Evaluation of Angiopoietin-2 Serum Level in Cirrhotic patients with High AFP with or without Hepatocellular Carcinoma

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Abstract: **Background**: Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the third most common cause of cancer-related death. Ang-2 values in patients with HCC suggesting that it might represent a useful marker for HCC and a complementary diagnostic tool **Methods:** This study was conducted on 74 patients, they were 47 male and 27 female and their ages were ranging from 36 to 78 years in Group1 and 31 to 70 years in Group 2. They were chosen from outpatient and inpatient of the Tropical medicine department in Shebin El – kom teaching hospital, in the period between February 2016 and February 2017. In addition, an informed consent was obtained before patients enter the study. They were divided into two groups: Group1 (HCC group): Included 60 patients with elevated AFP ($\geq$ 200 ng/dl), HCC on top of liver cirrhosis. Group 2(liver cirrhosis group): Included 14 patients with elevated AFP ($\geq$ 200ng / dl), liver cirrhosis without HCC. **Results:** The diagnostic accuracy of Ang-2 in detection of HCC was shown in receiver operator characteristic (ROC) curve analyses. Regarding the diagnostic accuracy of Ang-2 analysis, area under the curve (AUC) was 87. At a cut off value 2215 pg/ml, serum Ang-2 showed 93.33% sensitivity, 71.4% specificity, 93.33% PPV, 71.4% NPV with accuracy 89.18%. **Conclusion:** Serum Ang-2 is elevated in patients with cirrhosis and further elevated in patients with HCC, so its use as an independent tumor marker in the diagnosis of HCC is to be considered and the detection rates could increase when using both markers. These results suggest that Ang-2 was a potential diagnostic tumor marker for HCC, especially among high-risk group of patients. This value extends beyond the traditional tumor biomarkers as AFP, as it possess good prognostic value. Although, AFP has to be considered 'the golden standard' for HCC serum markers for years, in the view of our data and that of others; the usefulness of AFP testing for the population at risk should be seriously questioned. Ang-2 levels appear to be an additional tumor biomarker for HCC detection especially among high risk group of patients.

Key words: Hepatocellular carcinoma - Ang-2 - AFP.

1. Introduction:

HCC is a common problem worldwide which ranks the 5th and 7th most common cancer among men and women respectively **(Globocan, 2008 and Zhao et al., 2013).**

Hepatocellular carcinoma accounts for about 4.7% of chronic liver disease (CLD) patients in Egypt **(Hussein et al., 2008).** Early detection of patients with HCC is attractive because it gives better prognosis as HCC trends to grow slowly and stay confined to the liver. Early detection is possible if tumor markers and medical imaging were combined **(Okuda, 2000).**

Screening patients with chronic liver disease for hepatocellular carcinoma (HCC) causes significant cost and uses scarce medical resources**.** Screening strategies involve serum markers and imaging which include hepatic ultrasonography, computed tomography (CT), nuclear magnetic resonance (NMR) and angiography *(Colli et al, 2006).*

Accurate diagnosis of small liver nodules is of paramount importance. Until 2000, diagnosis was based on biopsy. This approach had some limitations related to feasibility due to location and risk of complications, such as bleeding or needle-track seeding *(Stigliano et al., 2007)*. In addition, achieving accuracy in differentiating between high-grade dysplastic nodules and early HCCs was complex, since stromal invasion, the most relevant criteria, is difficult to recognize even for an expert pathologist *(Roskams and Kojiro, 2010)*.

Recent studies reported high serum Ang-2 values in patients with HCC suggesting that it might represent a useful marker for HCC and a complementary diagnostic tool *(Scholz et al., 2007)*.

Aim of the work

The aim of this work was to evaluate angiopoietin-2 serum level in cirrhotic patients with high AFP to determine its accuracy as a diagnostic tool for HCC.

2. Subjectsand Methods

This study was conducted on 74 patients, they were 47 male and 27 female and their ages were ranging from 36 to 78 years in Group1 and 31 to 70 years in Group 2. They were chosen from outpatient and inpatient of the Tropical medicine department in Shebin El – kom teaching hospital, in the period between February 2016 and April 2017. In addition, an informed consent was obtained before patients enter the study.

They were divided into two groups:

Group1 (HCC group):

Included 60 patients with elevated AFP ($\geq$ 200 ng/dl), HCC on top of liver cirrhosis.

