Diagnostic Value of Serum Angiopoietin-2 in Patients with Hepatocellular Carcinoma

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Background and study aim: Hepatocellular carcinoma (HCC) is a hypervascular tumor and its progression is known to be closely related to angiogenesis. Angiopoietin-2 (Ang-2) is one of the angiogenic factors that may have a diagnostic value in HCC. Alpha-fetoprotein (AFP) serum levels are used for screening for HCC with limited success. This study aimed to evaluate diagnostic value in using angiopoietin-2 as a serum marker in HCC patients.

Patients and Methods: 50 patients with HCC (G1), 20 patients with liver cirrhosis (G2), and 20 healthy control persons (G3) were included in this study. Serum AFP and Ang-2 levels were measured by enzyme-linked immunosorbent assay.

Results: Serum Ang-2 levels in the HCC group (1523.54±886.46 pg/ml) was highly significantly elevated as compared to those with cirrhotic liver (222.55±153.60 pg/ml) and controls (138.35±54.09 pg/ml). The Ang-2 levels were significantly different between patients with liver cirrhosis and controls. In HCC patients, the serum Ang-2 levels in patients with portal vein (PV) thrombosis (n=7, 2164.0±960.85 pg/ml) and with large HCC (>5cm in diameter) (n=17, 2017.70±903.06 pg/ml) were significantly higher than those without PV thrombosis (n=43, 1274.47±727.56 pg/ml) and with small HCC (≤5cm in diameter) (n=33, 1268.97±773.93 pg/ml), while the serum AFP levels in patients with portal vein (PV) thrombosis (961.05±1007.70 ng/ml) and with large HCC (>5cm in diameter) (1000.81±1079.57 ng/ml) were not significantly higher than those without PV thrombosis (500.24±733.07 ng/ml) and with small HCC (437.87±611.02 ng/ml).

Conclusion: Combined measurement of serum AFP and Ang-2 significantly increases the sensitivity and specificity of HCC detection rather than using of AFP or Ang-2 separately.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide, and the third cause of cancer-related death **[1]**.

Development of HCC is known to be triggered by factors that lead to chronic hepatic injury and deregulation of the normal process of wound healing, which promote persistent stimulation of profibrotic and proangiogenic processes that lead to significant structural changes in the liver and functional changes in hepatic physiology **[2]**.

When tumor size reaches a critical size, the angiogenic switch enables the tumor to grow vessels that allow further growth and metastatic spread **[3]**. Hypoxia induces HIF-1α, a transcriptional regulator initiating synthesis of several proangiogenic factors **[4]**. Factors involved in neovessel formation in HCC include vascular endothelial growth factor (VEGF) and the angiopoietins (Ang) **[5]**. Angiopoietin-1 and -2 bind toTie-2, which is expressed on endothelial cells, in a competitive manner **[6]**. Ang-1 stabilizes vessels by recruiting smooth muscle cells whereas Ang-2 decreases stability and favors remodeling. Ang-2 seems not to

be directly regulated by hypoxic stimuli or HIF-1α **(7)**, but indirectly through VEGF and COX-2 **[8]**. In turn, Ang-2 and VEGF act synergistically on angiogenesis and tumor growth in HCC **[9]**. Ang-1 is expressed in HCC and in normal liver tissue, although upregulation of Ang-1 was also observed in HCC **[7]**. In contrast, Ang-2 expression is restricted to endothelial cells under physiologic conditions, and Ang-2 is over expressed in endothelial cells within tumors **[10]**, but de novo expression of Ang-2 by some tumors occurs **[11]**. Ang-2 is overexpressed in HCC, especially of the highly vascular type **[12]** and expression levels increase with dedifferentiation of tumors **[8]**.

PATIENTS AND METHODS

This study was carried out on 70 patients and 20 controls. An informed consent was obtained before patients enter the study.

They were divided into three groups :
Group 1: Included 50 patients with HCC on top of liver cirrhosis.

Group 2: Included 20 patients with liver cirrhosis.

Group 3: Included 20 healthy individuals. Patients of this study were selected from patients attending the outpatient clinic and/or inpatient department of Tropical Medicine, Minoufiya university hospital. They were 51 males (72.9%) and 19 females (27.1%) and their ages were ranging between 38–76 years, with mean (55.13±8.16) as well as, 20 healthy persons of matched age and sex as a control group. All patients and control were subjected to: Full and detailed history taking, complete clinical examination, routine laboratory investigation, viral markers, abdominal ultrasonography, triphasic CT (this was done for G1 only), quantitative measurement of serum alpha-fetoprotein and quantitative measurement of Ang-2 by ELISA kit.

