Alfred Augustus Glendening Jr. 1861-1907. *Cattle Watering, North Wales.* Oil on canvas, 16″ × 26″. Courtesy of the Callan Fine Art gallery, New Orleans, Louisiana.

Combining other serum tumor markers with AFP may improve the detection sensitivity for HCC.

Enhanced Detection of Hepatocellular Carcinoma

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Background: *Tumor markers in the early detection of tumors are promising tools that could improve the control and treatment of tumors. While alpha-fetoprotein (AFP) is a commonly used tumor marker in the detection of hepatocellular carcinoma (HCC), its sensitivity and specificity are insufficient to detect HCC in all patient samples. .*

Methods: *We compared AFP with serum levels of vascular endothelial growth factors (VEGF and VEGF-A), insulin-like growth factor-2 (IGF-II), and the activity of the lysosomal enzyme alpha-L-fucosidase (AFU) in the sensitivity of detection of HCC and cirrhosis in Egyptian patients.*

Results: *The sensitivity of tumor detection using AFP was 68.2%. This level of detection was increased to 88.6% when AFP was evaluated in conjunction with AFU. The combined use of AFP and VEGF increased the sensitivity of detection to 95.5% in patients with HCC. The combination of the three markers yielded 100% detection sensitivity. VEGF-A showed a low specificity (20%), and IGF-II showed extremely low sensitivity (4.5%).*

Conclusions: *We suggest that AFU or VEGF or both be measured with AFP to improve the detection sensitivity of HCC.*

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Abbreviations used in this paper: HCC = hepatocellular carcinoma, AFP = α-fetoprotein, AFU = alpha-L-fucosidase, ROC curve = receiver operating characteristic curve, VEGF = vascular endothelial growth factor.

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy worldwide and is the main cause of mortality in patients with chronic liver diseases.[1,2] For example, liver cirrhosis is a precancer condition that in many cases can develop into HCC. Therefore, cirrhotic patients are usually screened for HCC during their follow-up procedure.[3,4]

Tumor markers are potential screening tools that are widely used for early diagnosis of tumors.[2,5] Many research groups are evaluating the sensitivity of available tumor markers and also are investigating the development of novel markers.[6-9] The primary marker for HCC is α-fetoprotein (AFP), a single polypeptide chain glycoprotein. Generally, AFP shows acceptable sensitivity; however, AFP

is not secreted in all cases of HCC and may be normal in as many as 40% of patients with early HCC.[9,10]

We studied methods to improve the detection of HCC by measuring AFP in addition to other suggested biochemical factors for the same sample. Among these factors is α-L-fucosidase (AFU), a lysosomal enzyme present in all mammalian cells. AFU has been proposed as a tumor marker since many studies reported increased AFU serum levels in patients with cirrhosis and HCC.[3,11,12] At the same time, it is not correlated to AFP level in serum.[13]

We also investigated vascular endothelial growth factor (VEGF), the angiogenic glycoprotein, which was previously reported to express high serum levels in patients with HCC.[14,15] Since HCC is characterized by hypervascularity,[14,15] it is likely to produce angiogenic factors such as VEGF, causing proliferation of the hepatic sinusoidal endothelial cells.[16] We also studied VEGF-A, a subtype of VEGF. High VEGF-A levels have been reported in patients with hepatic ascites.[17]

We also examined the levels of the polypeptide hormone insulin-like growth factor II (IGF-II) in patients with cirrhosis and HCC. The level of IGF-II in serum showed a degree of sensitivity toward HCC. IGF-II levels have been reported as markedly lower than normal in patients with primary HCC.[18] Other studies reported an increase of IGF-II levels in early HCC in experimental animal models.[4,19] IGF-II is mainly produced by liver cells[19] and is probably affected by liver disorders.

