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Evaluation the Role of Amphiregulin protein in Hepatitis C virus patients

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Abstract

Background: Hepatocellular carcinoma (HCC) is the most common primary malignant liver cancer and the sixth most common form of cancer worldwide. The number of its patients are growing all over the world as it affects half a million patients yearly. The main indication of HCC is the secretion of Alfa Feto Protein which may be normal in only 40 % of its patients. Amphiregulin protein has been identified as one of the 10- gene signatures in close association with the occurrence of liver metastasis in colorectal cancer patients. The expression of AREG in normal livers is undetectable; however, it is induced during acute and chronic liver injury AREG is an early response growth factor during liver regeneration. It also contributes to the transformed phenotype of human hepatocellular carcinoma cells. **Objectives**: The aim of the study is biochemical investigation of amphiregulin protein in Hepatitis C virus patients. **Patients and Methods**: This study involved 90 participants in 3 groups: Group (1): Control group composed of 30 healthy subjects. Group (2): Cirrhosis group which is composed of 30 patients with chronic liver disease (cirrhosis). Group (3): HCC group which is composed of 30 patients with hepatocellular carcinoma on top of HCV-related liver cirrhosis. All patients were subject to an assessment of AFP and AREG. Besides, HCC patients went clinically through a full estimation of liver biochemical profile, viral indications, and finally US and triphasic abdominal CT. **Results**: There is a statistically significantly higher age, Amphiregulin, AST, and INR in HCC > cirrhosis > control. AFP was statistically significantly higher in HCC and cirrhosis vs. control. Though, AFP was higher in HCC vs. control, and this difference was not statistically significant. There is a statistically significantly lower serum albumin in HCC > cirrhosis > control. WBCs and platelet counts were statistically significantly lower in HCC vs. cirrhosis. There is statistically significantly higher ALT and total bilirubin in HCC and cirrhosis vs. control, and a statistically significantly lower hemoglobin level in HCC and cirrhosis vs. control. **Conclusion**: The result exhibit There is the diagnostic performance is good for AREG and when use to gather with AFP is perfect.

Keywords: Amphiregulin protein, Alpha feto protein, Inflammation, Hepatitis-B.

1. Introduction

Chronic liver disease (CLD) and cirrhosis account for 44,000 deaths in the United States and 2 million deaths worldwide each year, in addition to a high burden of disability and increased health care utilization.[1] HCC is a global dilemma the severity of which varies from one place to another. Concerning Egypt, the third most populous African country and the 15th internationally, the local health authorities view HCC as an imminent danger.

Over just ten years, the number of HCC patients doubled. Such a review is meant to compare the situation in Egypt to that in the rest of the world from a number of angles including the risk factors, precaution, checking and monitoring, diagnosis and treatment, and finally as a research strategy. Full

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awareness of such points would guide the efforts exerted by the authorities concerned to confront HCC both nationally and internationally.

AFP is the most frequently used biomarker for HCC.[3] Serum AFP is elevated in 60–80% of HCC patients and is helpful in screening and monitoring treatment responses.[4] European and Asian Pacific guidelines have recommended the use of an AFP level of 200 ng/ml as a reliable cut-off value for HCC diagnosis.[5,6] Although AFP can help to define the population at risk of HCC[7,8], it showed a suboptimal performance as a serological test for surveillance.

Amphiregulin (AREG) is a ligand of the Epidermal Growth Factor Receptor (EGFR) which has an essential role in cell proliferation, survival and migration. Two copies of AREG gene are identified in humans (AREGA and AREGB) and located at the EGF family gene cluster on the chromosome band.[9,10] Contrary to normal liver, Amphiregulin expression remarkably increases upon liver injury, which would play an outstanding role in cytoprotecting and regenerating liver tissues. [11,12]

Amphiregulin has been associated with resistance of liver cancer cells to chemotherapeutic agents as doxorubicin and cisplatin[37]. Most importantly, increased AREG expression in HCC cells was associated with resistance to sorafenib, the sole clinically approved agent for advanced HCC [40].

