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Highly sensitive alpha-fetoprotein, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein and des-gamma-carboxy prothrombin for hepatocellular carcinoma detection

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Abstract

Aim: Hepatocellular carcinoma (HCC) develops with high incidence in patients with chronic liver disease (CLD), and particularly in those with cirrhosis. Currently, diagnosis and surveillance are mainly based on imaging-methods. The aim of this study was to evaluate the diagnostic accuracy of highly sensitive measurement of alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-gamma-carboxy-prothrombin (DCP) alone and in combination, for early HCC detection. In addition, a recently proposed statistical model, including these three biomarkers plus gender and age, the GALAD model was applied.

Methods: In a total of 98 patients [44 CLD patients without HCC (23M, 21F; mean age 53.2 ± 13.4 years) and 54 patients with HCC (45M, 9F; 69.5 ± 9.8 years)] AFP, AFP-L3 and DCP levels were determined using a highly sensitive assay on μ TASWako i30 immuno-analyzer. Areas under the curve (AUC) were calculated and compared to assess diagnostic performance of the HCC biomarkers and of the GALAD model.

Results: AFP, AFP-L3 and DCP were significantly elevated in HCC compared to CLD patients ($p < 0.0001$). AUC values were 0.891, 0.867 and 0.870 respectively. The combination of the 3 biomarkers resulted in AUC=0.947 (Se=94.3%; 87.6% of patients correctly classified), whereas the GALAD model showed a AUC=0.976 (Se=96.3, specificity=84.1%, positive predictive value=88.1%; negative predictive value=94.9%; 89.8% of patients correctly classified).

Conclusions: These data confirm the elevated accuracy of highly sensitive methods for AFP, AFP-L3 and DCP quantitation. Moreover, the combination of these serological biomarkers and application of the GALAD model could improve HCC early detection and current surveillance efficiency.

Key words: alpha-fetoprotein, *Lens culinaris* agglutinin-reactive fraction of AFP, des-gamma-carboxy prothrombin, hepatocellular carcinoma, tumor marker

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignancy and the third cause of cancer-related death in the world,¹ representing a major global health problem. The principal risk factor for HCC development is cirrhosis, especially in patients with an underlying hepatitis B and C infection.² According to the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) clinical guidelines for HCC management, surveillance should be performed every 6 months in all at-risk patients using abdominal ultrasound (US), and diagnosis should be based on imaging methods, such as computed tomography (CT) and magnetic resonance imaging (MRI), and/or biopsy.^{3,4} Besides imaging methods, the Japan Society of Hepatology (JSH) guidelines suggest the use of tumor markers such as alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3) and des-gamma-carboxy-prothrombin (DCP) for surveillance programs and for early HCC detection.^{5,6} Moreover, in a recent study, Johnson PJ et al developed a model (GALAD) that is based on the objective measures of Gender, Age and three serological biomarkers, AFP, AFP-L3 and DCP for the detection of HCC, showing excellent diagnostic accuracy, even in patients with early disease as classified by Barcelona Liver Cancer Clinics (BCLC) staging system.⁷ Since recent technical improvements in the analytical methods of measuring AFP, AFP-L3 and DCP employing an advanced microfluidics-based separation technology have been developed,⁸ the aim of the present study was to evaluate the diagnostic accuracy of AFP, AFP-L3 and DCP alone or in combination and to test GALAD model performance for HCC detection in a cohort of Italian patients.

METHODS

Patients

This single-center cross-sectional study included 98 prospectively enrolled patients (68M, 30F; mean age 62.2 ± 14.1 years) with chronic liver disease (CLD) that underwent US screening for

hepatic nodular lesion. Final diagnosis of HCC was established by 4-phase multidetector CT scan or dynamic contrast-enhanced MRI showing arterial hypervascularity and washout in the venous/late phase.³ The degree of liver disease was classified according to clinical, serological and histological criteria where appropriate. Liver cirrhosis was diagnosed by liver biopsy or by laboratory data and imaging findings (US and transient elastography).^{9,10} All patients gave their written informed consent prior to recruitment. The study protocol was conformed to the principles of the Declaration of Helsinki and it was approved by the Institutional Ethics Committee.

Measurements of serological biomarkers

Blood samples were collected from each participant at the time of the scheduled outpatient visit. Sera were stored at -80°C and subsequently analyzed for the concentration of AFP, AFP-L3 and DCP using an automated immunoassay system assay on the μ TASWako i30 immuno-analyzer (Wako Chemicals GmbH, Neuss, Germany).^{11,12} Analytical assay sensitivities are 0.3 ng/mL for AFP and 0.1 ng/mL for DCP. AFP-L3 is reportable as ratio to total AFP if both AFP-L1 and AFP-L3 are ≥ 0.3 ng/mL. All measurements were carried out in the same sample and were performed in an outside laboratory blinded to diagnosis and details of the patients' clinical histories.

