Alpha-Fetoprotein, Alpha-Fetoprotein-L3, Protein Induced by Vitamin K Absence, Glypican 3 and Its Combinations for Diagnosis of Hepatocellular Carcinoma

Razvan Cerban¹, Carmen Ester¹, Speranta Iacob¹, Mihaela Ghioca¹, Liliana Paslaru², Radu Dumitru³, Mugur Grasu³, Georgiana Constantin², Irinel Popescu⁴ and Liliana Gheorghe¹

¹Centre for Digestive Diseases and Liver Transplantation, Fundeni Clinical Institute, Bucharest, Romania

²Department of Biochemistry, Fundeni Clinical Institute, Bucharest, Romania

³Departement of Interventional Radiology, Fundeni Clinical Institute, Bucharest, Romania

⁴Dan Setlacec Centre of General Surgery and Liver Transplantation, Fundeni Clinical Institute, Bucharest, Romania

Corresponding author:

Liliana Gheorghe, MD, PhD
Centre for Digestive Diseases
and Liver Transplantation,
Fundeni Clinical Institute, Bucharest
Romania
E-mail: drgheorghe@xnet.ro

ABSTRACT

Introduction: Despite its limitations, alpha-fetoprotein (AFP) is still the most common used serum marker for hepatocellular carcinoma (HCC). Alpha-fetoprotein-L3 (AFP-L3), protein induced by vitamin K absence (PIVKA-II) and Glypican-3 (GPC-3) have been proposed as complementary biomarkers but their role is still controversial.

Aims and Methods: We prospectively included 101 patients with HCC and 52 control patients with liver cirrhosis with the aim to investigate the diagnostic performance of AFP, AFP-L3, PIVKA-II, and GPC-3 as single markers or in combination for HCC diagnosis. To compare the diagnostic value in distinguishing the presence of HCC from chronic non-malignant liver disease, receiver operating characteristic (ROC) curves were constructed for each marker and for every combination of markers.

Results: When all biomarkers were individually analyzed, AFP-L3 had the highest area under the curve (AUC) (0.84), followed by AFP (0.79), PIVKA-II (0.75) and GPC-3 (0.73) for HCC diagnosis. The best sensitivity (84.7%) was for AFP L3 at a cut-off >13.5ng/mL and the best specificity (93.9%) was for AFP at a cut-off >18.9 ng/mL. For combinations of two biomarkers, the AUC was highest (0.87) for AFP and AFP-L3. The combination of all four biomarkers resulted in a much better sensitivity (88.1%) and specificity (93.9%) than each of the markers individually ($p = 0.01$).

Conclusion: AFP-L3 was the most useful single marker for HCC diagnosis, and the combination of AFP, AFP-L3 and PIVKA-II could maximize the diagnostic performance. Efforts to seek novel combination of biomarkers for HCC should be continued.

Key words: Alpha-fetoprotein, Alpha-fetoprotein-L3, protein induced by vitamin K absence, Glypican-3, Hepatocellular carcinoma

Abbreviations:

AFP - alpha-fetoprotein;
HCC - hepatocellular carcinoma;
AFP-L3 - Alpha-fetoprotein-L3;
PIVKA-II - protein induced by vitamin K absence;
GPC-3 - Glypican-3;
ROC - receiver operating characteristic;
AUC - area under the curve;
LC - liver cirrhosis;

INTRODUCTION

Primary liver cancer is one of the main cause of cancer-related death and its incidence has steadily risen over the last 20 years (1). Hepatocellular carcinoma

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(HCC) accounts for more than 90% of all liver cancers, being responsible for more 600 000 new cases every year worldwide (2).

The cumulative risk at five years for the HCC development in liver cirrhosis patients has been shown to range between 5% and 30% (3). Although there have been advances in diagnosis and treatment modalities, the survival rates of HCC patients has not improved significantly. According to reports, the survival rate at five years of patients with HCC, in the United States remains below 12% (4).

Diagnosis of HCC in an early stage, followed by surgical resection or liver transplantation offers a good chance for long-term survival of the patients. About 30% of patients with HCC are diagnosed early enough to undergo a curative treatment.