Subjects And Methods

Study Design:

This study is meant to evaluate amphiregulin protein in Hepatitis C virus patients.

Setting:

Patients were recruited from in-patient clinics of the National Liver Institute, Faculty of Medicine, Menoufia University, Menoufia, Egypt.

Study Population

This study was performed on 90 participants. Their diagnosis had laboratory, clinical, and radiological dimensions. They all clearly agreed to participate in this research.

The patients were divided into three groups:

- Hepatocellular carcinoma group: 30 patients with an average age (range: 36-76 years)
- Liver cirrhosis group: 30 patients with liver cirrhosis with mean age ± SD = 55.75 ± 7.70 (range: 40-69 years)
- Control group: 30 healthy volunteers (ages ranging from 42-61years), whose liver biochemistry is normal and no hepatitis signs shown.

Exclusion criteria of cases

1- They had a background marked by liver transplantation, or ever received any treatment for HCV or HCC, or had any other type of cancer, or presented by renal insufficiency.
2- patients who previously suffered from different forms of solid tumors.
3- Mixed HCC-cholangiocarcinoma.
4- Patients with a history of HBV, Nonalcoholic fatty liver disease and auto-immune hepatitis.

1. Laboratory investigation:
   - Liver function tests (serum albumin, serum bilirubin, prothrombin time (INR), serum creatinine, Alanine aminotransferase& Aspartate aminotransferase).
   - Anti HCV and HCV PCR.
   - Serum level of Alpha feto protein.
   - Serum AREG concentration was measured using a commercialy available kit from (My BioSource, San Diego, CA, USA) according to the manufacturer`s recommendations.

Statistical Methods

Data were statistically analyzed with Statistical Package for the Social Sciences (SPSS) version 21. The normality of data was first tested with one-sample Kolmogorov-Smirnov test. To compare the parametric data of two groups, Student t test was used while Mann-Whitney was used for the non-parametric. On the other hand, analysis of variance (ANOVA) test was used to compare the parametric data related to the means of more than two groups while Kruskal Wallis test was used for the non-parametric. It is noteworthy that specificity and sensitivity at various cutoff points are important. They are tested by receiver operating characteristic (ROC) Curve.P. value >0.05. Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) is given by these equations.[13]

Results

Notes: Test of significance for categorical data is Chi-square or Fisher's exact test (FET). Comparisons of column proportions were presented as small letters, similar letters = Insignificant difference, and different letters = Significant difference. Test of significance for age, and serum creatinine is One-way ANOVA, and for other quantitative data is Kruskal-Wallis H-test. Pairwise comparisons were presented as small letters, similar letters = Insignificant difference, and different letters = Significant difference.

Table 1 shows a statistically significantly higher age, Amphiregulin, AST, and INR in HCC > cirrhosis > control. AFP was statistically significantly higher in HCC and cirrhosis vs. control. Though, AFP was higher in HCC vs. control, this difference was not statistically significant. This table also shows a

statistically significantly lower serum albumin in HCC > cirrhosis > control. WBCs, and platelet counts were statistically significantly lower in HCC vs. cirrhosis. This table also shows a statistically significantly higher ALT and total bilirubin in HCC and cirrhosis vs. control and a statistically significantly lower hemoglobin level in HCC and cirrhosis vs. control.

Table 2 indicates that there is an important statistical positive relationship between AFP, and Amphiregulin, a statistically significant positive relation between both AFP and amphiregulin, and age, ALT, serum creatinine, serum total bilirubin, INR, and FBG, and a statistically significant positive relation between Amphiregulin, and AST. This table also shows an important statistical negative relationship between both AFP, and Amphiregulin, and hemoglobin level, platelet count, and serum albumin.

Table 3 indicates that there is an important statistical positive relationship between AFP, and Amphiregulin, a statistically significant positive relation between both AFP and amphiregulin, and age, ALT, serum creatinine, serum total bilirubin, INR, and FBG, and an important statistical positive relationship between Amphiregulin, and AST. This table also shows an important statistical negative relationship between both AFP, and Amphiregulin, and hemoglobin level, platelet count, and serum albumin.