In all of these markers, we determined serum levels for 13 normal individuals, for 20 patients with cirrhosis, and for 44 patients with HCC. An extensive statistical analysis of given data was used to calculate the sensitivity, specificity, and diagnostic accuracy for each marker. A combined evaluation was also carried out for AFP with AFU and VEGF. The receiver operating characteristic (ROC) curve was used for further evaluation and comparison of data.[20,21]

Materials and Methods

Blood samples were collected from patients at the National Cancer Institute (NCI) of Cairo during a 1-year period. Patients were diagnosed according to radiological imaging, laboratory tests, and clinical investigation following the institutional protocol. Individual patient profiles were collected from medical records to determine the clinical stages according to the TNM classification system of the International Union Against Cancer (UICC). Table 1 presents the characteristics of the investigated groups — normal individuals, cirrhotic patients, and HCC patients.

Samples were collected using venipuncture technique in glass test tubes and were left to clot for a period of 30 minutes at 37°C. Samples were then centrifuged at 3,000 RPM for 10 minutes. The supernatant sera were collected and stored at -80°C.[20,22] All samples were studied following the completion of the collection period; however, AFU activity was assayed within 30 days after collection.[12] This step was taken to avoid any possible alterations in AFU activity after this period.[13]

Table 1. — Profiles of Investigated Groups

Characteristic	Control Subjects (n = 13)	Cirrhotic Patients (n = 20)	HCC Patients (n = 44)
Mean age (yrs)	46 ± 3.9	50 ± 2.3	55 ± 1.5
Sex:			
M	8	12	34
F	5	8	10
Child-Pugh class:			
A		11	19
B	—	6	19
C		3	6
UICC stage:			
I			8
II	—	—	28
III			8
Tumor size:			
>5 cm	—	—	16
<5 cm			28

UICC = Union Internationale Contre le Cancer (International Union Against Cancer)

A commercially available microparticle enzyme immunoassay was used to determine the serum level of AFP expressed in ng/mL. Serum AFU activity was assayed as described by Giardina et al[12] in sera stored at -20°C within 30 days after collection. Enzyme activity is expressed as nanomoles of p-nitrophenyl-α-L-fucopyranoside cleaved at ng/mL at 37°C. Serum levels of total VEGF and its subtype VEGF-A, expressed in pg/mL, were determined using enzyme-linked immunosorbent assay kits. Serum levels of IGF-II, also expressed in ng/mL, were determined using immunoradiometric assay kits.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS Version 7.5). Data were expressed as either mean value ± standard error or median + range. The relationship between continuous variables was analyzed by Pearson's correlation coefficient. Median values of continuous variables were compared using the nonparametric Kruskal-Wallis test, followed by Duncan's multiple range test. The significance level was set at $P<.05$. Sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy were calculated according to the following formula (in which a = true-positive cases, b = false-positive cases, c = false-negative cases, and d = true-negative cases)[23]:

Sensitivity = $a/(a + c)$, specificity = $d/(b + d)$
Diagnostic accuracy = $(a + d)/(a + b + c + d)$
Positive predictive value = $a/(a + b)$
Negative predictive value = $d/(c + d)$

The ROC curves were constructed by calculating the sensitivities and specificities at several cutoff points. The

cutoff point was calculated as mean + two standard deviations[12] (mean – two standard deviations in case of IGF-II) of the normal group.

Results

Median Values of Markers in Patients and Control Subjects

Table 2 presents the median values of AFP, AFU, VEGF, VEGF-A, and IGF-II. A median value was used to express the levels of these investigated markers due to their wide range of individual values. The median level of AFP in HCC patients was significantly higher than in control subjects and cirrhotic patients. This result was expected because AFP is considered the main marker for HCC.[1-5,7-10,12,13,19-21] AFU also showed higher activity in HCC patients compared with the other two groups. Serum levels of VEGF and its subtype VEGF-A showed significant differences among the three investigated groups. The median level of IGF-II was significantly reduced in cirrhotic and HCC patients compared to control subjects. This is a characteristic feature of IGF-II.

Table 3 presents the median values of the investigated markers for critical controls (cirrhotic patients) with high AFP levels and HCC patients with low AFP levels. In cirrhotic patients with high AFP levels (ie, false positive for HCC), a correct diagnosis was reached using AFU, VEFG, or IGF-II. The level of these markers confirms the absence of HCC according to the cutoff values presented in Table 4. Similarly, Table 3 shows that the levels of AFU, VEGF, and VEGF-A are above their cutoff values for HCC patients with low levels of AFP. This also indicates the presence of HCC despite the low AFP levels.