This is partially due to the limited ultrasound access for many patients, combined with a poor performance of the currently used tumor marker - α -fetoprotein (AFP), which can lead to a delay in diagnosis. Additional sensitive serum markers are definitely needed in order to improve the early diagnosis in individuals at risk for developing HCC.

Serum AFP, considered by far the most extensively-used HCC biomarker, has a relatively low diagnostic sensitivity, due to the fact that its levels are elevated not only in patients with HCC but also and in those with chronic liver disorders. In subjects diagnosed with chronic hepatitis C, the most prevalent type of viral hepatitis in Romania, AFP is often elevated in the absence of HCC, but has a normal value in as many as 50% of patients with HCC (5).

Study of the chemical structure of AFP revealed that there are different sugar components of the bonds that determine their binding capacity to Lens culinaris agglutinin (6). Lens culinaris agglutinin-reactive AFP (AFP-L3) is the major glycoform in the serum of patients with HCC and is more specific than AFP (7). The AFP-L3 isoform has also been shown to be associated with more aggressive HCCs, and to predict a worse outcome (8).

Protein induced by vitamin K absence or antagonist-II (PIVKA-II) is an abnormal prothrombin that can be detected at higher levels in the serum of patients diagnosed with HCC. Since the initial report by Liebman et al. (9), PIVKA-II has been established as a marker with high specificity for HCC and a predictor of the prognosis of patients with HCC (6,10,11). Several studies have shown that performing a combined measurement of PIVKA-II and AFP resulted in a sensitivity ranging from 50% to 90%, and a specificity ranging from more 50% to almost 99% for early HCC diagnosis, these values were

superior to those for either biomarker alone (12,13).

For early HCC, measurement of both tumor markers is recommended, since PIVKA II is a more specific marker compared with AFP (14). A high PIVKA-II level implies a poor prognosis, and a slight increase in its concentration after therapy could suggest recurrence.

Glypican-3 (GPC-3) is a heparin sulfate proteoglycan that was evaluated as a biomarker for the HCC diagnosis, based on the fact that it was detected in liver cancer cells and not in benign liver tissues (15). It was reported that serum levels of GPC-3 above 300 ng/L had a sensitivity of 47.0% and specificity of more 90% for diagnosing HCC (16). In addition, the diagnostic accuracy of GPC-3 was shown to be increased when tested in combination with AFP (17).

The objective of the current study was to determine the diagnostic performance of the four biomarkers for HCC detection by analyzing the sensitivity and specificity of each single biomarker and their combination in a cohort of HCC patients and a control group of patients with liver cirrhosis (LC).

MATERIALS AND METHODS

Patients

A total of 153 patients (101 HCC and 52 LC) were enrolled at Fundeni Clinical Institute from March 2016 to December 2018.

All patients were newly diagnosed with HCC, without any previous HCC treatment and none had extrahepatic malignancy at the time of their diagnosis. Plasma samples were obtained from all the patients included in the study and from patients that underwent resection or liver transplant also tissue samples were collected for future studies. HCC was diagnosed based on typical imaging findings as defined by the EASL guidelines (18). Barcelona Clinic Liver Cancer (BCLC) classification system was used for HCC staging (5).

The diagnosis of LC was established using one or more findings of portal hypertension: 1) liver with a cirrhotic aspect associated with splenomegaly on imaging examinations (ultrasonography, computed tomography or magnetic resonance), 2) presence of esophageal or gastric varices on endoscopy 3) thrombocytopenia (platelet < 100,000/mm), 4) presence of ascites, and 5) evidence of hepatic encephalopathy. All patients with LC included in the control group underwent imaging examination to exclude the presence of HCC.

All clinical and laboratory data were prospectively collected for all the patients and included liver trans-

aminases, bilirubin, albumin, creatinine, platelet count, INR hepatitis B and C serologies.

This study was conducted with approval from the Ethics Committee of the Fundeni Clinical Institute. An informed consent in written was obtained from all included participants.