Statistical analysis:
Collected Data was analyzed using SPSS computer software, version 15, and expressed as a number and a percentage for qualitative variables and as a mean ± SD for quantitative variables.

RESULTS

There was high statistical significant difference as regard levels of Ang-2 between HCC and cirrhosis patients and controls.There was also high statistical significant difference as regard level of AFP between all groups (Table 1).

There was high statistical significant difference as regard level of Ang-2 in large sized tumors than small sized one in HCC, while, there was no significant difference between large and small sizes HCC as regard mean value of AFP (Table 2).

There was statistical significant difference as regards Ang-2 levels in HCC with portal vein thrombosis than those with patent portal vein (Table 3).

There was no statistical significant difference as regards levels of AFP in HCC with portal vein thrombosis than those with patent portal vein.

ROC analysis displayed 98% sensitivity and 100% specificity for Ang-2 in the discrimination between HCC patients and healthy controls at a cutoff level 315.5pg/L (Table 4).

Discrimination between cirrhotic and control individuals by Ang-2 serum levels increased somewhat with the progression of cirrhosis. Mean Ang-2 serum levels were significantly different between cirrhotic patients Child C and controls. Mean Ang-2 serum levels were with a high significant difference between patients with HCC in all Child categories, cirrhotic patients and control (Table 4).

Detection rates have been increased from 64.3% when using AFP alone to 82.9% when using both markers (Table 5).

Combined use of AFP and Ang-2 increased the sensitivity to 100% and specificity to 60%.

Table (1) : Comparison between the studied groups as regards AFP and angiopiotin-2

	G1 N = 50	G2 N = 20	G3 N = 20	Test of significance	P value
AFP X ± SD Median	629.27±834.83 254.5	10.98±1.82 13.19	1.38±0.51 1.10	5.88 6.48 4.36	<0.001[1] <0.001[2] <0.001[3]
Ang-2 X ± SD Median	1523.54±886.46 1166.5	222.55±153.60 170	138.35±54.09 121	6.23 6.40 1.29	<0.001[1] <0.001[2] >0.05[3]

AFP= Alpha-fetoprotein, Ang-2= Angiopoietin-2, x=mean, SD= standare of deviation
1 = Comparison between G1 and G2
2 = Comparison between G1 and G3
3 = Comparison between G2 and G3

This table shows that, there was highly statistical significant difference as regards levels of Ang-2 between G1 & G2 and G1 & G3.

This table shows that, there was high statistical significant difference as regards level of AFP between all groups.

Table (2) : Relationship between the tumor size, Ang-2 and AFP in G1

	Tumor size (CT)		Mann Whitney U test	P value
	≤ 5 Cm N = 33	> 5 Cm N = 17		
AFP X ± SD Median	437.87±611.02 254	1000.81±1079.57 538	0.79	>0.05
Ang-2 X ± SD Median	1268.97±773.93 1112	2017.70±903.06 2215	2.92	<0.001

This table shows that, there was highly statistical significant difference as regards level of Ang-2 in large sized tumor than small sized ones in G1, while there was no significant difference between large and small sizes HCC as regard mean value of AFP.

Table (3) : Relationship between Portal vein and Ang-2 and AFP in G1

	Portal vein (CT)		Mann Whitney U test	P value
	Patent N = 43	Thrombosed N = 7		
AFP X ± SD Median	500.24±733.07 215	961.05±1007.70 455.5	1.78	>0.05
Ang-2 X ± SD Median	1274.47±727.56 1122.5	2164.0±960.85 2171	2.68	<0.05

This table showed statistical significant difference as regards Ang-2 levels in G1 with portal vein thrombosis than those with patent portal vein.
This table showed that, there was no statistical significant difference as regards levels of AFP in G1 with portal vein thrombosis than those with patent portal vein.

Table (4) : Diagnostic performance of AFP and Ang-2 in diagnosis of HCC:

Parameter	Discrimination between	AUC	Cut-off value	Sensitivity	Specificity	P value
Ang-2	Cirrhosis and control	0.62	99	75%	25%	>0.05
	Child A and control	0.35	70	68%	24%	<0.05
	Child B and control	0.49	99	70%	25%	>0.05
	Child C and control	0.82	128	88.9%	60%	<0.05
	HCC and control	0.99	315.5	98%	100%	<0.001
	HCC and cirrhosis	0.98	400.5	98%	85%	<0.001
	Child A and cirrhosis	100	617.5	100%	100%	<0.001
	Child B and cirrhosis	0.95	661.5	91.7%	100%	<0.001
	Child C and cirrhosis	0.98	400.5	100%	85%	<0.001
AFP	Cirrhosis and control	0.90	1.84	85	80	<0.001
	Child A and control	0.97	1.95	100	85	<0.05
	Child B and control	0.80	1.535	87.5	75	<0.05
	Child C and control	0.96	2.08	88.9	95	<0.001
	HCC and control	0.99	5.55	96.0	100	<0.001
	HCC and cirrhosis	0.86	10.78	78%	70%	<0.001
	Child A and cirrhosis	0.76	10.78	75%	70%	<0.05
	Child B and cirrhosis	0.84	7	91.7%	60%	<0.05
	Child C and cirrhosis	0.89	16	83.3	75%	<0.001