Table 4 shows that AFP at cutoff value >10 can discriminate cirrhosis from control, and at cutoff value >9 can discriminate HCC from control, but it was not able to discriminate HCC from cirrhosis.

Table 5 shows that amphiregulin at cutoff value ≤23.97 can discriminate cirrhosis from control.

Table 1: Comparisons of the studied parameters between the three groups

Parameter	Group (1)	Group (2)	Group (3)	Test of significance	
Categorical	N (%)			χ^2	P value
Sex Male Female	 19 (63.3%) a 11 (36.7%) a	 18 (60%) a 12 (40%) a	 27 (90%) b 3 (10%) b	7.897	0.019
Residence Rural Urban	 18 (60%) a 12 (40%) a	 0 (0%) b 30 (100%) b	 18 (60%) a 12 (40%) a	FET	<0.001
Current smoking	10 (33.3%)	12 (40%)	11 (36.7%)	0.287	0.866
Diabetes	0 (0%) a	0 (0%) a	12 (40%) b	FET	<0.001
Hypertension	0 (0%) a	0 (0%) a	5 (16.7%) b	FET	0.010
Exposure to canal water	0 (0%) a	14 (46.7%) b	18 (60%) b	FET	<0.001
History of previous surgery	0 (0%) a	0 (0%) a	15 (50%) b	FET	<0.001
History of blood transfusion	0 (0%)	0 (0%)	3 (10%)	FET	0.104
Edema of lower limb	0 (0%)	0 (0%)	2 (6.7%)	FET	0.326
Ascites	0 (0%) a	0 (0%) a	4 (13.3%) b	FET	0.032
Splenomegaly	0 (0%) a	16 (53.3%) b	7 (23.3%) a	FET	<0.001
Quantitative	Mean ± SD			F value	P value
Age (years)	31.4 ± 6.1 a	49.7 ± 11.5 b	60.8 ± 6.4 c	93.529	<0.001
Serum creatinine (mg/dl)	0.78 ± 0.21	0.80 ± 0.14	0.86 ± 0.19	1.702	0.188
Quantitative	Median (25^{th} percentile – 75^{th} percentile)			H [2]	P value
AFP (ng/ml)	3.7 (3.1-5.7) a	25.5 (18.8-32.3) b	45.9 (6.2-503.5) b	44.621	<0.001
Amphiregulin (pg/ml)	13.7 (10.7-15.1) a	23.9 (20.2-25.7) b	42.1 (29.8-110.7) c	79.160	<0.001
ALT (U/L)	24 (19.0-29.5) a	37.0 (29.0-82.2) b	41.0 (28.7-68.3) b	25.366	<0.001
AST (U/L)	26.5 (19.0-29.5) a	31.5 (26.0-57.3) b	47.5 (37.0-70.5) c	36.754	<0.001
Hemoglobin level (g/dl)	14.4 (13.1-16.2) a	13.5 (11.5-14) b	13 (10.6-14.6) b	12.247	0.002
WBC count (*10^3 per µL)	6.5 (4.9-8.3) a, b	6.6 (5.9-8.2) a	5.5 (4.4-6.6) b	6.179	0.046
Platelet count (*10^3 per µL)	282 (188-372) a	251 (171-304) a	125 (88-154) b	34.281	<0.001
Serum total bilirubin (mg/dl)	0.48 (0.3-0.7) a	0.94 (0.78-1.1) b	1.2 (0.7-1.4) b	31.225	<0.001
Serum direct bilirubin (mg/dl)	0.22 (0.2-0.43)	0.31 (0.23-0.33)	0.25 (0.2-0.5)	2.332	0.312
Serum albumin (g/dl)	4.3 (4.1-4.5) a	3.8 (3.6-3.9) b	3.3 (3.1-3.7) c	42.555	<0.001
INR	1.04 (1-1.12) a	1.1 (1-1.2) b	1.15 (1.1-1.35) c	18.253	<0.001
FBG (mg/dl)	84 (72-89) a	98 (93.8-103.3) b	89.5 (82.5-97) c	32.437	<0.001