Sample storage and assays

A peripheral blood sample was collected from each patient at the time of the initial diagnosis for the HCC group or during the clinic visit for the control group. Until measurement, serum aliquots were stored at -35°C.

Serum AFP, AFP L3, PIVKA-II and GPC-3 levels were measured in the same samples. Serum AFP and PIVKA-II were determined by use of automated chemiluminescent microparticle immunoassays (ARCHITECT AFP 3P36; ARCHITECT PIVKA-II 2P4) according to the manufacturer's instructions, utilizing the Abbott™ Architect System 1000 analyser.

AFP-L3 and GPC-3 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) in accordance with the instructions of the manufacturer (AFP L3 - Catalog No: E-EL-H0289; GPC-3 Catalog No: E-EL-H1712, ElabScience Co. Wuhan China). The measurement range was 0.63 to 40 ng/mL for AFP-L3 and 160 to 10000 pg/mL for GPC-3.

Adequate standard curves were calculated for each ELISA plate that was used. In the cases where AFP level of a sample was >2000ng/mL or the PIVKA-II level was >30,000mAU/mL, the original sample was diluted according to the instructions of the manufacturer. All tests were performed at the Biochemistry Department of Fundeni Clinical Institute by the same group of experienced laboratory technicians, who were not informed on clinical data of any patient. Refreezing of thawed plasma samples was not performed.

Statistical analysis

The data are presented as mean $\pm$ SD and median (range) or as relative frequencies (%) when appropriate. We used Mann-Whitney test and the χ^2 test to perform the comparison of continuous and dichotomous variables between the two groups. For correlations between tumor markers, Pearson's correlation coefficient was calculated.

For all of the tests of significance a p value lower than 0.05 was regarded as statistically significant.

To compare the diagnostic performance of each marker and every combination of markers, receiver

operating characteristics (ROC) curves were constructed. We determined the optimal cut-off value by calculating the Youden index for each ROC curve. Differences between the area under the ROC curve (AUC) of each biomarker for distinguishing between HCC and LC patients and their 95% confidence intervals (CI) were calculated. We considered a positive combination if any biomarker in the combination was positive.

Statistical analyses were performed using Medcalc ver. 13.3.3.0 software (Medcalc Software).

RESULTS

Both clinical and biological characteristics of the study population (n=153) are presented in *table 1*. There was a higher proportion of males (p=0.04) and the subjects tended to be older (p=0.03) in the group of patients diagnosed with HCC, compared with the LC control group. In our study all patients with HCC had cirrhosis as their underlying liver disease. There was no difference in the serum levels of alanine aminotransferase (ALT) and of total bilirubin between the two groups of patients.

The most common etiology in both study groups was HCV infection, followed by hepatitis B infection. No significant differences according to Child-Pugh class was observed between the two groups of patients.

Considering the fact that serum levels of biomarkers were sometimes extremely high, we decided to measure median and interquartile range (IQR) values to allow more accurate comparisons between values and avoid misleading mean values. Median serum values for all four biomarkers were observed to be significantly higher in subjects with HCC than in the LC group.

Each studied biomarker was observed to have a different serum level distribution and range. We performed an analysis of the correlation coefficients to identify statistically significant correlations between the markers. The correlation coefficient for AFP and PIVKA-II was 0.107 (*fig. 1*), whereas that for AFP and GPC-3 was 0.102 (*fig. 2*). We demonstrated a good correlation between AFP and AFP-L3, with a coefficient of 0.51 (*fig. 3*).

Biomarkers performance evaluation

Receiver operator characteristic curves (ROC) for HCC diagnosis using total AFP, AFP-L3, PIVKA-II and GPC-3 are shown in *fig. 4*.