Table 3. — Median Values of Tumor Markers for Critical Controls Classified by AFP Level

Marker	Cirrhosis With High AFP Above Cutoff of 19.2 ng/mL (n = 5)	HCC With Low AFP Below Cutoff of 19.2 ng/mL (n = 14)
AFU nmol/mL/hr (range)	**108.3** (116.7–550.0)	**315.8** (135.0–833.3)
VEGF pg/mL (range)	**147.1** (80.2–503.0)	**1517.7** (168.9–6178.2)
VEGF-A pg/mL (range)	64.7 (51.9–104.7)	**85.2** (22.6–229.6)
IGF-II ng/mL (range)	**252.6** (189.0–355.6)	392.6 (189.5–685.7)

The bold numerals indicate successful decision.

We investigated the correlation of the five biochemical markers to tumor size, TNM grade, Child-Pugh class, and liver function test. The only correlated parameters were found in HCC for AFU vs bilirubin (r = –0.32, P<.05) and IGF-II vs albumin (r = 0.37, P<.05) and bilirubin (r = –0.31, P<.05). It should be noted that we found no correlation between the markers in each of the investigated groups. Thus, each marker provides independently different information and therefore is expected to increase the diagnostic accuracy if multiple markers are used for detection.

Individual Values of Markers

The individual values of AFP, AFU, VEGF, VEGF-A, and IGF-II are presented in Fig 1. A logarithmic scale was used for the y-axis in AFP due to its wide range of values. The horizontal dotted line in each graph represents the cutoff value calculated as noted earlier. Overlapping may hide few individual dots in almost all graphs.

Table 2. — Median Values of Tumor Markers in Investigated Groups

Marker	Control Subjects (n = 13)	Cirrhotic Patients (n = 20)	HCC Patients (n = 44)	*P* Value
AFP ng/mL (range)	7.5 [a] (1.5–13.5)	12.0 [a] (1.3–460.0)	116.0 [b] (1.5–7801.0)	.001
AFU nmol/mL/hr (range)	125.0 [a] (23.3–186.7)	172.5 [a] (41.7–550.0)	420.8 [b] (135.0–1150.0)	.001
VEGF pg/mL (range)	250.0 [a] (125.0–320.0)	350.2 [b] (80.2–875.1)	1366.9 [c] (168.9–7267.9)	.001
VEGF-A pg/mL (range)	36.0 [a] (23.9–56.1)	76.1 [b] (51.9–104.7)	84.7 [c] (22.6–271.0)	.001
IGF-II ng/mL (range)	806.3 [a] (209.3–1347.8)	246.0 [b] (189.0–727.7)	373.7 [b] (189.5–685.7)	.001

The letters a, b, and c indicate statistically different medians according to Duncan's multiple range test.

ROC Curves for Markers

Fig 2 shows the ROC curves demonstrating the validity of each marker in the differentiation between each two of the three groups (control, cirrhosis, and HCC). For a certain marker, a value less than 0.7 for the area under the curve means that it is not possible to differentiate between the two compared groups using this marker.[24] The evaluation of a marker using the ROC curve has the advantage of analyzing two investigated groups over the whole range of sensitivities and specificities. The judgment on the validity of differentiation between the investigated groups using the area under ROC curves is in most cases in agreement with the statistical decision based on median and range (Table 2).

Combination of Markers Increases the Diagnostic Accuracy

Table 4 presents the calculated sensitivities, specificities, diagnostic accuracy, and positive and negative

Table 4. — Sensitivity, Specificity, Diagnostic Accuracy, and Positive or Negative Predictive Values for the Investigated Markers at Optimal Cutoff Values