Statistical analysis

AFP, AFP-L3 and DCP values are expressed in medians and ranges. Kruskal-Wallis test was used to evaluate differences in HCC biomarkers levels among groups, while Fisher's exact test was performed to analyze categorical data. To evaluate diagnostic performance of AFP, AFP-L3 and DCP alone or in combination, area under the curve (AUC), sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) were assessed by using receiver operating characteristic (ROC) curves analysis. A logistic regression analysis was performed to evaluate the association of each biomarker with demographical and clinical factors. A

p-value <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc software, version 9.2.1.0.

RESULTS

Patient's demographic and clinical characteristics are reported in Table 1. The population studied comprised of 44 CLD patients without HCC (23M, 21F; mean age 53.2 ± 13.4 years) and 54 patients with diagnosis of HCC (45M, 9F; 69.5 ± 9.8 years). The etiology of liver disease among HCC patients was mainly viral (80%) followed by alcoholic and nonalcoholic steato-hepatitis, whereas etiology of liver disease in CLD group was exclusively viral. Almost all patients in the HCC group had cirrhosis (96%), while only the 31.8% of CLD patients showed histological or clinical signs of cirrhosis. The majority of HCC patients (85.2%) were diagnosed early stage (0 and A) according to BCLC staging system.

Median levels for AFP, AFP-L3 and DCP were significantly different between patients with and without HCC ($p < 0.0001$) (Figure 1) (Table 2). To evaluate whether different demographic and clinical factors could affect AFP, AFP-L3 and DCP performance, a logistic regression analysis was performed. Gender, age and underlying etiology of liver disease had no influence on the levels of the measured biomarkers ($p > 0.05$). Regarding severity of liver disease, only a weak association was found between the presence of cirrhosis and AFP-L3 values (OR=1.1753, 95%CI 1.0121-1.3648; $p = 0.034$). Conversely, either DCP and AFP-L3 resulted associated with HCC diagnosis (OR=9.1218, 95%CI 1.8085-46.0087, $p = 0.007$ and OR=1.2283, 95%CI 1.0281-1.4675, $p = 0.023$; respectively), whereas AFP showed just a trend (OR=1.1063, 95%CI 0.9972-1.2272; $p = 0.056$).

ROC curves were calculated to evaluate diagnostic accuracy for AFP, AFP-L3 and DCP alone or in combination to distinguish CLD from HCC (Figure 2). AUC values, Se, Sp, PPV, NPV and the corresponding cut-off are reported in Table 3.

The GALAD model, including gender, age, AFP-L3, AFP and DCP, was tested on our data set and showed a high diagnostic accuracy for HCC diagnosis (Table 4). Furthermore a comparison of the

diagnostic performance between each biomarker and the GALAD model was performed.

Differences between AUC values and corresponding statistical significance are reported in Table 5.

DISCUSSION

In the present study AFP, AFP-L3 and DCP showed significantly different median values between CLD and HCC patients and a high diagnostic performance when used as single biomarker (AUC=0.891, AUC=0.870 and AUC=0.867, respectively), allowing patients correct classification in at least 80% of subjects. Moreover, the combination of the 3 biomarkers significantly improved the diagnostic accuracy (AUC=0.947), enhancing Se in HCC detection (Se=94.3%; 87.6% of patients correctly classified).

AFP in particular, has been widely used as biomarker for HCC though several studies showed a lack of adequate Se and Sp for effective surveillance and diagnosis inasmuch that AASLD guidelines state that surveillance should rely on US examination only, whereas EASL guidelines adopt also AFP for surveillance purposes.^{13,14} In fact, several studies reported Sp values approaching 90% for all 3 biomarkers, but significant lower Se (<50%), in particular for early HCC detection.¹⁵ Ultrasound used as surveillance test showed Se values ranging from 58% to 89%,^{16,17} albeit being less effective for early-stage HCC detection (Se=63%).¹³ Conversely, JSH guidelines recommend to conduct surveillance programs using a combination of tumor biomarkers such AFP, AFP-L3 and DCP in addition to US for early HCC detection in patients with hepatitis B and C virus-related CLD. Moreover, the strategy to adopt US and AFP has been shown to be helpful and cost-effective particularly in patients with cirrhosis.^{18,19}

It is well known that older age and male gender are important risk factors that correlate with HCC development.²⁰ These parameters have been included in the GALAD model developed by Johnson et al that showed a higher diagnostic accuracy for HCC detection than the combination of AFP, AFP-L3 and DCP in our cohort of patients (Δ AUC=0.029, $p=0.028$). Moreover, considering that the majority of HCC patients enrolled in our study were stage 0 (27.8%) or stage A (57.4%) according

to BCLC staging system, the performance we observed suggests that this model could be suitable for early HCC detection despite a validation in a larger cohort of patients is required.

A limitation of our study could be the difference in etiology and in cirrhosis prevalence between the CLD and HCC patients group. These features, together with the high occurrence of early stage tumors in the HCC group and with the treatment status of HBV- CLD patients (all under nucleos(t)ide analogue treatment), could explain both the lower median values of AFP, DCP and AFP-L3 and the low cut-off found in these cohort. Even if demographic characteristics were not properly balanced between CLD and HCC patients, when we evaluated whether these differences could impact HCC biomarkers performance, we found no effect.