Group 2(liver cirrhosis group):

Included 14 patients with elevated AFP ($\geq$ 200ng / dl), liver cirrhosis without HCC.

Exclusion criteria:

1. Inflammatory or septic conditions as spontaneous bacterial peritonitis.

2. Focal hepatic lesions other than HCC (cholangiocarcinoma, hemangioma, hepatoblastoma or metastatic focal lesions).

3. Carcinoma elsewhere.

All patients were subjected to the following:

1- Thorough history taking.

2- Full clinical examination.

3- Routine laboratory investigations:

a- Complete blood picture.

b- Liver function tests: Alanine transaminase (ALT), Aspartate transaminase.

(AST), alkaline phosphatase (ALP), serum bilirubin (direct and indirect) and serum albumin.

c- Prothrombin time and concentration (PT and PC) and international normalized ratio (INR).

d- Serum creatinine.

e- Hepatitis markers: Hepatitis B surface antigen (HBs Ag)and anti-HCV antibody.

4- Imaging studies:

a- Abdominal and pelvic ultrasonography:

Abdominal and pelvic ultrasonography was performed for all groups.

Patients were examined using a real time machine.

Liver was assessed for size, texture, the presence of focal lesions and their detailed description as regards number, size, site, echogenicity was reported, portal vein diameter, presence of portal vein thrombosis and thickening of portal tracts as an indicator for schistosomal hepatic fibrosis. Also the presence of ascites, spleen size, the presence of lymph nodes and abdominal masses.

b- Triphasic abdominal and pelvic CT scanning:

Triphasic abdominal and pelvic CT was done to all patients in HCC group for the diagnosis of hepatic focal lesions with specific features of HCC. To assess the size of the liver, its outline (normal or wavy outline like in cirrhosis), focal lesion (site, size, arterial contrast uptake and washout in venous phase), spleen (size), portal vein (patent or thrombosed), biliary system and abdominal lymph nodes.

c- Plain chest x-ray postero-anterior and left lateral view for G1 and G2.

5- Tumor markers:

a) A F P

b) Angiopoitein-2

A 5 ml blood sample was drawn from each patient. Blood samples were centrifuged and serum stored at –20°C until tested for Ang-2.

- Measurement of serum Ang-2(pg/dl):

Principle of the test:

The raybio human angiopoietin-2 ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human angiopoietin-2 in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for human angiopoietin-2 coated on a 96-well plate. Standards and samples are pipetted into the wells and angiopoietin-2 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human angiopoietin-2 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells.

The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of angiopoietin-2 bound.

The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm.

Reagents:

1. Angiopoietin-2 microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human angiopoietin-2.

2. Wash buffer concentrate (20x) (Item B): 25 ml of 20x concentrated solution.

3. Standards (Item C): 2 vials, recombinant human angiopoietin-2.

4. Assay diluent (Item E): 15 ml of 5x concentrated buffer. For Standard/Sample (serum/plasma samples/cell culture medium/urine) diluent.

5. Detection antibody angiopoietin-2 (Item F): 2 vial of biotinylated antihuman angiopoietin-2 (each vial is enough to assay half microplate).

6. HRP-Streptavidin concentrate (Item G): 200 μl 200x concentrated HRP conjugated streptavidin.

7. TMB one-step substrate reagent (Item H): 12 ml of 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution.

8. Stop solution (Item I): 8 ml of 0.2 M sulfuric acid.

Storage: May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at –80°C) after reconstitution. Opened microplate Wells or reagents may be store for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20°C.

Avoid repeated freeze-thaw cycles.

Additional materials required:

1. Microplate reader capable of measuring absorbance at 450 nm.

2. Precision pipettes to deliver 2 μl to 1 ml volumes.

3. Adjustable 1-25 ml pipettes for reagent preparation.

4. 100 ml and 1 liter graduated cylinders.

5. Absorbent paper.

6. Distilled or deionized water.

7. Log-log graph paper or computer and software for ELISA data analysis.

8. Tubes to prepare standard or sample dilutions.

Assay procedure:

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 μl standard or sample to each well.

Incubate 2.5 hours at room temperature or over night at 4°C.

3. Add 100 μl prepared biotin antibody to each well.

Incubate 1 hour at room temperature.

4. Add 100 μl prepared Streptavidin solution.

Incubate 45 minutes at room temperature.

5. Add 100 μl TMB one-step substrate reagent to each well.

Incubate 30 minutes at room temperature.

6. Add 50 μl stop solution to each well.

Read at 450 nm immediately.