Marker(s)	Cutoff	Sensitivity (%)	Specificity (%)	Diagnostic Accuracy (%)	Positive Predictive Values (%)	Negative Predictive Values (%)
AFP	19.8	68.2	75.0	70.3	85.7	48.3
AFU	213.0	81.8	55.0	73.4	80.0	42.1
VEGF	355.2	86.4	60.0	78.1	82.6	33.3
VEGF-A	53.0	95.5	20.0	71.9	72.4	66.7
IGF-II	198.4	4.5	90.0	31.3	4.5	30.0
AFP and AFU	*	88.6	85.0	93.8	93.5	5.6
AFP and VEGF	*	95.5	85.0	93.8	93.5	5.6
AFP, AFU, and VEGF	*	100.0	95.0	96.9	97.7	5.0

* See individual cutoff.

predictive values for the investigated markers at the optimal cutoff. The values were based on the differentiation between cirrhosis (as a high-risk group) and HCC. The values for the combined detection using more than one marker are also presented. The combined detection using two markers, for example, assumes that the tumor is detected if any of the two markers (or both of them) yields a positive result.

The detection using a combination of AFP (the reference marker) and AFU produced better sensitivity (88.6%) and specificity (85%) compared to their individual sensitivities (68.2% and 81.8%, respectively) and specificities (75% and 55%, respectively). The combined detection using AFP and AFU also enhanced the diagnostic accuracy, positive predictive values, and reduction in the negative predictive values compared with the individual detection. The combinations of AFP and VEGF also produced enhancement of sensitivity (95.5%) and specificity (85%) compared to their individual sensitivities (68.2% and 86.4, respectively) and specificities (75% and 60%, respectively). The same combination improved the diagnostic accuracy and positive predictive values, and reduced the negative predictive values compared to the individual values. If the detection involved the three markers (AFP, AFU, and VEGF), a maximum sensitivity of 100% was achieved. Moreover, a considerable enhancement in specificity, diagnostic accuracy, and positive predictive values and a slight reduction in the negative predictive values were observed. As a result of the very low specificity of VEGF-A (20.0%) and the extremely low sensitivity of IGF-II (4.5%), their combined detection with AFP was not considered.

Table 5 shows the variation of detection sensitivity according to tumor stage. In general, the detection sensitivity of stage III was in most cases greater than stage II, and that of stage II was in most cases greater than stage I, as expected. For early detection purposes, most markers