AUC=area under curve
ROC analysis revealed discrimination between HCC, cirrhotic and control individuals

Table (5) :Detection of hepatocellular carcinoma in patients with liver cirrhosis using Ang-2 or AFP or both serum measurements:

	Elevated		Normal	
	No	%	No	%
Both Ang-2 and AFP elevated	39	55.7		
Ang-2 elevated	13	18.6		
AFP elevated	6	8.6		
Normal both Ang-2 and AFP			12	17.1

When the optimal cutoff level of Ang-2 for discrimination between HCC and cirrhosis (400.5 pg/L) and the established cutoff for AFP (10.78 pg/L) were applied, both markers were elevated in only 55.7% of patients. Either marker alone was elevated in 8.6% (AFP) or 18.6% (Ang-2), whereas 17.1% of HCC patients had normal values for Ang-2 and AFP.
Detection rates could have been increased from 64.3% when using AFP alone to 82.9% when using both markers.

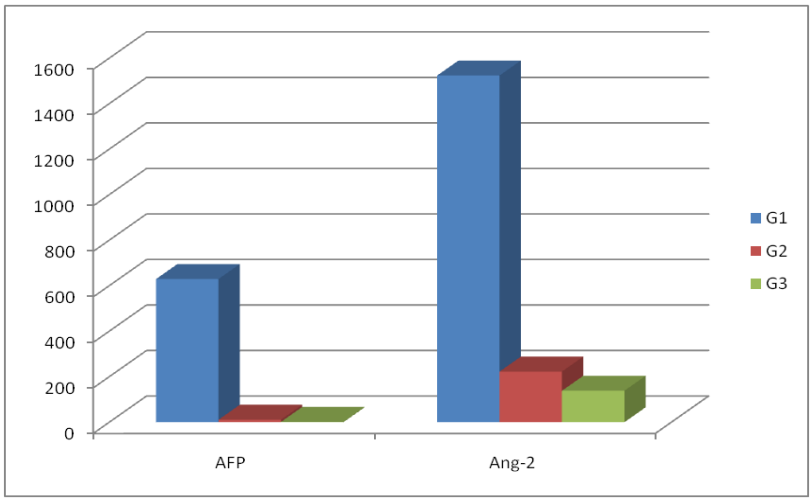

Fig. (1) : Comparison between the three studied groups as regards AFP and Ang-2.

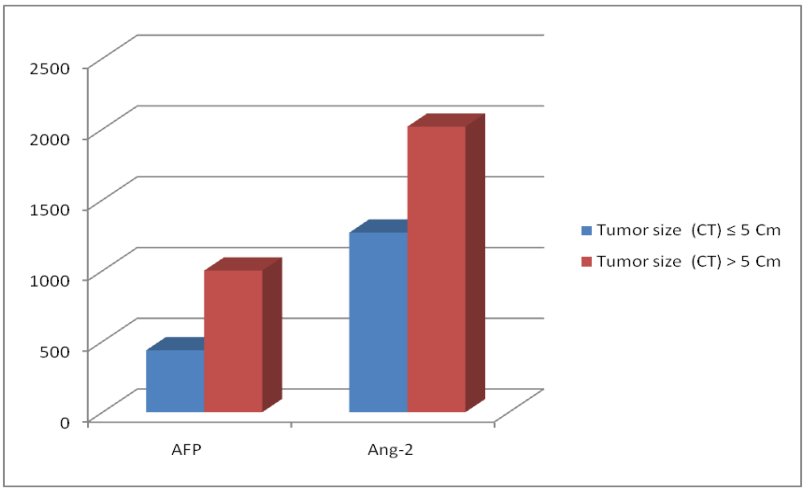

Fig. (2) : Relationship between the tumor size, Ang-2 and AFP in G1.

