Evaluation of Alpha-fetoprotein and Prothrombin Induced by Vitamin K Absence for Hepatocellular Carcinoma Diagnosis in Patients Admitted to a Large Transplant Center

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ABSTRACT

Background & aims: Prothrombin induced by vitamin K absence-II (PIVKA II) is a diagnostic marker and a major prognostic factor for hepatocellular carcinoma (HCC) with limited experience in European patients. The aim of this study was to investigate the clinical utility of simultaneous measurement of alphafetoprotein (AFP) and PIVKA II for hepatocellular carcinoma (HCC) diagnosis.

Methods: In a Romanian cohort, we performed a case-control study to compare the performances of AFP and PIVKA-II serum levels for HCC diagnosis. To determine the diagnostic value of both biomarkers in distinguishing HCC from liver cirrhosis, receiver operating characteristic (ROC) curves were constructed for each biomarker and their combination.

Results: From January 2016 to December 2017, 54 cirrhotic patients and 59 HCC cases were prospectively included. ROC curve analysis showed that 63 mAU/mL and 6 ng/mL were the optimal cut-off values for PIVKA-II and AFP respectively for diagnosis of HCC in our cohort. For HCC diagnosis in patients with AFP levels <20 ng/mL, the combination of AFP and PIVKA II had a specificity of 93.8% vs. 68.8 for AFP (AUC 0.846 vs. 0.732 respectively) ($p=0.03$). For the diagnosis of early HCC, the combination of AFP and PIVKA-II had a sensitivity of 72.7% and a specificity of 90.9% vs. 69% and 75% for AFP (AUC 0.867 vs. 0.825, respectively).

Conclusion: The diagnostic performance of a combination of AFP and PIVKA II is better to that of either marker alone, in the diagnosis of HCC especially in patients with AFP levels <20 ng/mL and in patients with early tumors.

Key words: Alpha-fetoprotein, Prothrombin induced by vitamin K absence, Hepatocellular carcinoma

Abbreviations:

PIVKA II - Prothrombin induced by vitamin K absence-II

HCC - hepatocellular carcinoma

AFP - alphafetoprotein

HCV - hepatitis C virus

LC - liver cirrhosis

BCLC - Barcelona Clinic Liver Cancer

CI - confidence intervals

IQR - interquartile range

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related death worldwide. It is considered to be responsible for more than

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600 000 new cases and 500 000 deaths every year worldwide (1).

Treatment methods which can be curative, like surgical resection or liver transplantation are available only for early stage HCC patients, but most HCCs are diagnosed at an advanced stage. Therefore, early diagnosis which primarily depends on specific serum tumor markers and liver imaging tests is the key to improving the prognosis of HCC (2).

Since the 1970s, serum α -fetoprotein (AFP) is the most widely-used HCC biomarker currently used by many physicians, in clinical practice for HCC diagnosis (3). The diagnostic sensitivity of AFP especially for small HCCs is rather low, with levels being elevated both in HCC patients and in 20% to 40% of those with chronic hepatitis or cirrhosis and tend to fluctuate alongside with underlying inflammatory activity (4). In patients with hepatitis C virus infection (HCV), which is the main cause of liver cirrhosis and the leading indication for liver transplantation in Romania, (5) AFP is often elevated in the absence of HCC, but has a normal value in as many as 50% of patients with HCC (6).

The current Western guidelines (7,8) have excluded the measurement of AFP for HCC diagnosis, because of its limited value in detecting HCC especially during the early disease stages, with a sensitivity of around 60% at a cut-off value of 20 ng/mL (8,9,10) and low specificity (11).

PIVKA II is an abnormal form of prothrombin protein, with no coagulation function, that is present at high levels in the serum of HCC patients (12).

Since the initial report by Liebman et al (13), PIVKA II has been considered to be a highly specific biomarker for diagnosis as well as a predictor of prognosis in patients with HCC (9,14,15).

Several studies demonstrated that measurement of PIVKA II in conjunction with AFP has a sensitivity ranging from 47.5% to 94.0%, and a specificity ranging from 53.3% to 98.5% for early HCC diagnosis, and that the combination is superior to either marker used alone (16,17). Pote et al. also showed PIVKA II utility for the diagnosis of early HCC and demonstrated that it can predict microvascular invasion (18).