Table 2: Relation of AFP and biochemical parameters

Parameter	AFP		
	r_s	P value	
AFP (ng/ml)	-	-	
Amphiregulin (pg/ml)	0.631	<0.001	Moderate
Age (years)	0.570	<0.001	Moderate
ALT (U/L)	0.342	0.001	Weak
AST (U/L)	0.088	0.410	Weak
Hemoglobin level (g/dl)	-0.217	0.040	Weak
WBC count (*10^3 per µL)	-0.036	0.736	Weak
Platelet count (*10^3 per µL)	-0.379	<0.001	Weak
Serum creatinine (mg/dl)	0.257	0.014	Weak
Serum total bilirubin (mg/dl)	0.422	<0.001	Weak
Serum direct bilirubin (mg/dl)	0.161	0.129	Weak
Serum albumin (g/dl)	-0.526	<0.001	Weak
INR	0.236	0.025	Weak
FBG (mg/dl)	0.298	0.004	Weak
Meld score	Min. – Max. 15.0 – 52.0 Mean ± SD. 27.63 ± 9.52 Median (IQR) 24.0(24.0 – 26.0)	<0.001*	

Notes: r_s = Spearman's relation coefficient.

Table 3: Relation of Amphiregulin, and biochemical parameters

Parameter	Amphiregulin		
	r_s	P value	
AFP (ng/ml)	0.631	<0.001	Moderate
Amphiregulin (pg/ml)	-	-	
Age (years)	0.731	<0.001	Strong
ALT (U/L)	0.484	<0.001	Weak
AST (U/L)	0.329	0.002	Weak
Hemoglobin level (g/dl)	-0.319	0.002	Weak
WBC count (*10^3 per µL)	-0.198	0.061	Weak
Platelet count (*10^3 per µL)	-0.611	<0.001	Weak
Serum creatinine (mg/dl)	0.196	0.064	Weak
Serum total bilirubin (mg/dl)	0.537	<0.001	Moderate
Serum direct bilirubin (mg/dl)	-0.005	0.960	Weak
Serum albumin (g/dl)	-0.674	<0.001	Weak
INR	0.391	<0.001	Weak
FBG (mg/dl)	0.238	0.024	Weak
Meld score	Min. – Max. 15.0 – 52.0 Mean ± SD. 27.63 ± 9.52 Median (IQR) 24.0(24.0 – 26.0)	<0.001*	

Notes: r_s = Spearman's relation coefficient.

Table 4: ROC curve analysis for AFP cutoff values in discriminating the three groups

Discrimination	**Cutoff**	**AUC**	**95% CI**	**SE**	**P value**
Cirrhosis vs. control	>10	0.976	0.898-0.998	0.0246	<0.001
HCC vs. control	>9	0.888	0.780-0.955	0.0421	<0.001
HCC vs. cirrhosis	>42	0.613	0.479-0.736	0.0841	0.178

Notes: AUC = area under the ROC curve. SE = standard error.

Table 5: Diagnostic performance for amphiregulin to discriminate cirrhosis (n = 30) from control (n = 30)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Amphiregulin	0.701	0.008*	0.554 – 0.848	≤23.97	66.67	73.33	71.4	68.7

Notes:
AUC: Area Under a Curve
p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value
PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

Table 6 shows that amphiregulin at cutoff value ≤14.71 can discriminate HCC from control. Table 7 shows that amphiregulin at cutoff value ≤14.71 can discriminate HCC from cirrhosis. Table 8 shows that Diagnostic performance for Amphiregulin and AFP to discriminate cirrhosis from control. Table 9 shows that diagnostic performance for Amphiregulin and AFP to discriminate HCC from control. Table 10 shows that diagnostic performance for Amphiregulin and AFP to discriminate HCC from cirrhosis

Table 6: Diagnostic performance for amphiregulin to discriminate HCC (n = 30) from control (n = 30)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Amphiregulin	0.710	0.005*	0.563 – 0.857	≤14.71	70.0	67.67	75.0	71.9

Notes:
AUC: Area Under a Curve p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

Table 7: Diagnostic performance for amphiregulin to discriminate HCC (n = 30) from cirrhosis (n = 30)

