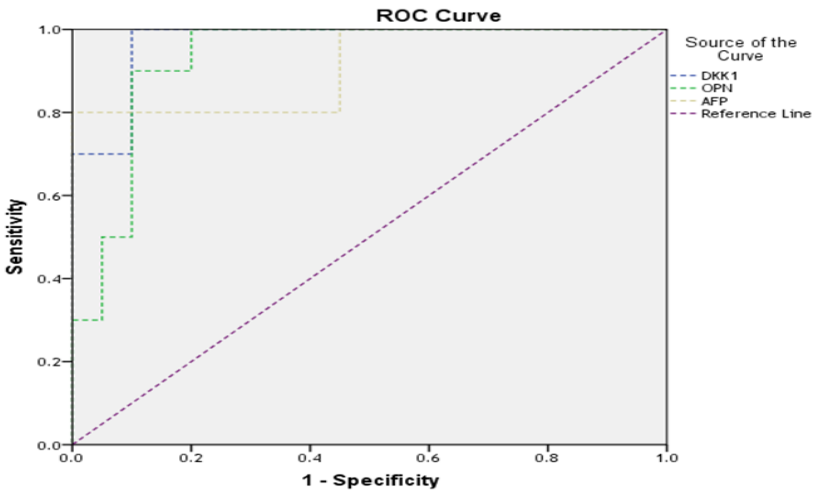

Figure (1): ROC Curve for detection of HCC markers cut off.

DISCUSSION

Hepatocellular carcinoma is an increasingly prevalent clinical problem worldwide and is the third most common cause of cancer-related death.[26]

Owing to the lack of reliable clinical HCC markers, fewer than 20% of patients are diagnosed at a stage where curative treatment can be performed. In most cases HCC is diagnosed at a late stage, and often arises in a background of chronic liver disease and cirrhosis. Therefore, the prognosis of patients with HCC is generally poor with the 5-year survival rate for this malignancy is depressingly low, ranging from 4-6% in different countries.[27]

Studies on OPN tissue expression have shown that OPN is elevated in a number of tumors compared with normal specimens. Moreover, intensity of OPN expression appears to correlate with patients' survival and clinicopathological data.[28]

The aim of the present study is to evaluate the role of plasma OPN level and Dickkopf-1(DKK1) as potential markers of HCC among HCV infected patients, compared to AFP. Also, its relationship with clinicopathological features of HCC patients.

In our study, the age of HCC patients ranged from 40 to 75years with a mean± (SD) was 64.50±8.15 years and this is probably attributed to the duration of the underlying liver disease but there was no significant difference as regard age. These results were in agreement with Salem et al.[29] in which the age of HCC patients ranged from 39 to70 years with a mean of 56.7 ± 8.9 years. In Keddeas and Abo-shady[30] the age of the patients with HCC ranged from 40 to 72 years, also Di Bisceglie[31] stated that HCC is reported to develop in the fifth decade. The same results were reported by Johnson[32] who found that the average age of patients ranged from fifth to sixth decades of life.

As regards the sex of patients, in our study the male to female ratio in HCC Group was 1.5: 1(60% males and 40% females with HCC). The reasons for higher rates of liver cancer in males may be explained by differences in exposure to risk factors. However, sex hormones and other x-linked genetic factors may also be important but there was no significant difference as regard sex. It has been speculated that estrogens and androgens could modulate hepatocarcinogenesis and explain the higher incidence of HCC in men.[33]

These results were in agreement with those obtained by Salem et al[29] who reported in HCC patients a male: female ratio was 2: 1, this male predominance was also observed by

Goldman and Ausiello[34] who reported a male: female ratio 2:1 up to 4:1. In Keddeas and Abo-shady[30] it was reported that HCC is three times more common in men than women.

Our results show that there were highly significant differences among groups as regard OPN, Dickkopf1 and AFP markers highest in HCC followed by CLD followed by normal levels in control groups.

The mean of DKK1 in control, CLD, HCC was 1.28± 0.383, 1.37± 0.414, and 2.58± 0.510 respectively, and P value = 0.000.

The mean of OPN in control, CLD, HCC was 31.15± 15.031, 153.60± 93.931, 349.83± 183.912 respectively, P value = 0.000.

The mean of AFP in control, CLD, HCC was 1.110± 0.224, 34.06± 43.702, 4666.01± 3938.67 respectively, P value = 0.000.

The sensitivity of DKK1, OPN and AFP was 86.7%, 90.0% and 80.0% respectively. When AFP was combined with DKK1 sensitivity increased to 83.3%. When AFP was combined with OPN sensitivity decreased to 76.7%. When OPN was combined with DKK1 sensitivity was 83.3%.

The specificity of DKK1, OPN and AFP was 88.3%, 90.0% and 55.0% respectively. When AFP combined with DKK1 specificity increased to 93.3%. When AFP combined with OPN specificity increased to 95.0%. When OPN was combined with DKK1 specificity increased to 96.6%.

These results were similar with those of Salem et al[29] who found that: significant elevation of plasma osteopontin levels and AFP levels in HCC patients than HCV patients' levels and lower levels in normal control group.