In conclusion, our data gather further evidence for the use of highly sensitive methods for AFP, AFP-L3 and DCP quantitation for HCC detection. The combination of these biomarkers in addition to state-of-the-art ultrasound imaging may prove more powerful to identify patient at risk of HCC in surveillance protocols.

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Table 1 Characteristics of patients with hepatocellular carcinoma and chronic liver disease

	All patients	CLD	HCC
Demographics			
Number of patients	98	44	54
Age (years), mean $\pm$ SD	62.2 $\pm$ 14.1	53.2 $\pm$ 13.4	69.5 $\pm$ 9.8
Gender (M/F)	68/30	23/21	45/9
Etiology			
HCV	45 (45.9%)	9 (20.5%)	36 (66.7%)

	HBV	36 (36.7%)	31 (70.5%)	5 (9.3%)
	HCV + HBV	2 (2.0%)	0	2 (3.7%)
	HBV + HDV	4 (4.1%)	4 (9.0%)	0
	Alcohol	9 (9.2%)	0	9 (16.6%)
	NASH	2 (2.0%)	0	2 (3.7%)
Biochemistry				
	AST (IU/L), median (range)	38 (14-327)	24 (14-109)	64 (17-327)
	ALT (IU/L), median (range)	36 (9-382)	26 (9-200)	48 (12-382)
	Albumin (g/dL), median (range)	4.1 (2.3-5.1)	4.4 (3.5-5.1)	3.9 (2.3-4.9)
	PLT (10 ⁹ /L), median (range)	129 (32-352)	175 (78-352)	106 (32-256)
Cirrhosis		66 (67.3%)	14 (31.8%)	52 (96.3%)
BCLC staging				
	0	/	/	15 (27.8%)
	A	/	/	31 (57.4%)
	B	/	/	8 (14.8%)
	C	/	/	0
	D	/	/	0

ALT, alanine aminotransferase; AST, Aspartate aminotransferase; BCLC, Barcelona Liver Cancer Clinics; CLD, chronic liver disease; F, female; HCC, hepatocellular carcinoma; M, male; PLT, platelet count; SD, standard deviation.

Table 2 AFP, DPC and AFP-L3 values in hepatocellular carcinoma and chronic liver disease cohorts

	CLD	HCC	p
AFP (ng/mL), median (range)	2.3 (1.0 - 25)	13.4 (2.4 - 5785.0)	<0.0001
DCP (ng/mL), median (range)	0.26 (0.10 - 1.45)	1.13 (0.10 - 87.49)	<0.0001
AFP-L3 (%), median (range)	1.0 (1.0 - 15.1)	5.9 (1.0 - 81.6)	<0.0001

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; CLD, chronic liver disease; DCP, des-gamma-carboxy-prothrombin; HCC, hepatocellular carcinoma.

Table 3 AUC values, Se, Sp, PPV and NPV of AFP, DCP and AFP-L3 alone or in combination.

	AUC	Cut-off	Se	Sp	PPV	NPV	Correctly classified
AFP (ng/mL)	0.891	5.3	81.1	86.4	87.8	79.2	79.4%
DCP (ng/mL)	0.870	0.4	77.8	90.9	91.3	76.9	79.6%
AFP-L3 (%)	0.867	1.0	84.9	88.6	90.0	83.0	84.5%
AFP + AFP-L3 + DCP	0.947	/	94.3	86.4	89.3	92.7	87.6%

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; AUC, area under the curve; DCP, des-gamma-carboxy-prothrombin; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

Table 4 AUC values, Se, Sp, PPV and NPV at different cut-off levels of GALAD model

AUC	Cut-off	Se	Sp	PPV	NPV	FN	FP	Correctly classified
0.976	-2.94	100	77.3	84.4	100	10	0	89.8%
0.976	-2.59	96.3	84.1	88.1	94.9	7	3	89.8%
0.976	0.16	57.4	100	100	65.7	0	23	76.5%

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; AUC, area under the curve; DCP, des-gamma-carboxy-prothrombin; FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

Table 5 AUC values comparison between AFP, AFP-L3, DCP and GALAD model

	DCP	AFP-L3	AFP + DCP + AFP-L3	GALAD model
AFP	$\Delta\text{AUC}=0.024$ p=0.611	$\Delta\text{AUC}=0.024$ p=0.551	$\Delta\text{AUC}=0.056$ p=0.063	$\Delta\text{AUC}=0.085$ p=0.011
DCP	/	$\Delta\text{AUC}=0.003$ p=0.996	$\Delta\text{AUC}=0.080$ p=0.002	$\Delta\text{AUC}=0.106$ p=0.001
AFP-L3	/	/	$\Delta\text{AUC}=0.080$ p=0.022	$\Delta\text{AUC}=0.109$ p=0.002
AFP + DCP + AFP-L3	/	/	/	$\Delta\text{AUC}=0.029$ p=0.028

AFP, alpha-fetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; AUC, area under the curve; DCP, des-gamma-carboxy-prothrombin.

Fig. 1 AFP, DCP and AFP-L3 values in hepatocellular carcinoma and chronic liver disease cohorts

Fig. 2 ROC curves showing the performance of AFP, AFP-L3 and DCP alone and in combination