Calculation of results:

Calculate the mean absorbance for each set of duplicate standards and samples and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

Sensitivity:

The minimum detectable dose of Angiopoietin-2 is typically less than 10 pg/ml.

Specificity:

Cross reactivity: This ELISA kit shows no cross-reactivity with the following cytokines tested: Human angiogenin, angiopoietin-1, BDNF,

BLC, ENA-78, FGF-4, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-.

8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10,

GCSF, GM-CSF, IFN-g, Leptin (OB), MCP-1, MCP-3, MDC, MIP-1a,

MIP-1 b, MIP-1_, MMP-1, -2, -3, -10, PARC, RANTES, SCF, TARC, TGF-b, TIMP-1, TIMP-2, TNF-a, TNF-b, TPO and VEGF.

Statistical Analysis

The data were coded, entered and processed on computer using *SPSS* (version 18). The results were represented in tabular and diagrammatic forms then interpreted.

Mean, standard deviation, range, frequency, and percentage were use as descriptive statistics.

The following test was done:

- **Chi-Square test** X^2 was used to test the association variables for categorical data.

- **Student's-test** was used to assess the statistical significance of the difference between two population means in a study involving independent samples.

- **Pearson correlation (r)**: is a test used to measure the association between two quantitative variables.

- **Receiver Operating Characteristic curve analysis (ROC-curve) analysis:**

✓ *Sensitivity:* Probability that the test results will be positive when the disease is present (true positive rate, expressed as a percentage).

✓ *Specificity:* Probability that the test results will be negative when the disease is present (true negative rate, expressed as a percentage).

P value was considered significant as the following:

* $P > 0.05$: Non significant.

* $P \leq 0.05$: Significant.

(Levesque, 2007)

3. Results:

This table showed the descriptive data of HCC group compared to Non HCC in relation to sex. HCC was 42 (70%) males and 18 (30%) females. NO HCC was 5 (35.7%) males and 9 (64.3%) females. There was statistically significant difference between two groups as regard to sex (p=0.01).(**Tab:1**).

This table showed the descriptive data of HCC group compared to Non HCC in relation to age. The age of HCC group ranged between 36 to 78 years with mean age 59.86 year and standard deviation 9.27. The age of Non HCC group ranged between 31 to 70 years with mean age 52.42 year and standard deviation 12.90. There was statistically significant difference between two groups as regard to age (p=0.01). **(Table 2).**

Table (1): Comparison between HCC and Non HCC as regard to sex.

			HCC	Non HCC	X2	P. value
Sex	Female	N.	18	9	5.75	0.01
		%	30.0%	64.3%		
	Male	N.	42	5		
		%	70.0%	35.7%		
	Total	N.	60	14		
		%	100.0%	100.0%		

Table (2): Comparison between HCC and Non HCC as regard to age.

	HCC				Non HCC				t. test	P. value
	Range		Mean	S.D	Range		Mean	S.D		
	Min.	Max.			Min.	Max.				
Age	36	78	59.86	9.27	31	70	52.42	12.90	2.49	0.015

This table showed the descriptive data of HCC group compared to Non HCC in relation to AFP. AFP of HCC group ranged between 400 to 50000 with mean 3063.63 and standard deviation 6956.46 AFP of Non HCC group ranged between 202 to 916 with mean 310.14 and standard deviation 184.03. There was statistically significant difference between two groups as regard to AFP (p=.003). (**Table 3**).

Table (3): Comparison between HCC and Non HCC as regard to AFP.

	HCC				Non HCC				t. test	P. value
	Range		Mean	S.D	Range		Mean	S.D		
	Min.	Max.			Min.	Max.				
AFP	400	50000	3063.63	6956.46	202	916	310.14	184.03	1.473	0.003

This table showed the descriptive data of HCC group compared to Non HCC in relation to Angiobiotin-2. Angiobiotin-2 of HCC group ranged between 5.90 to 2824 with mean 2130.86 and standard deviation 589.09. Angiobiotin-2 of Non HCC group ranged between 5.90 to 2304 with mean 611.26 and standard deviation 997.97. There was statistically significant difference between two groups as regard to Angiobiotin-2 (p=.000). **(Table 4).**

Table (4): Comparison between HCC and non HCC as regard to Angiopoietin-2.