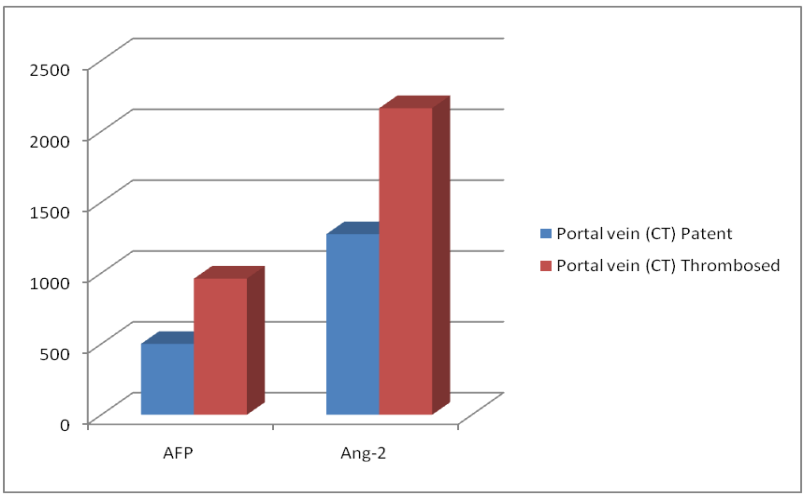

Fig. (3) : Relationship between Portal vein patency, Ang-2 and AFP in G1

DISCUSSION

Angiogenesis is essential for tumor growth and metastasis **[13]**. It is a multifactorial phenomenon: Many factors have been reported to be involved in this complicated process **[14]**. The ANG-TIE-2 system, which includes two major mediators, ANG-1 and ANG-2, is a new, important family for the regulation of angiogenesis and vascular integrity **[15].** Although, only a few reports are available on the role of ANGs in neoplastic angiogenesis **[16]**, their roles in embryonic development or normal tissues have been well described in several papers **[6,17**].

The primary marker for HCC is alpha-fetoprotein (AFP), generally, it shows acceptable sensitivity. However, AFP is not secreted in all cases of HCC and may be normal in about 40% of patients with early HCC **(18)**.

This work aimed to study methods which improve the early detection of HCC by measuring serum Ang-2 as well as, AFP to improve the diagnostic ability.

On using the ROC curve to reach the value of the best sensitivity and specificity of AFP; it has been shown that, the sensitivity and specificity of AFP varied with the different cutoff values used. At a cutoff value of 10.78 ng/ml, the sensitivity was 78% and the specificity was 70%. Bruix and Sherman **[2]** reported the diagnostic cutoff of HCC at 200 ng/ml. In our study, when using this cutoff, the sensitivity declined to 24% while the specificity was 100%. This finding was comparable to that of Oka et al. **[19]** who reported low sensitivity (13%) and a specificity of 97% at AFP values over 200 ng/ml. When we further increased the cutoff of serum AFP> 400 ng/ml, the specificity increased and the sensitivity decreased to 10% which was close to the results obtained by Rapaccini et al. **[20]** who reported a sensitivity of 7.2% at a cutoff > 400 ng/ml.

In this study, AFP was elevated (>200 ng/ml) in only 23.3% of HCC patients and this was in agreement with Huo et al. **[21]** who concluded that, serum AFP level was a weak diagnostic predictor in HCC patients.

Our results revealed that there was a statistically highly significant elevation (P<0.001) in the mean serum Ang-2 in HCC group (1523.54 ± 886.46 pg/ml) when compared to cirrhosis group (222.55 ± 153.60 pg/ml) and control group (138.35 ± 54.09 pg/ml).

These results are consistent with Scholz et al. **[22]** who reported a statistically highly significant elevation of Ang-2 serum levels in cirrhosis patients when compared to control persons. Although, there are few data available regarding the expression of angiopoietins in cirrhosis in humans, many theories could be speculated in explanation:

Ang-2 is released in inflammatory conditions such as, chronic HCV infection **(22)**. Salcedo et al. **[23]** reported that, chronic HCV patients showed elevated serum baseline VEGF and Ang-2 levels. After treatment by interferon alpha 2b plus ribavirin, both factors were decreased, whereas, antiangiogenic Tie-2 was increased, indicating a shift toward an "anti-angiogenic" profile of serum markers in chronic HCV patients.

These data may argue against inflammation as the sole reason for elevated Ang-2 levels in serum of cirrhotic patients and emerge the fibrosis process as an extra explanation **[22]**. Other similar findings include generation of neo-vessels in primary biliary cirrhosis patients accompanied by the increased expression of VEGF, Ang-2 and Tie-2 **[24]**. Taken together, these published data suggest a causative role of angiogenic factors such as Ang-2, in the remodeling of the cirrhotic liver.

ROC analysis displayed 98% sensitivity and 100% specificity for the discrimination between HCC patients and healthy controls at a cutoff level of 315.5pg/L (AUC 0.99).