	AUC	p	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
Amphiregulin	0.663	0.030*	0.514 – 0.813	≤14.71	70.0	70.0	70.0	70.0

Notes:
AUC: Area Under a Curve p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

Table 8: Diagnostic performance for Amphiregulin + AFP to discriminate cirrhosis (n = 30) from control (n = 30)

	AUC	p	95% C.I	Sensitivity	Specificity	PPV	NPV
Amphiregulin + AFP	0.993	<0.001*	0.979 – 1.008	80.0	96.67	24.0	82.9

Notes:
AUC: Area Under a Curve p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

able 9: Diagnostic performance for Amphiregulin + AFP to discriminate HCC (n = 30) from control (n = 30)

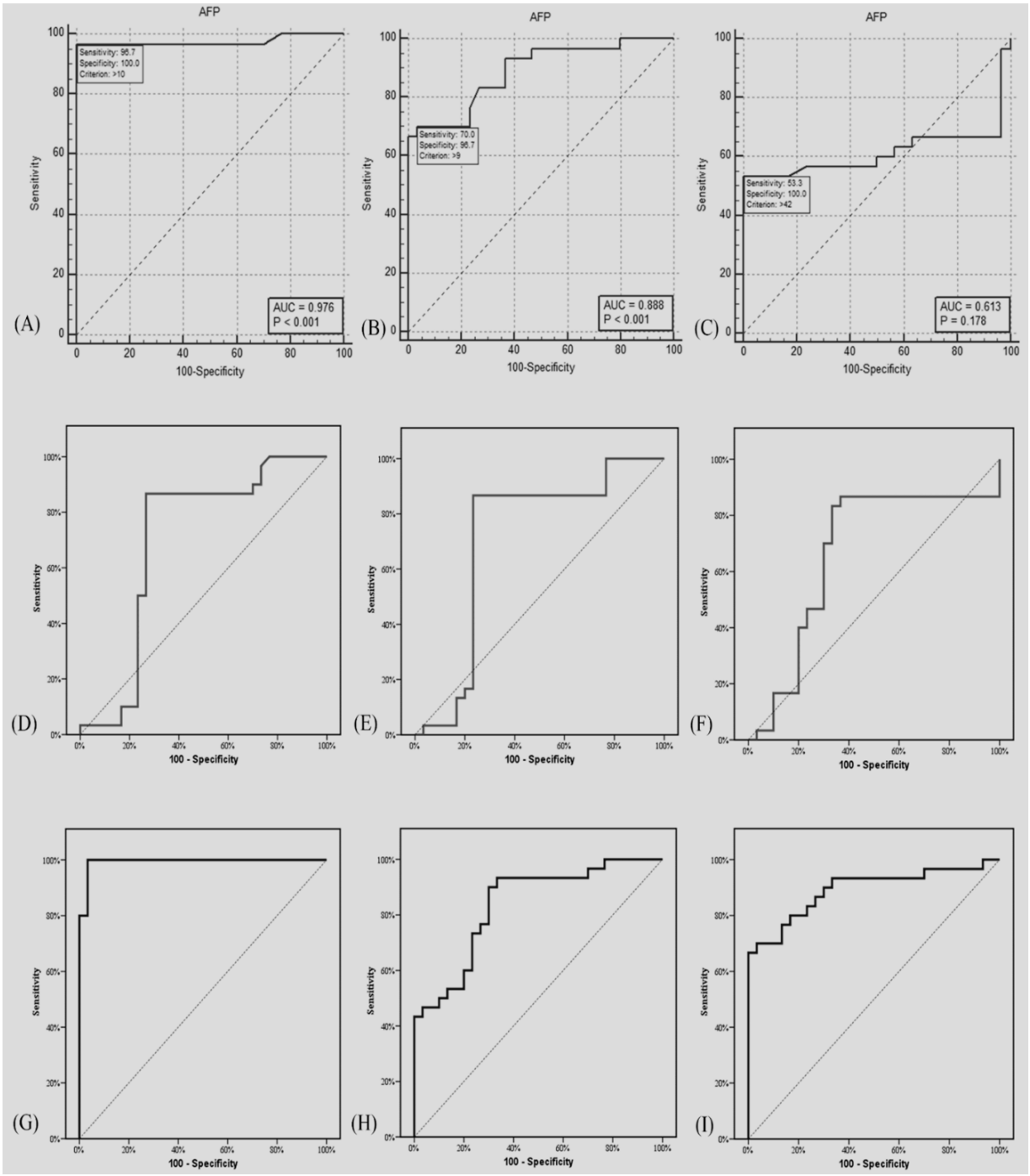
	AUC	p	95% C.I	Sensitivity	Specificity	PPV	NPV
Amphiregulin + AFP	0.838	<0.001*	0.738 – 0.938	80.0	70.0	72.7	77.8