For these reasons Japanese guidelines on HCC management recommended the use of the combination of AFP and PIVKA II for routine follow up of patients with a high risk of HCC development (19). The cut-offs of AFP >200 ng/mL and DCP >40 mAU/mL are considered by the same Japanese guidelines to be highly suggestive for HCC diagnosis.

Despite other reports (20, 21,22) also demonstrating that a combined analysis of AFP and PIVKA II led

to better detection of HCC, there are still some controversies regarding the diagnostic accuracy of PIVKA II and AFP combination.

Many studies mostly from Asia have compared the utility of tumor markers for HCC diagnosis, but a head-to-head comparison of the diagnostic utility of AFP, PIVKA II and their combination in a Eastern European cohort has not been reported yet.

The aim of this study was to evaluate the diagnostic performance of AFP, PIVKA II and their combination among patients with HCC admitted to a transplant center.

MATERIALS AND METHODS

Patients

We conducted a case control study at Fundeni Clinical Institute from January 2016 to December 2017.

A total of 59 patients with HCC and 54 controls with liver cirrhosis (LC) were enrolled. All HCC patients were newly diagnosed. Plasma samples were obtained from all the patients included in the study regardless if they belonged to the case or the control group. HCC was diagnosed based on histology or typical image characteristics as defined by the EASL and AASLD guidelines (7,8) Barcelona Clinic Liver Cancer (BCLC) system was used for HCC staging (8).

LC was diagnosed by one or more findings of portal hypertension including: cirrhotic appearance of the liver with splenomegaly on imaging studies, thrombocytopenia (platelet < 110,000/mm³), finding esophageal varices on endoscopy, detection of ascites, or presence of hepatic encephalopathy. All LC patients underwent imaging studies to exclude HCC.

Clinical and laboratory data were prospectively collected for all subjects and included liver transaminases, bilirubin, albumin, creatinine, platelet count, INR hepatitis B and C serologies.

For this study, liver disease etiology was matched as much as possible between the HCC and the LC groups.

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

Two peripheral blood samples were collected in EDTA tubes from each patient at diagnosis for the HCC group or at the time of the visit in our clinic for the control group. Aliquot samples were stored at -80°C

until measurement.

Serum AFP and PIVKA-II levels were measured for each sample by the same experienced laboratory technician, who was blind to all clinical data. For the frozen samples that were thawed we did not perform further refreezing.

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 iSystem 1000 analyser in the Department of Biochemistry at Fundeni Clinical Institute, Bucharest. For each ELISA plate used, an adequate standard curve was calculated.

Statistical analysis

All enrolled patients were included in the final analysis. Continuous variables were presented as means + SD (standard deviation) or as median when suitable. Categorical variable were reported as percentages.

For evaluating the differences between patients in the HCC group and patients in the LC group we used a chi-square test for classified variables and Mann-Whitney test was performed for continuous non-parametric variables. Significance was considered for $p < 0.05$.

For the evaluation of the diagnostic performance, receiver operating characteristics (ROC) curves and

binary logistic regression analyses were constructed for each biomarker and for their combination. The optimal cut-off value was considered by determining the Youden index for each ROC curve using a single marker. To identify the relationships between the biomarkers a correlation analysis was performed. For distinguishing between HCC and LC patients, the differences between the area under the ROC curve (AUC) for each biomarker and their 95% confidence intervals (CI) were calculated. Evaluation of the correlation of selected variables was performed using Pearson's correlation coefficient.

Statistical analyses were performed using Medcalc ver. 13.3.3.0 software.

RESULTS

Both the clinical and biological characteristics of the study population ($n=103$) are presented in *table 1*. The HCC patient group had a higher proportion of males ($p=0.04$) and was older ($p=0.03$) than the LC control group. All HCC patients had underlying liver cirrhosis. HCV infection was the most common etiology in both groups, followed by hepatitis B infection. There were no significant differences according to Child-Pugh class between the two patient groups.