Fouad et al[35] also reported that there was statistically significant increase in the serum OPN levels in the HCC group compared to the benign chronic liver disease groups (HCV without cirrhosis, HCV with cirrhosis, Fatty liver disease), healthy subjects, OPN was superior to AFP in the selective detection, diagnosis of HCC and in predicting liver cirrhosis.

El-Din Bessa et al[36] also found that: plasma levels of OPN and AFP in HCC cirrhotic patients (n= 30) being significantly higher than in cirrhotic patients without HCC (n=30) and healthy controls (n=30).

The same was demonstrated in a study by Kim et al[13] who determined plasma levels of OPN, AFP, in a group of 62 HCC patients, in 60 patients with chronic liver diseases, and in 60 healthy control individuals showing that plasma OPN levels in the HCC patients were significantly higher than those patients with CLD or of a healthy control group.

In our study the median plasma OPN level, in our patients, with small tumor size<5cm was 237.33ng/mL, with large tumor size >5cm was 398.04ng/mL and this was statistically significant.

This was in agreement with Salem et al[29] who found that tumors < 3 cm, present in 40% of patients, showed median plasma OPN level 140 with a range of (100 - 336 ng/mL), and tumors ≥ 3 cm, present in 60% of patients, showed median plasma OPN level 229 with a range of (131 - 438 ng/mL) (P value: 0.28). Zhang et al[37] also found that tumors ≤ 5 cm showed median plasma OPN level 176.90 ng/mL and tumors > 5 cm showed median plasma OPN level 172.92 ng/mL. However Abu El Makarem et al[38] reported that the median plasma OPN level in tumors< 5cm was 510 ng/mL and in and tumors ≥ 5cm was1230 and this was statistically significant.

The present study showed OPN was significantly higher among cases with lymph node metastasis than those with no metastasis. These results were in accordance with Abu El Makarem et al[38] who reported that the median plasma OPN level in patients with lymph node metastasis (1423ng/mL) was higher than patients with no lymph node metastasis (497ng/ml).

In our study, there was a statistical significant positive correlation between the levels of AFP and OPN(r=0.474& P=< 0.001) and this was in agreement with that of Salem et al[29] who found that there was significant positive correlation between OPN and AFP and similarly Zhang et al[37] found that the plasma OPN level positively correlated with the serum AFP concentration. However, Sun et al[39] found that the correlation between plasma OPN and serum AFP was insignificant & therefore, they had stated that plasma OPN levels might be helpful for the diagnosis of HCC in the patients with non-diagnostic AFP level.

In our study, the sensitivity, specificity, PPV and NPV of plasma OPN levels in HCC patients were 90, 90, 81.8, and 94.7respectively at a cut-off value >164.5. AUC for OPN was 0.930 with CI (0.879 – 0.981).

For AFP at a cut-off value>7.15ng/ml; the value of sensitivity, specificity, PPV and NPV of plasma AFP levels in HCC patients relative to the CLD group were 80.00 %,45.0 %,47.05 and 84.6 respectively. AUC for AFP was0.910 with CI (0.840 – 0.980).

Results of our study were in agreement with the study done by El-Din Bessa et al[36] who reported that, the sensitivity and specificity of OPN for HCC diagnosis were 88.3% and 85.6%, respectively, at a cut-off value of 9.3 ng/mL with OPN having a greater AUC value (0.918) than AFP (0.712). Also Kim et al[13] found that the diagnostic sensitivity and specificity of OPN for HCC was 87% and 82%, respectively (cut-off value: 617.6 ng/mL) with OPN had a greater AUC value (0.898) than AFP (0.745).

Many studies reported better diagnostic accuracy of OPN over AFP in HCC diagnosis. Abohalima and Salem[40] found that OPN AUC for HCC diagnosis was 0.991 (95% CI: 0.948 to 1.000) and it differed significantly (p= 0.01) from AFP AUC (0.889, 95% CI: 0.810 to 0.943). At a cutoff value of OPN > 178 ng/ml, the test had sensitivity of 98% and specificity of 96% while AFP at a cutoff value of >185 ng/ml had sensitivity and specificity of 86% and 94% respectively in HCC diagnosis.

Abu El Makarem et al[38] reported AUC for OPN was (0.998; 95% CI: 0.952-1) which was significantly (p= 0.0001) higher than that yielded by AFP (0.91; with 95% CI: 0.826-0.961). The sensitivity and specificity of plasma OPN were 97.67% and 100%, at a cut-off value of 300 ng/ml. For AFP at a cut-off value > 43 ng/mL; the values of sensitivity, specificity, were 74.4% and 100% respectively.