Notes:
AUC: Area Under a Curve p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05

Table 10: Diagnostic performance for Amphiregulin + AFP to discriminate HCC (n = 30) from cirrhosis (n = 30)

	AUC	p	95% C.I	Sensitivity	Specificity	PPV	NPV
Amphiregulin + AFP	0.892	<0.001*	0.806 – 0.978	80.0	83.33	82.8	80.6

Notes:
AUC: Area Under a Curve p value: Probability value
CI: Confidence Intervals
NPV: Negative predictive value PPV: Positive predictive value
*: Statistically significant at p ≤ 0.05



DISSCUSION

Liver cancer is the fifth most common cancer globally and the second most frequent cause of cancer-related death worldwide.[14] This dismal outcome may be a result of various reasons including the absence of early detection techniques, ineffective therapies, and metastasis recurrence. [15,16] Late detection results in inability to follow up effective treatment, such as liver resection, transplantation, or local ablation, for a big number of patients. [17]

The currently Liver ultrasound with/out Alfa-fetpprotein (AFP) is the HCC examination tool used nowadays. It largely depends on the experience of the operator. and machine quality especially in obese patients with nonalcoholic steatohepatitis. Moreover, early detection of small tumors through US may be hindered by cirrhotic background. [18]

AFP is the most frequently used biomarker for HCC.[19] Serum AFP is elevated in 60–80% of HCC patients and is helpful in screening and monitoring treatment responses.[20] European and Asian Pacific guidelines have recommended the use of an AFP

level of 200 ng/ml as a reliable cut-off value for HCC diagnosis.[5,6]

Although AFP can help to define the population at risk of HCC [7,8], it showed a suboptimal performance as a serological test for surveillance.
One major limitation of AFP is its low specificity. AFP levels may be elevated in benign chronic liver diseases which may be due to exacerbation of HCV or HBV infection.[21,22] Furthermore, serum AFP shows suboptimal performance in distinguishing HCC from intrahepatic cholangiocarcinoma[23], which would critically impact the misdiagnosed patients as resection is the optimum treatment option for HCC not intrahepatic cholangiocarcinoma[24], another limitation is low sensitivity as HCC patients do not usually show AFP overexpression. About 80% of small HCCs may not exhibit elevated AFP levels.[25] A molecular subclass of aggressive HCCs (S2 class, EpCAM- positive) shows elevated AFP level in only about 10-20% of patients at early stage.[26,27] That was in accordance with the present results which showed that AFP, using a cut-off value of 200 ng/ml, has a low sensitivity (56.4%) in diagnosing HCC patients from cirrhotic patients and healthy control. In addition, 24 cases (43.6%) among 55 HCC cases of the present study were AFP-negative.

According to the above, there is an imperative need to search for more accurate and trustworthy biomarkers that can be used alone or complementary to US for early detection of HCC which would greatly affect the patient's survival.

The present study aims to assess the detection role of Alpha-fetoprotein (AFP) and amphiregulin (AREG) as HCC serum biomarkers. It also attempted to uncover their relation to HCC patients clinicopathological parameters. Therefore, serum concentrations of the two parameters were measured in 55 HCC patients along with 15 healthy controls as well as 20 cirrhotic patients to nullify the impact of cirrhosis on the studied markers.