	HCC				Non HCC				t. test	P. value
	Range		Mean	S.D	Range		Mean	S.D		
	Min.	Max.			Min.	Max.				
Angiopoietin-2	5.90	2824	2130.86	589.09	5.90	2304	611.26	997.97	7.51	0.000

The Ang-2 levels were positively correlated with ALT and AST. The Ang-2 levels were negatively correlated with platelets and prothrombin concentration in HCC group **(Tab: 5).**

**Table (5): Correlation between Angiopoietin-2 and other variablesin HCC.**

Correlation	Pearson's correlation	
	r	P
PCR * Angiopoitein-2	0.116	0.379
plat * Angiopoitein-2	-0.283	0.028
hemoglobin * Angiopoitein-2	-0.093	0.479
Prothrombin concentration* Angiopoitein-2	-0.138	0.050
albumin * Angiopoitein-2	0.084	0.525
ALT * Angiopoitein-2	0.090	0.049
AST * Angiopoitein-2	0.189	0.034
Total bilirubin * Angiopoitein-2	0.071	0.589
direct * Angiopoitein-2	0.043	0.746
AFP * Angiopoitein-2	0.172	0.189
ALK phosphatase * Angiopoitein-2	0.131	0.319

The diagnostic accuracy of Ang-2 in detection of HCC was shown in receiver operator characteristic (ROC) curve analyses. Regarding the diagnostic accuracy of Ang-2 analysis, area under the curve (AUC) was 87. At a cut off value 2215 pg/ml, serum Ang-2 showed 93.33% sensitivity, 71.4% specificity, 93.33% PPV, 71.4% NPV with accuracy 89.18%. **(Table 6), Figure (1).**

Table (6): Validity of Ang-2 levels in diagnosis of HCC

Tumor Markers	AUC	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%	Cut off
Ang-2 pg/ml	87	93.33%	71.4%	93.33%	71.4%	89.18%	2215
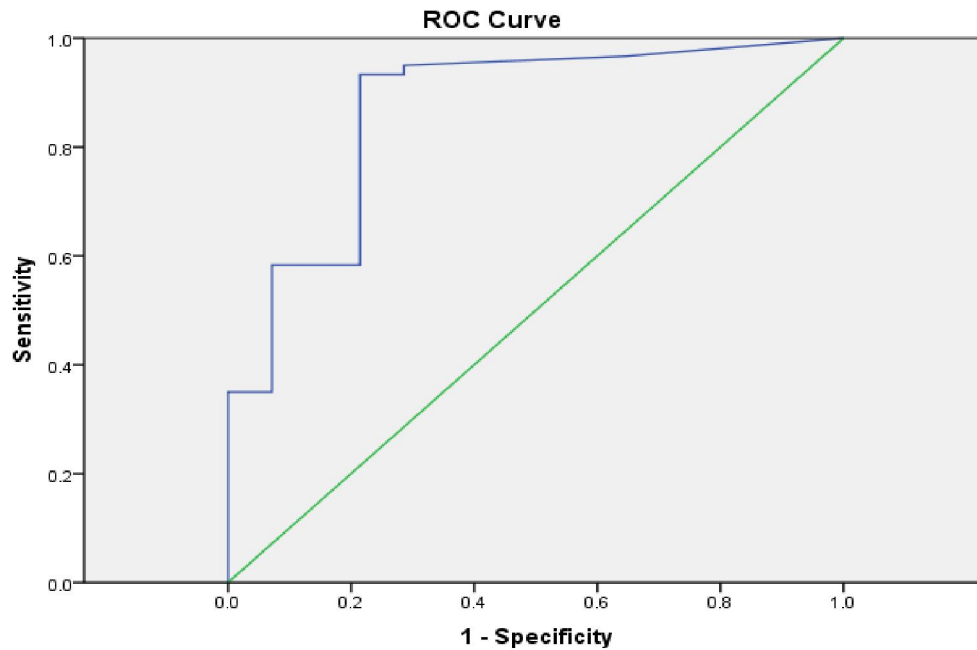
Figure (1). ROC curve for Ang – 2 in diagnosis of HCC

4. Discussion:

This study showed that, AFP of HCC group ranged between 400 to 50000 with mean 3063.63 and standard deviation 6956.46. AFP of Non HCC group ranged between 202 to 916 with mean 310.14 and standard deviation 184.03. There was statistically significant difference between two groups as regard to AFP (p=.003).

This agrees with (**Elzefzafy et al., 2009)** which showed, as comparing among two patient groups (HCC group and cirrhosis group without HCC), There was statistically significant difference between two groups as regard to AFP (p <.05).