For the 4 biomarkers individually, in distinguishing between HCC and chronic liver disease, AFP L3 showed the highest area under the ROC curve (AUC) (0.842,

Table 1 - Characteristics of the patients

	HCC (n=101)	LC (n=52)	p value
Age, mean years	60,3 ± 8,52	59,4 ± 8,4	p=0.03
Gender, n (male)	67 (66.3%)	27 (51.9%)	p=0.04
Etiology			
HCV	63 (62.4%)	30 (57.7%)	p=0.86
HBV	24 (23.7%)	13 (25%)	
Alcohol	12 (11.9%)	7 (13.5%)	
Others	2 (1.9%)	2 (3.8%)	
Child-Pugh class			
A	78 (77.2%)	30 (57.7%)	p=0.24
B	17 (16.8%)	12 (23.1%)	
C	6 (5.9%)	10 (19.3%)	
Platelet, x10 ³ /L	99 (74.50-139.50)	81 (67.0-133.0)	p=0.36
Albumin, g/L	3.7 (3.08-4.0)	3 (2.7-3.5)	p=0.01
Bilirubin, mg/dL	1 (1-1.75)	1.2 (1.10-1.85)	p=0.23
ALT, U/L	54 (37-109)	49 (32-115)	p=0.65
INR	1.25 (1.00-1.70)	1.20 (1.05-1.45)	p=0.93
MELD score	10 (8-12)	11 (8.25-15.00)	p=0.18
AFP, ng/mL	18 (6.63-104.56)	4.75 (3.21-6.70)	p<0.0001
AFP L3, ng/mL	23.7 (16.53 - 40.00)	12 (6.97 - 14.35)	p<0.0001
PIVKA II, mUA/mL	188.4 (81.36-723.21)	63 (34.73-160.03)	p<0.0001
GPC-3, pg/ml	601 (475.75 - 1989.00)	415 (250.00 - 527.75)	p<0.0001

Data are expressed as mean ± SD, or median (interquartile range) for quantitative variables and as number or cases (percentage) for qualitative variables.

LC=liver cirrhosis; HCC=hepatocellular carcinoma; HBV=hepatitis B virus; HCV=hepatitis C virus; ALT=alanine aminotransferase; INR=international normalized ratio; MELD=model for end-stage liver disease; AFP=alpha-fetoprotein; PIVKA-II=protein induced by vitamin K absence; GPC-3=glypican-3

Figure 1 - Linear correlation between AFP and PIVKA II **Figure 2 - Linear correlation between AFP and GPC-3** **Figure 3 - Linear correlation between AFP and AFP L3** **Figure 4 - ROC curves for the diagnosis of HCC using AFP, AFP-L3, PIVKA II and GPC-3**

95% confidence interval [CI] 0.763-0.911), followed by AFP (0.798, 95% confidence interval [CI] 0.738-0.881). As a single marker GPC-3 had the lowest AUC (0.730, 95% confidence interval [CI] 0.670 to 0.831) (*table 2*). There was no significant differences of the AUC among the individual biomarkers.

The optimal cut-offs that resulted from the Youden index for AFP, AFP-L3, PIVKA-II and GPC-3 were 18.9 ng/mL, 13.5 ng/mL, 63 mAU/mL, and 466 pg/mL respectively. Of the 101 serum samples from patients diagnosed with HCC, 50 samples (49.5%) were positive for AFP, 78 samples (77.2%) were positive for AFP-L3, 48 samples (47.5%) were positive for PIVKA-II and 46 samples (45.5%) were positive for GPC-3. As diagnostic markers for HCC, AFP > 18.9 ng/mL was the most specific (92%) but with a lower sensitivity than AFP L3 (52.5% vs. 87.7%).

When we studied the diagnostic utility of the combination of two biomarkers, AFP > 18.9 ng/mL combined with AFP L3 > 13.5 ng/mL showed the highest area under the ROC curve (AUC) (0.872, 95% confidence interval [CI] 0.799 to 0.930), an increased sensitivity (74.5%), and a similar specificity as compared to AFP alone. The combination with the greatest specificity using two biomarkers was AFP-L3 >13.5

ng/mL combined with PIVKA-II > 63 mAU/mL (specificity of 96.9%, sensitivity of 71.2%) (*table 2*).