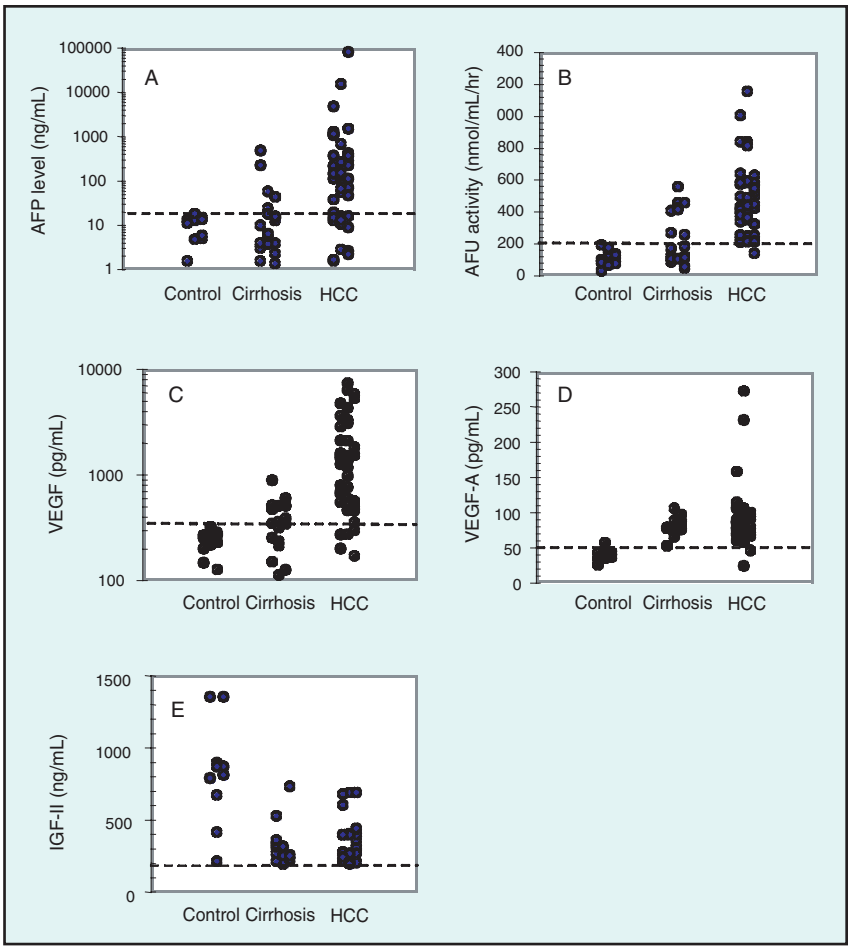

Fig 1. — A scatter diagram showing the individual values of investigated markers in patients and control subjects. The horizontal lines represent the optimal cutoff values.

showed high detection sensitivity for stage I (except AFP and IGF-II), reaching a maximum of 87.5% for either AFU or VEGF-A. A maximum of 100% detection for any of the three stages was reached using the combination of three markers (AFP, AFU, and VEGF). The combination of two markers (AFP and AFU or AFP and VEGF) increased the sensitivity of detection of any stage, reaching a maximum of 100% for stage III.

Discussion

The sensitivity of the marker AFP, the "gold standard" for the detection of HCC as reported by many groups, varies around a value of approximately 65%.[9,12,25,26] This means that 35% of examined HCC patients may be considered false negatives. Thus there is a need for the enhancement of the detection of HCC using AFP. Our study investigated the concept of combined detection using several markers in order to support the detection of HCC using AFP. Some groups also investigating this concept reported promising results.[12,13]

The samples collected for our study contained a considerable number of early UICC stages and Child-Pugh class. These are most important for the validity of our results in the purpose of early detection. The other stages and grades are also represented with adequate number of samples (Table 1). Stage IV was not included because it has no significance in the diagnosis of HCC.

Table 5. — Sensitivity of Investigated Markers in the Detection of HCC According to UICC Stage

Marker(s)	Stage I (n = 8)	Stage II (n = 28)	Stage III (n = 8)
AFP	62.5%	67.8%	75.0%
AFU	87.5%	75.0%	100.0%
VEGF	75.0%	85.7%	100.0%
VEGF-A	87.5%	100.0%	87.5%
IGF-II	0.0%	3.6%	12.5%
AFP + AFU	87.5%	85.7%	100.0%
AFP + VEGF	87.5%	96.4%	100.0%
AFP + AFU + VEGF	100.0%	100.0%	100.0%

AFP >19.8 ng/mL
AFU activity >213.0 nmol/mL/hr
VEGF >355.2 pg/mL
VEGF-A >53.0 pg/mL
IGF-II <198.4 ng/mL