This study showed that, Angiopoietin-2 of HCC group ranged between 5.90 to 2824 with mean 2130.86 and standard deviation 589.09. Angiopoietin-2 of Non HCC group ranged between 5.90 to 2304 with mean 611.26 and standard deviation 997.97. There was statistically significant difference between two groups as regard to Angiopoietin-2 (p=.000).

This agrees with (**Elzefzafy et al., 2009)** which showed, as comparing among two patient groups (HCC group and cirrhosis group without HCC), There was statistically significant difference between two groups as regard to Angiopoieten-2 (p <.01).

This agrees also with (**Youssef et al., 2015)** which found, as comparing among two patient groups

(HCC group and cirrhosis group without HCC), statistically significant difference was recorded as regard to Angiopoietin-2 (p < 0.001).

Also, this finding is in agreement of **(Bergers & Benjamin, 2003)** and (**Scholz et al., 2007**) who stated that HCCs are hypervascularized tumors and generation of new arterial vessels is a prerequisite for their survival. The induction ofneoangiogenesis is mainly driven by hypoxia, leading to activation of severalangiogenic pathways, including the angiopoietin/Tie-2 pathway.

Zhang et al; (2006) observed that Ang-2 mRNA was significantly upregulatedin HCC compared with the nontumorous liver tissue, and has been regardedas a contributor to recurrence, metastasis and poor prognosis of HCC.

The Ang-2 levels were positively correlated with ALT and AST. The Ang-2 levels were negatively correlated with platelets and prothrombin concentration in HCC group.

This agrees with (**Elzefzafy et al., 2009)** which showed that the Ang-2 levels were positively correlated with ALT and AST and negatively correlated with prothrombin concentration in HCC group..

The diagnostic accuracy of Ang-2 in detection of HCC was shown in receiver operator characteristic (ROC) curve analyses. Regarding the diagnostic accuracy of Ang-2 analysis, area under the curve (AUC) was 87. At a cutoff value 2215 pg/ml, serum Ang-2 showed 93.33% sensitivity, 71.4% specificity, 93.33% PPV, 71.4% NPV with accuracy 89.18%.

So this lead to that best use or validity of Ang-2 was in diagnosis of HCC with high validity and overall accuracy. These results are consistent with (**Hunter et al., 2010)** who found that, a sensitivity and specificity of 70% and 80% respectively. Serum Ang-2 was significantly elevated in HCC patients with portal vein thrombosis than those without. There was a significant positive correlation between the number of hepatic focal lesions and the serum level of Ang-2 and concluded that the combined use of the two markers (AFP and Ang-2) led to an increase in the sensitivity of AFP from 53.3% to 83.3%. And also concluded that the serum Ang-2 is elevated in patients with cirrhosis and further elevated in patients with HCC, so its use as an independent tumor marker in the diagnosis of HCC is to be considered and the detection rates could increase when using both markers.

5. Conclusion:

From the results and discussion of this study, it can be concluded that:

- Serum Ang-2 levels were elevated in HCC patients than patients with liver cirrhosis.

- Also Ang-2 is relatively comparative to AFP in detection of HCC among high risk groups and superior to AFP.

- The diagnostic accuracy of Ang-2 in detection of HCC was shown in receiver operator characteristic (ROC) curve analyses. Regarding the diagnostic accuracy of Ang-2 analysis, area under the curve (AUC) was 87. At a cut off value 2215 pg/ml, serum Ang-2 showed 93.33% sensitivity, 71.4% specificity, 93.33% PPV, 71.4% NPV with accuracy 89.18%.

- So this lead to that best use or validity of Ang-2 was in diagnosis of HCC with high validity and overall accuracy.

- Also concluded that the serum Ang-2 is elevated in patients with cirrhosis and further elevated in patients with HCC, so its use as an independent tumor marker in the diagnosis of HCC is to be considered and the detection rates could increase when using both markers.

- These results suggest that Ang-2 was a potential diagnostic tumor marker for HCC, especially among high-risk group of patients. This value extends beyond the traditional tumor biomarkers as AFP, as it possess good prognostic value. Although, AFP has to be considered 'the golden standard' for HCC serum markers for years, in the view of our data and that of others; the usefulness of AFP testing for the population at risk should be seriously questioned. Ang-2 levels appear to be an additional tumor biomarker for HCC detection especially among high risk group of patients.

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