In contrary to our results: The plasma levels of OPN show low diagnostic accuracy for HCC compared to AFP. However, OPN may have a complementary role in diagnosing HCC in patients with non-diagnostic levels of AFP.[41]

DKK1 has been proposed to be a potential new biomarker in several types of cancers. The diagnostic accuracy of DKK1 as a serum biomarker for HCC in a large-scale, multicenter study. Their study demonstrated that serum DKK1 was high in both sensitivity and specificity in diagnosing HCC, especially early-stage HCC and α-fetoprotein-negative HCCs. The

authors also demonstrated that combination of serum α-fetoprotein with serum DKK1 could further improve the diagnostic accuracy. Overall, their findings revealed the importance and significance of DKK1 in HCC diagnosis.[42]

There was highly significant difference between patient and control groups as regard DKK1 and this result was in line with that of who reported high expression of DKK1 in HCC.[43]

In our study our patients were classified into two groups according to size of lesion: first group the size of lesion<5 cm included 9/30patients, second group the size of lesion >5cm included 21/30 patients.

There was no significant difference among patient group as regard size of lesion.

This result was in line with that of Yu et al[24] who reported that there is no correlation between DKK1-positivity and tumor size. On the other hand Shen et al[42] stated that there is correlation between serum DKK1 level and a larger tumor size (≥5 cm).

Serum DKK1was documented as a marker for detection of early HCC in HCV infected patients. Significant reduction of DKK1 5 days after curative resection indicated that it can be used as a follow up marker for recurrence in surgically resected HCV induced HCC patients.[44]

Our result was in line with that of Shen et al[42] who reported that serum DKK1 had a high diagnostic accuracy for early-stage HCCs. The diagnostic accuracy of DKK1 in a-fetoprotein (AFP)-negative patients was also high. More importantly, DKK1 was found to be a good marker in diagnosing early-stage HCC in patients with negative AFP. Furthermore, serum DKK1 had significant power to distinguish HCC patients from those with non-malignant chronic liver diseases, as serum DKK1 level in HCC patients with cirrhosis was significantly higher than that in patients with cirrhosis alone. Serum DKK1, as compared with AFP, may be a more reliable biomarker for HCC diagnosis.

Also this result goes in hand with the results of Gomceli et al[45] who reported that DKK1 may have a substantial role is in patients where AFP levels are negative or equivocal such as the case in chronic liver disease.

On contrary to our results Yang et al[46] stated that dickkopf-1 (Dkk1) was significantly elevated in nodular HCC (multiple lesion) with high metastatic potential compared to solitary HCC (solitary lesion).

Fatima et al[47] found that in comparison to serum α-fetoprotein (AFP) level, which remains the gold standard for HCC diagnosis, high serum DKK1 levels have higher diagnostic value for HCC, especially for AFP-negative HCC, and can distinguish HCC from non-malignant chronic liver diseases.

Liaw and Chu[48] stated that measurement of serum DKK1 has diagnostic value for HCC better than that of AFP, especially for patients with AFP-negative status and early-stage HCC. 30–40% of all patients with HCC are AFP negative, and diagnosis and assessment of treatment response are difficult with current methods. Thus, combined testing of DKK1 and AFP concentrations in serum could improve results.

AFP concentrations are raised in 11–58% of patients with chronic hepatitis or cirrhosis in the absence of HCC. Therefore, measurement of DKK1 in serum can help to make a differential diagnosis of HCC in patients in these high-risk populations.

Yu et al[24] found that although elevated levels of AFP remain the gold standard for screening HCC, there are, however, a subgroup of patients who have HCC and normal levels of AFP. When patients were stratified according to AFP levels, DKK1 over expression demonstrated worse prognosis for AFP-normal HCC patients, suggesting that DKK1 may serve as a prognostic marker for this group of patients.

This result was in line with that of Shen et al[42] who reported that serum DKK1 had a high diagnostic accuracy for early-stage HCCs and single HCCs ≤2 cm. The diagnostic accuracy of DKK1 in a-fetoprotein (AFP)-negative patients was also high. More importantly, DKK1 was found to be a good marker in diagnosing early-stage HCC in patients with negative AFP. Furthermore, serum DKK1 had significant power to distinguish HCC patients from those with non-malignant chronic liver diseases, as serum DKK1 level in HCC patients with cirrhosis was significantly higher than that in patients with cirrhosis alone. Serum DKK1, as compared with AFP, may be a more reliable biomarker for HCC diagnosis.

Our study shows that there was high levels of OPN and DKK1 in metastatic cases compared to non-metastatic ones with statistical significant difference p=0.00.

Our result was in agreement with Cao et al[49] who reported that OPN plays key roles in HCC stemness and metastasis. Also Bhattacharya et al[50] reported that OPN was also a powerful promoter for HCC metastasis.

Also Kuang et al[51] reported that DKK-1 expression has a significant positive association with HCC progression, metastatic potential and also observed similar influence of DKK-1 on 293 cells.

CONCLUSION

1. OPN and DKK1can Be used for diagnosis of HCC and differentiation between HCC and CLD.
2. OPN and DKK1have higher sensitivity and specificity than AFP and can be used for early diagnosis of HCC.
3. This is the first study does combination between OPN and DKK1 for diagnosis of HCC and this combination had increased both sensitivity and specificity for detection of HCC.
4. OPN and DKK1can be used for differentiation between metastatic and non-metastatic HCC.
5. We recommend for study of OPN and DKK1 on a large number of patients in order to reach to optimum cut off values to start implication of OPN and DKK1 in diagnosis of HCC.

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