Because the serum levels of biomarkers were sometimes extremely high, we measured median and interquartile range (IQR) values to allow more accurate comparisons and avoid the extreme outliers that can

Table 1 - Clinical characteristics of the study population

	HCC (n=59)	LC (n=54)	p value
Age, median years (range)	60.3 $\pm$ 8.52	59.4 $\pm$ 8.4	$p=0.03$
Gender, n (male)	39 (66.1%)	28 (51.9%)	$p=0.04$
Etiology			
HCV	37 (62.7%)	31 (57.4%)	$p=0.86$
HBV	14 (23.7%)	13 (24.1%)	
Alcohol	7 (11.9%)	8 (14.8%)	
Others	1 (1.7%)	2 (3.7%)	
Child-Pugh class			
A	46 (77.9%)	31 (57.4%)	$p=0.24$
B	10 (16.9%)	13 (24.1%)	
C	3 (5.1%)	10 (18.5%)	
Platelet, $\times 10^3/L$	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	1.8 ($\pm$ 1.4)	4.8 ($\pm$ 3.7)	$p < 0.0001$
ALT, UI/L	54 (37-109)	49 (32-115)	$p=0.65$
INR	1.3 ($\pm$ 0.3)	1.6 ($\pm$ 0.5)	$p=0.006$
MELD score	10.4 ($\pm$ 3.2)	14.1 ($\pm$ 5.2)	$p < 0.0001$
AFP, ng/mL	18 (6.63-104.56)	4.75 (3.21-6.70)	$p < 0.0001$
PIVKA II, mUA/mL	188.4 (81.36-723.21)	63 (34.73-160.03)	$p < 0.0001$

Data are expressed as mean $\pm$ SD, or median (interquartile range) for quantitative variables and as number or cases (percentage) for qualitative variables, ALT=alanine aminotransferase, HCV=hepatitis C virus, HBV=hepatitis B virus, INR=international normalized ratio, PIVKA II=Prothrombin induced by vitamin K absence-II, AFP=alpha-fetoprotein

produce misleading mean values. Median serum AFP and PIVKA-II levels were found to be significantly higher in patients with HCC than in the LC group (AFP, 18 (IQR 6.63-104.56) vs. 4.75 (IQR (3.21-6.70) ng/mL; PIVKA- II 188.4 (IQR 81.36-723.21), vs 63 (IQR 34.73-160.03) mUA/mL. There was no correlation between the serum levels of AFP and PIVKA II ($r=0.107$) (fig. 1).

Staging of HCC was done using BCLC system. BCLC staging was 0 in 16.9%, A in 38.9%, B in 20.3%, C in 18.6% and D in 5.1% of the HCC patients. Subgroup analysis of cases showed that patients with BCLC C and D vs. 0, A, B had a significant higher serum, PIVKA II levels (779.6 vs. 160.5 mAU/mL, $p=0.01$) (fig. 2). For AFP there was no statistically significant difference between patients with BCLC grade of C and D vs. 0, A and B ($p=0.78$).

Biomarkers performance evaluation

The performance of AFP, PIVKA II and their combination for diferenciating HCC from LC was evaluated on all pacients included in study and on two subgroups of HCC cases. A comparison of AUC, sensitivity, and specificity for differentiating HCC from LC in a subgroup of HCC patients with AFP level <20 ng/mL and in another subgroup of patients with early stage HCC were performed. Early stage HCC included BCLC stage 0 and A patients.

Performance assessment on all patients included in the study showed that the combination of AFP + PIVKA II had the best AUC (0.862, 95% CI 0.774 – 0.926) for HCC diagnosis, compared with the AUC for AFP (cut-off: 6 ng/mL) alone of 0.831 (95% CI 0.734 – 0.899) and for PIVKA-II (cut-off 63 mAU/mL), 0.759 (95% CI 0.658 – 0.842) (fig. 3).

Figure 1 - Linear correlation between AFP and PIVKA II

At the cut-off value of 63 mAU/mL PIVKA II was proved to be more sensitive (81.4%) but less specific than AFP (table 2).

In our cohort, 36 (61%) HCC and 46 (85.2%) LC patients had a plasma AFP <20 ng/mL. As a single marker PIVKA II > 63 mAU/mL showed the best AUC (0.751, 95% CI 0.621 – 0.840) (fig. 4) with a sensitivity of 81.4% and a specificity of 62.5% (table 2). The combination of both biomarkers, (AFP > 6ng/mL and PIVKA-II > 63 mAU/mL) showed an AUC of 0.862 which is statistically significant better than for AFP alone ($p=0.03$).

In the present study, 33 (55.9%) HCC patients were diagnosed at an early stage (BCLC 0 or A). AFP as a single marker for the diagnosis of early HCC, had the best (AUC 0.825 95% CI 0.712 – 0.908) with a sensitivity of 69.7% and a specificity of 75.7% (AFP > 6 ng/mL). AFP was most specific as a single marker (75.7%). Although PIVKA-II had a greater sensitivity than AFP for early HCC diagnosis, the specificity was lower (60.6%) (PIVKA >63mAU/mL) than that of AFP. The combination of both biomarkers, (AFP > 6ng/mL and PIVKA-II > 63 mAU/mL) showed an AUC of 0.867 which is better than each marker alone but the difference was not statistically significant (fig. 5) (table 2).