As to triple biomarker combinations, the AFP > 18.9 ng/mL and AFP L3 > 13.5 ng/mL and PIVKA-II > 466 mAU/mL combination showed the best sensitivity (84.7%) with a specificity of 90.9 lower than that of the combination of AFP L3 and PIVKA-II. The combination of all four biomarkers (AFP, AFP L3, PIVKA-II and GPC-3), improved the sensitivity (83.1%) compared and the best two-marker combination but with a lower specificity (93.9%).

DISCUSSION

Early HCC diagnosis is essential to ensure that treatments with a curative intent can be carried out to improve the long-term prognosis and survival of patients (19).

The latest Western guidelines no longer recommends AFP measurement for evaluation of patients for the presence of HCC, because of its limited value in HCC detection, due to the sensitivity of about 60% at a cut-off value of 20 ng/mL (18,20,21,22) and low specificity (23). To overcome this, investigators have examined other biological molecules in an attempt to

Table 2 - Diagnostic value of AFP, AFP-L3, PIVKA-II, GPC-3 and their combinations in discriminating HCC from LC

Number of markers	Combination	Total subjects (n = 153)				
		Sn	Sp	PPV	NPV	AUC
Single	AFP	52.54	93.94	93.9	52.5	0.798
	PIVKA-II	81.36	60.61	78.7	64.5	0.752
	AFP L3	84.75	75.76	86.2	73.5	0.842
	GPC	76.27	66.67	80.4	61.1	0.730
Double	AFP + PIVKA	74.58	87.5	91.7	65.1	0.852
	AFP + AFP L3	74.58	92.94	95.7	67.4	0.872
	AFP + GPC3	74.58	90.91	93.6	66.7	0.85
	PIVKA + AFP L3	71.19	96.97	97.7	65.3	0.859
	PIVKA+ GPC3	74.58	91.93	95.7	67.4	0.866
	AFP L3 + GPC 3	79.66	87.88	92.2	70.7	0.862
Triple	AFP + PIVKA + AFP L3	84.75	90.91	94.3	76.9	0.916
	AFP + PIVKA + GPC3	86.44	87.88	92.7	78.4	0.905
	AFP + AFP L3+ GPC3	76.27	96.97	97.8	69.6	0.896
	PIVKA + AFP L3 + GPC3	77.97	96.97	97.9	71.1	0.90
Quadruple	AFP+PIVKA+AFP L3+GPC 3	88.14	93.94	96.3	81.6	0.917

Cut-off values for each biomarker were: AFP >18.9 ng/mL, AFP L3 >13.5 ng/ml, PIVKA-II > 63 mAU/ml, GPC > 466 pg/mL

LC = liver cirrhosis; HCC = hepatocellular carcinoma, AFP = alpha-fetoprotein; PIVKA-II = protein induced by vitamin K absence; GPC-3 = glypican-3, Sn = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; AUC = area under the curve

find better diagnostic markers for HCC.

It was demonstrated in previous studies that PIVKA-II could be useful for the early diagnosis of small HCC tumors (24). In addition, several papers most of them from Asia have shown that combining AFP-L3 with PIVKA-II could improve the rate of HCC detection (8,25, 26). Lim et al showed that combining AFP, AFP-L3, and PIVKA-II can improve the diagnostic performance for HCC among subjects with LC in comparison with each biomarker used individually (27).

The aim of the current study is to evaluate if AFP L3, PIVKA-II and GPC-3 could be useful as diagnostic biomarkers for HCC in comparison to AFP in for the first time in Romanian patients. In order to achieve this, we performed a case-control study with the aim to identify new valuable diagnostic biomarkers that can be used in daily practice.

Chronic hepatitis C is considered to be the major cause of cirrhosis and HCC in Western countries (28). In our study, chronic HCV hepatitis was the most frequent cause in both HCC and LC control group. The results from our study are therefore likely to be representative of the total Romanian population.

Considering the fact that HCC development is a major cause of death in patients with LC (29) and most HCC patients have underlying LC, we decided to include in our study only LC patients as a control group.

We have evaluated the utility of AFP, AFP-L3, PIVKA-II and GPC-3 for HCC diagnosis, by performing a direct comparison individually and in combination, and found that AFP-L3 was the best individual marker for differentiating between HCC and LC (sensitivity 84.7%, specificity 75.7%, AUC 0.842, 95% CI 0.763 - 0.918). There was no statistical significant difference between AFP-L3 and the other biomarkers.