In the present study, AFP showed a significantly elevated level in HCC group when compared to cirrhotic patients and control subjects. In addition, AFP level in cirrhotic group was significantly higher than control group. These results are in accordance with others.28.29

In the present study, some cases of cirrhotic patients showed increased serum AFP above the normal limit without liver cancer. This may be due to increased hepatocyte regeneration after HCV-induced cellular death.30 Fluctuating AFP levels in cirrhotic patients may reflect HCV exacerbation and flaring of the underlying liver disease.31

In addition, high serum AFP level in HCC patients can be explained by the multiple roles of AFP in HCC progression associated with cellular proliferation, angiogenesis as well as apoptosis.32,33 It has been also reported that AFP can block the apoptotic pathway in HCC by binding to caspase-3.34 In addition, AFP has an immunosuppressive effect via inhibiting the proliferative ability of T-lymphocytes and natural killer cells.35

The present study unveiled an important correlation in HCC patients between serum AREG level and portal vein thrombosis besides metastasis. As a downstream target of yes associated protein (YAP), AREG was YAP responses main mediator. Such responses include cell migration and proliferation. 36 Increased AREG expression as a result of mitochondrial dysfunction and over-production of reactive oxygen species induces HepG2 cell migration and chemoresistance.37,38 Castillo et al. realized that AREG encourages a feature of fierce and metastatic phenotype of cancer cells, i.e. the growth of HCC cells away from anchorage. 39

The findings of the study demonstrated that the AUC of serum AREG was 0.701. Separating cirrhosis from control, AREG had 66.67% sensitivity and 73.33% specificity at a cut- off point of ≤23.97 pg/ml. In addition, the results revealed that the AUC of serum AREG was 0.710. At a cut- off point of ≤14.71 pg/ml, the sensitivity of AREG was 70.0% and its specificity was 67.67% at the time of discriminating HCC from control.

Moreover, the results indicated that the AUC of serum AREG was 0.663. Discriminating HCC from cirrhosis at a cut- off point of ≤14.71 pg/ml, both the sensitivity and specificity of AREG were 70.0%. Combining AREG with AFP, the AUC increased to 0.993, which was accompanied by an increase in sensitivity to 80.0% and specificity to 96.67% upon differentiating cirrhosis from control.

In the same vein, the AUC increased to 0.993 as a result of combining AREG with AFP. The sensitivity also increased to 80.0% and specificity to 96.67% just after separating cirrhosis from control. similarly, carrying out both AREG and AFP resulted in an increase of AUC to 0.838 with an increase in sensitivity to 80.0% and specificity to 70.0% during the distinction of HCC from control. Combining AREG and AFP, the AUC also increased to 0.892 with increased sensitivity to 80.0% and specificity to 83.33% when distinguishing HCC from cirrhosis.

Finally, the previous date uncovered that the diagnostic performance of amphiregulin only in HCC patients was good as follows: AREG showed a sensitivity of 70.0% and specificity 67.67%, but such performance developed when AREG was used together with AFP and changed from good to excellent as the sensitivity reached 80.0% and specificity reached 70.0%.

As well, the previous date showed that the diagnostic performance of amphiregulin only in cirrhotic patients was good as follows: the sensitivity of AREG was 66.67% and its specificity was 73.33%. When AREG was used together with AFP,

the performance changed to excellent as the sensitivity was 80.0% and specificity was 96.67%.

The results of this study revealed that:

Statistically, there are significantly higher age, Amphiregulin, AST, and INR in HCC > cirrhosis > control. Besides, AFP was significantly higher in HCC and cirrhosis vs. control. It was also higher in HCC vs. control. However, the difference was not statistically significant.

Furthermore, the serum albumin in HCC > cirrhosis > control was statistically sharply lower. WBCs and platelet counts were also statistically fundamentally lower in HCC vs. cirrhosis. To the contrary, there were significantly higher ALT, and total bilirubin in HCC and cirrhosis vs. control. As for the hemoglobin level in HCC and cirrhosis vs. control, it was significantly lower.

The result exhibit that the diagnostic performance of AREG is good. However, it improves and becomes perfect when it is used together with AFP.

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