DISCUSSION

Early HCC diagnosis is of great importance to ensure that curative interventions can be applied to improve the prognosis and survival of patients (23). In addition to ultrasound imaging and other methods, clinicians greatly depend on serum markers, such as AFP, for HCC diagnosis, especially in areas where advanced diagnostic technologies are not readily available.

Figure 2 - Box plot of PIVKA II levels according to BCLC stage

HCC marker	AUC	95% CI	P (DeLong test)		
AFP	0.831	0.734 - 0.899	P = 0.32	P = 0.04	P = 0.23
PIVKA II	0.759	0.658 - 0.842			
AFP + PIVKA II	0.862	0.774 - 0.926			

Figure 3 - ROC curve for all HCC cases

Although AFP is used by physicians for assessing HCC besides imaging techniques, it is not a completely reliable marker and it is not recommended by current guidelines due to low sensitivity and specificity. This makes AFP insufficient for early diagnosis with subsequent loss of the possibility for an effective HCC treatment.

The aim of our study was to determine whether a combination of AFP and PIVKA II could be useful for

HCC diagnosis compared to the traditional AFP for the first time in Romanian patients.

Since HCC development is one of the most frequent causes of death in LC patients (24) and many patients with HCC have underlying LC, we took the decision to include only LC patients as a control group.

In the current study HCC patients were older and more often males than patients who did not have HCC. Male predominance in HCC development may be

Table 2 - Diagnostic value of AFP, PIVKA-II, and their combination in discriminating HCC from LC in different study groups

HCC marker	Sn	Sp	PPV	NPV	AUC
HCC vs. LC - all patients					
AFP > 6ng/mL	78.0%	75.8%	85.2%	65.8%	0.831
PIVKA II > 63mAU/mL	81.4%	60.6%	78.7%	64.5%	0.759
AFP + PIVKA II	74.6%	87.5%	91.7%	65.1%	0.862
HCC vs. LC - patients with AFP < 20ng/mL					
AFP > 6ng/mL	75.1%	68.8%	73.0%	64.1%	0.732
PIVKA II > 63mAU/mL	80.6%	62.5%	70.0%	72.4%	0.751
AFP + PIVKA II	66.7%	93.8%	88.9%	72.5%	0.846
HCC vs. LC - early stage HCC patients					
AFP > 6ng/mL	69.7%	75.7%	74.2%	71.4%	0.825
PIVKA II > 63mAU/mL	76.7%	60.6%	65.8%	72.6%	0.710
AFP + PIVKA II	72.7%	90.9%	88.9%	78.9%	0.867

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

HCC marker	AUC	95% CI	P (DeLong test)		
AFP	0.732	0.618 to 0.837	P = 0.93	P = 0.06	P = 0.03
PIVKA II	0.751	0.621 to 0.840			
AFP + PIVKA II	0.846	0.726 to 0.915			

Figure 4 - ROC curve for HCC cases with low AFP

explained by hepatocyte androgenic stimulation and by greater exposure to toxins (25).

Chronic hepatitis C is thought to be the main cause of cirrhosis and HCC in Western countries (26). In our

study, chronic HCV hepatitis was the most common cause in both HCC and LC control group. The results from our study are therefore likely to be representative of the total Romanian population.

HCC marker	AUC	95% CI	P (DeLong test)		
AFP	0.825	0.712 - 0.908	P = 0.15	P = 0.004	P = 0.37
PIVKA II	0.710	0.585 - 0.815			
AFP + PIVKA II	0.867	0.755 - 0.935			

Figure 5 - ROC curve for early stage HCC

In the present study we demonstrated that increased serum concentration of both AFP and PIVKA II were significantly correlated with the diagnosis of HCC. This finding is similar to other authors who reported that PIVKA II produced by HCC cells is an invasive and proangiogenic factor that stimulates tumoral growth (14,27).

In accordance to other previous papers, in our study group there was no correlation between the serum levels of PIVKA II and AFP which let us to evaluate the diagnostic performance of their combination.

The serum concentration of PIVKA II increased at higher BCLC staging, but AFP levels had no correlation with tumor stage. This finding might be explained by the fact that, as previously reported, tumor diameter is significantly correlated with PIVKA II levels (28).