ROC analysis revealed that, Ang-2 levels discriminated with 98% sensitivity and 85% specificity between patients with HCC and cirrhotic patients at a cutoff level of 400.5pg/L (AUC 0.98).

These results are consistent with Scholz et al. **[22]** who reported a sensitivity of 70.56% and specificity of 73.28% when the high cutoff value of Ang-2 was used (12350pg/ml), the sensitivity and specificity were 40% and 100% respectively. These results demonstrate that the high cutoff value of serum Ang-2 may show better results of specificity than the best cutoff value of (315.5 pg/ml) at the expense of much decrease of sensitivity value.

This study revealed a significantly higher Ang-2 level in combined HBV&HCV induced HCC than HCV and HBV alone. Yates et al. **[25]** concluded that, infection with HCV and HCV-HBV double infection, but not HBV alone, is strongly correlated with HCC in Egypt. Scholz et

al. **[22]** was in agreement with our results, they demonstrated that Ang-2 serum levels are elevated in patients with liver cirrhosis independent of the cause of cirrhosis.

There is statistical significant difference between Child A cases and Child C cases among HCC group as regards level of AFP, as was the case in Scholz et al. **[22]**. Our study revealed no statistical significant difference between Child classification and Ang-2 serum levels however Ang-2 level was higher in child C and child B than child A. Scholz et al. **[22]** suggested that, Ang-2 production in the cirrhotic liver is mediated by inflammatory as well as, fibrous tissue but not hepatocytes. This fact explains the close relationship between Ang-2 levels and severity of liver cirrhosis.

Although, Scholz et al. **[22]** had reported no significant correlation between serum Ang-2 levels and Child classification, they found that, discrimination between cirrhotic and control individuals by Ang-2 serum levels improved somewhat with the progression of cirrhosis. These interesting data are in need to be further studied to assess the significance of Ang-2 as a serum marker in liver cirrhosis and illustrate its role in liver remodeling process.

The present study revealed no statistical difference of AFP in HCC group as regard tumor size. This is in agreement with Sato et al. **[26]** who concluded that, the rise in the serum AFP level did not usually correlate with the tumor size. This could be explained by the fact that tumor differentiation and its ability to secrete AFP are more important than the tumor size in determining the level of AFP produced by HCC. In contrast, Nomura et al. **[27]** found that, serum level of AFP correlates with the size of the tumor.

Nomura et al. **[27]** concluded that some HCC are AFP-negative tumors at an early stage and may turn into AFP-producers later on in advanced stages, where others may not produce AFP throughout the clinical course.

Our results showed a significantly higher level of Ang-2 in large sized tumor (>5cm) (2017.70 ± 903.06) than small sized ones (≤5 Cm) (1268.97 ± 773.93) in HCC group, while there is no significant difference between large and small sizes HCC as regard mean value of AFP. Scholz et al. **[22]** had reported that, Ang-2 levels varied somewhat depending on tumor size, although, not statistically significant (P= 0.067 for the comparison of tumors >80 mm with tumors <30 mm and P = 0.073 for the comparison of tumors of 31–80 mm with tumors <30 mm. AFP levels did not depend on tumor size.

Our results showed significantly higher Ang-2 levels in HCC cases with portal vein thrombosis (2164.0 ± 960.85) than those with patent portal vein (1274.47 ± 727.56). Li et al. **[28]** reported a high expression of Ang-2 mRNA (using immuno-histochemistry) in HCC cases with portal vein tumor thrombosis in comparison with those without; concluding that Ang-2 can promote tumor thrombus formation by modulating angiogenesis.

In our results, the combined use of the two markers (AFP and Ang-2) led to an increase in the sensitivity of AFP from 64.3% to 83%. These results were in agreement with Scholz et al. **[22]** who compared the ability of Ang-2 serum levels to predict HCC with elevated AFP in patients with established HCC and in patients with cirrhosis but without HCC. Whereas, both markers were elevated in 50% of patients, either one of the markers alone was elevated in 18.6% and 8.6% of patients, respectively. Scholz et al. **[22]** concluded that, detection rates could increase from 64.3% when using AFP alone to 82.9% when using both markers.

CONCLUSION:

From this study, it was concluded that combined measurement of serum AFP and Ang-2 significantly increases the sensitivity, and specificity in detection of HCC in cirrhotic patients rather than using of AFP or Ang-2 separately. Ang-2 may be a helpful tool in the diagnosis of vascular invasion in patients with HCC, as it was significantly elevated in HCC patients with portal vein thrombosis when compared to those without.

Conflicts of interest: None
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Ethical approval:Approved.

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