PIVKA-II is a precursor of prothrombin and its synthesis is induced by vitamin K deficiency. Tumor liver cells have lower levels of vitamin K than normal cells and this fact explains for the extremely low levels of PIVKA-II in healthy individuals. Additionally PIVKA-II is elevated not only in HCC subjects, but also in chronic hepatitis or liver cirrhosis patients, which limits its diagnostic accuracy (30). In our study we found that as a single marker PIVKA II had a sensitivity of 81.3% compared to 52.5% for AFP for diagnosis of HCC. Several studies have demonstrated that PIVKA-II is detected in AFP-seronegative HCC patients and offers better sensitivity than AFP for HCC diagnosis, which is consistent with our findings (31,32).

Since it was initially reported to be positive in up to 50% of subjects with HCC, but undetectable in controls without liver disease, GPC-3 has been investi-

gated as a diagnostic biomarker for HCC (33). Although initially it was considered that GPC-3 could replace or complement AFP, later studies have yielded variable results (34). In our study the sensitivity, specificity, and AUC of GPC-3 were comparable to previous results and the cut-off value (466pg/mL) determined by ROC analysis was different than other reported values, which range widely from 3.9 pg/mL to 300 ng/ mL (35). In our analysis GPC-3 had the lowest AUC of all studied biomarkers (0.730, 95% CI) and as a single marker, failed to be useful for HCC diagnosis compared to AFP.

Among all combinations of two biomarkers, AFP >18.9 ng/mL and AFP-L3 >13.5 ng/mL had the highest AUC (0.872, 95% CI), with a sensitivity of 74.58% and a specificity of 92.94%.

Triple panels did improve the diagnostic performance compared to the best results achieved from using two biomarkers. Performing the combined measurement of AFP, AFP L3 and PIVKA-II resulted in the best AUC, (sensitivity 84.75%, specificity 90.91%, AUC 0.916, 95% CI 0.852 - 0.940). Adding GPC-3 to the combination only marginally increased the sensitivity (88.14%), specificity (93.94%) and the AUC (0.917, 95% CI) of the panel but with a cost increase.

A diagnostic biomarker for HCC should be highly sensitive, should differentiate HCC from other liver diseases, and should be detected in the plasma at high levels even in early stages. In our study, serum AFP, AFP L3, PIVKA-II and GPC-3 failed to achieve these standards as single markers, but their combination greatly improved HCC detection rate.

One limitation of the present study is that all the samples were collected at single center, and so the results that we obtained need external validation. However, repeated experiments for internal validation were strictly performed and in all of these cases, we used new aliquots of stored samples.

Another limitation of this study is that we used only one commercially available ELISA assay for each of the studied biomarkers. The enrolled patients with HCC were heterogeneous in regard to liver disease etiology, tumor stage and underlying liver function, which reflects real-world practice, but this fact may lead to variability. Considering the fact that clinical utility of a biomarker panel should be considered after taking into account cost-effectiveness, further analyses are necessary to determine the proper number and combination of biomarkers.

Future studies should be performed in order to clarify several issues. By using larger sample sizes, more accurate estimates for the sensitivity and specificity of the panel will be established. Since levels of the

markers are much higher in HCC patients than in non-HCC patients and healthy individuals, different cut-off levels can be tried to further improve the specificity.

CONCLUSION

In conclusion, AFP and AFP-L3 seems to be the most valuable two marker combination for the diagnosis of HCC, as shown by comparative analyses of AFP, AFP-L3, PIVKA-II, and GPC-3, especially in a HCV-predominant HCC population. Persistent inconsistency in plasma GPC-3 levels questions its utility as a diagnostic biomarker for HCC compared to AFP. PIVKA-II was demonstrated to be a useful complementary marker if it is associated with AFP and AFP L3. Prospective studies to find the most efficient and cost-effective biomarker panel are needed.

Conflict of interest

All author declare that they have no conflict of interest.

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