The cut-off values used for each biomarker were fixed based on the value that resulted in the largest AUC. Although in each subgroup analysis the cut-offs were slightly different, to avoid confusion from changing the cut-offs for different clinical scenarios we took the decision to use the same cut-off value for each biomarker for easier use in real-world practice.

For HCC diagnosis, the optimal cut-off value of PIVKA-II, which resulted after ROC curve analysis was 63 mAU/mL. According to previous studies, the optimal cut-off value for PIVKA-II as a diagnostic biomarker for HCC, has been estimated to range from 30 to 42 mAU/mL and its variability may be due to the preexisting liver disease (18, 22).

The cut-off value of PIVKA-II for HCC diagnosis in the present study is higher than previous papers. An explanation might be the fact that since serum PIVKA-II levels in patients with HCC will gradually increase with liver disease progression, the inclusion of more advanced patients as opposed to other studies may account for the different optimal diagnostic cut-off value in our study as compared to previous papers.

We were able to demonstrate that PIVKA-II (cut-off: 63 mAU/mL) had a higher sensitivity but a lower specificity than AFP in all the study groups. The combination of AFP and PIVKA II at the above cut-off increased overall specificity and positive predicted value at the expense of sensitivity.

In the subgroup of patients with AFP < 20 ng/mL, PIVKA II showed the best sensitivity (80.6%) and the best AUC (0.751, 95% CI), as a single marker. Accuracy of PIVKA II was slightly superior to AFP in this subgroup of patients but without statistical significance.

Previous studies have shown that PIVKA-II could be helpful for the early diagnosis of small HCC tumors (29,30).

The combination of AFP >6 ng/mL and PIVKA II >63 mAU/mL had the highest AUC in the early HCC patients subgroup (0.867, 95% CI), with a sensitivity of 72.7% and a specificity of 90.9% (table 2). For early HCC diagnosis PIVKA II had a sensibility of 76.7% vs. 69% for AFP, but the specificity was lower, 60.6% vs 75.7% for AFP. These findings are consistent with previous studies which demonstrated that for this group of patients PIVKA II had a better sensibility but worse specificity than AFP (9).

Our results are slightly different from a French paper [18] in which the authors demonstrated that for early HCC patients, PIVKA II had a better diagnostic performance than AFP, with a sensitivity of 77% (vs. 75.7%) and specificity of 82% (vs. 61%), at a cut-offs values of 42 mAU/mL for PIVKA II and 5.5 ng/mL for AFP.

PIVKA II testing is convenient, inexpensive, and might be a valuable addition to current options for the HCC diagnosis, but clinical decision should be made taking into account that various factors might influence the diagnostic performance of this biomarker. Since levels of both AFP and PIVKA II are higher in HCC patients than in non-HCC patients and healthy individuals, different cut-off levels can be tried to further improve their specificity.

In our study both biomarkers and their combination were demonstrated to have a moderate accuracy for HCC diagnosis. The present study demonstrated, by performing a head-to-head comparison of the two diagnostic markers of HCC, that AFP was the best single marker and the combination of AFP with PIVKA-II has the best diagnostic performance, in all study groups, compared to either marker alone.

This study was subject to several limitations. One limitation was that the samples were obtained from our center only, and therefore our results require external validation. Repeated experiments for internal validation were carried out strictly. Refreezing of thawed plasma was avoided.

Another limitation of this study is that the study population was small, and we used only one commercially available ELISA PIVKA II assay. The enrolled patients with HCC were heterogeneous in regard to underlying liver disease etiology, status and tumor stage which reflects real-world practice, but this fact may also lead to variability. Finally, the aim of the present study was to compare the clinical utility of AFP and PIVKA II for HCC diagnosis with easy-to-use cut-off values, not to evaluate their performance for surveillance.

CONCLUSION

In conclusion, PIVKA II as a single marker does not appear to be significantly superior to AFP for HCC diagnosis in our cohort of patients. The combination of AFP and PIVKA II may serve as a valuable addition to current imaging modalities for HCC diagnosis, because it greatly improves the diagnostic performance for HCC especially in patients with low AFP levels and in patients with early stage tumors.

Future studies performed on larger cohorts are needed to confirm our results, with the need to establish the most efficient and economical current biomarker combination.

Conflict of interest

All author declare that they have no conflict of interest.

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