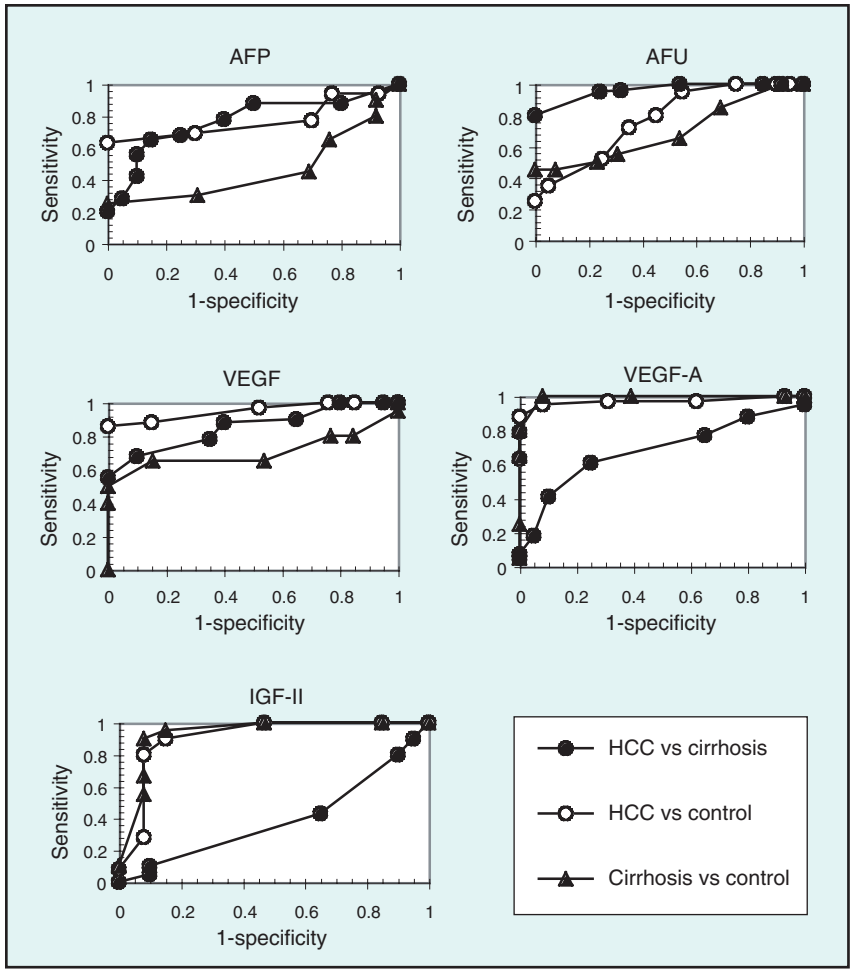

Fig 2. — Receiver operating characteristic (ROC) curves for investigated markers. Sensitivity = true-positive rate, specificity = false-positive rate.

The choice of markers in our study was AFP, which is the main tumor marker of HCC. AFU, which is involved in the catabolism of fucose-containing glycoconjugates, was also studied. AFU has shown remarkable sensitivity towards HCC and cirrhosis.[3,12,13] The choice of VEGF was based on the fact that it is an essential angiogenic factor that promotes the production of new vasculature for development of HCC.[14-16] As a result, correlating its serum level to HCC was a subject of interest.[14] VEGF-A is a subtype of VEGF. It induces the extravasation of plasma proteins such as fibrinogen, which, when deposited in the extracellular matrix, may help the formation of tumor stroma and new capillaries.[17] The present work examined the possibility of gaining useful information regarding HCC using VEGF-A. Finally, IGF-II, which is mainly produced by liver cells and transcribed in many primary HCC cell lines, has been reported to be a possible biological marker in the early detection of HCC.[18,19]

The values of cirrhotic patients as examined by all markers (except IGF-II) were always midway between the control and HCC groups (Table 2).[2]

This could be attributed to the fact that approximately half of cirrhosis cases develop into HCC.[1,2] The values obtained from all of the investigated markers were useful for the diagnosis of HCC from control subjects. AFP, AFU, VEGF, and VEGF-A were able to identify HCC patients from cirrhotic patients, while VEGF, VEGF-A, and IGF-II were able to distinguish cirrhotic patients from normal individuals. These results show that there are always useful features in some markers that are not present in others. Consequently, a combined evaluation would provide broad information in determining diagnostic procedures and treatment decisions. At the same time, using more than one marker to evaluate a specific sample achieves increased sensitivity (up to 100%), specificity (up to 95%) and diagnostic accuracy (up to 97%) (Table 4). Long-term screening of cirrhotic patients using AFU and VEGF in addition to AFP and comparison with other biochemical and diagnostic imaging data would be useful to further evaluate the veracity of the current results.

The areas under ROC curves were useful in the elucidation of the validity of a specific marker in distinguishing between each pair of samples (Fig 2). These results matched well with the results obtained from the analysis of median and range (Table 2). It may be reasonable to apply this method for future evaluation of tumor markers. The correlation of AFU and IGF-II with bilirubin and the correlation of IGF-II with albumin are also useful pieces of information.

Conclusions

Our study of 77 individuals, including control subjects and patients with cirrhosis or HCC, demonstrated that the use of combinations of multiple investigative markers, particularly AFU and VEGF combined with AFP, improves the detection of all stages of HCC. The combination of AFP, AFU, and VEGF resulted in a sensitivity of 100%, a specificity of 95%, and a diagnostic accuracy of 97%. The possibility of distinguishing HCC from cirrhosis, as a high-risk group, offers hope for the early detection of HCC.

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