Analysis of serum α-fetoprotein-L3% and des-γ carboxyprothrombin markers in cases with misleading hepatocellular carcinoma total α-fetoprotein levels

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Received October 3, 2012; Accepted November 12, 2012

DOI: 10.3892/or.2012.2147

Abstract. Serum fraction of α-fetoprotein L3 (AFP-L3%) and des-γ carboxyprothrombin (DCP) are proposed serum markers for the diagnosis of hepatocellular carcinoma (HCC). We evaluated their performance in two patient populations with total AFP levels non-diagnostic for HCC. From a cohort of 150 consecutive patients with HCC, 60 patients with total AFP <200 ng/ml were identified. Additionally, 50 patients with elevated AFP and no radiological evidence of HCC, for at least one year of follow-up, were included. AFP-L3% and DCP were measured by the Liquid Phase Binding Assay System (LiBASys). In cases where AFP-L3% was undetectable, a more sensitive method based on-chip electrokinetic reaction was applied. AFP-L3% was found to be positive in 22 (36.7%) of patients with HCC and 6 (12%) of non-HCC patients. DCP was found to be positive in 26 patients with HCC (43%) and in none of the non-HCC patients. Thirty-six out of sixty (60%) patients with HCC were positive for either AFP-L3% or DCP. With the on-chip technology, AFP-L3% was found to be positive in 10 patients with HCC and in 5 patients without HCC, who tested negative by LiBASys. The final sensitivity of combined AFP, AFP-L3% and DCP testing, in the entire cohort of patients with HCC, was 84%. The specificity of AFP-L3% and DCP in the studied population was 78.5 and 100%, respectively. The addition of AFP-L3% and DCP increased the sensitivity and specificity of total serum AFP for the diagnosis of HCC. The on chip AFP-L3% assay was more sensitive but less specific compared to LiBASys.

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Abbreviations: AFP-L3%, α-fetoprotein L3%; DCP, des-γ carboxyprothrombin; HCC, hepatocellular carcinoma; LCA, lens culinaris agglutinin

Key words: α-fetoprotein, α-fetoprotein-L3%, des-γ carboxyprothrombin, hepatocellular carcinoma, tumor marker

Introduction

Hepatocellular carcinoma (HCC) is a major cause of mortality, with a high prevalence in Asia and Africa and an increase in the incidence rates in Western countries (1). Patients with chronic hepatitis B or C virus infection as well as patients with liver cirrhosis of different etiologies are at high risk for development of liver cancer. Monitoring of these patients requires liver ultrasonography (US) every 6 months, for early detection of HCC (2,3). The sensitivity of US varies depending on the size of the lesion and the skills of the operator. For small lesions <1 cm in diameter, the sensitivity is 50%; it increases to 70% for lesions approximately 1 cm and reaches 90% when >5 cm, with specificity between 48 and 94% (4). Other radiographic tests with greater sensitivity and specificity are not suitable for monitoring. Contrast-enhanced CT or gadolinium-enhanced MRI are usually performed to evaluate suspicious lesions detected by US (5-8).

The most widely used serum marker for HCC is α-fetoprotein (AFP). It demonstrates low specificity (76%), which increases when elevated cut-off levels are used, but exhibits concomitant loss of sensitivity (maximum 60%) (9-11). Therefore, 40% of patients diagnosed radiographically with HCC have low serum AFP levels. Additionally, AFP can be found to be elevated in patients who are at high risk for HCC and have no radiological evidence of a liver tumor, due to temporal activation of the AFP gene (12). Due to this poor performance, previous recommendations (2) have been altered and serum AFP testing is not currently included in the instructions for HCC surveillance (3).

Furthermore, for the screening and evaluation of the disease, novel HCC markers have been clinically implemented. AFP-L3, an AFP glycoform with strong binding capacity for lens culinaris agglutinin (LCA), has been used in Japan as a specific marker for early HCC diagnosis for several years (13). Another marker which has also been shown to be present in 50-60% of patients with HCC is des-γ carboxyprothrombin (DCP or PIVKA-II), a prothrombin form lacking carboxylation (14-16). The performance characteristics of these new HCC markers might be differentiated by ethnicity, etiology, stage of liver disease and total AFP levels (12,17,18). In Greece, as well as in other areas of the Mediterranean basin, where the

Table I. Characteristics of studied patients with misleading total AFP.

	Patients with HCC			Patients without HCC		
	Male	Female	Total	Male	Female	Total
Number (N)	48	12	60	15	35	50
Median age in years (range)	66.5 (48-85)	70 (38-79)	67 (38-85)	48 (34-73)	67 (40-82)	63 (34-82)
Multifocal HCC (%)	13 (27)	5 (42)	18 (30)	0 (0)	0 (0)	0 (0)
HBV (%)	28 (58)	1 (8)	29 (48)	3 (20)	4 (11)	7 (14)
HCV (%)	7 (15)	7 (58)	14 (23)	3 (20)	12 (34)	15 (30)
Alcohol (%)	2 (4)	0 (0)	2 (3)	1 (7)	0 (0)	1 (2)
Other (%)	11 (23)	4 (33)	15 (25)	8 (53)	19 (54)	27 (54)
Median AFP ng/ml (range)	8 (1.7-173)	12.5 (2.0-128)	8.5 (1.7-173)	13.6 (10.2-30)	15.5 (10.4-140)	15 (10.2-140)

AFP, α-fetoprotein; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

etiology of HCC is primarily chronic viral hepatitis (1,19), the implementation of these tests has not been studied extensively.

In our study, we evaluated the combined testing of AFP-L3 and DCP as HCC markers in patients with serum AFP levels that were not in accordance with radiological findings. We investigated one group of patients with definite HCC characterized by either low or moderately elevated total AFP and a second group of patients with mild to moderate AFP elevation which showed no evidence of HCC when evaluated by extensive imaging.

Materials and methods

Patients. One hundred and fifty consecutive patients were hospitalized or seen in the Liver Unit of Hippokration Hospital, Athens, with the diagnosis of HCC, during the period 2009-2010. From this cohort, 60 patients (40%) had serum total AFP <200 ng/ml. At the same time, 50 patients with persistently elevated AFP >10 ng/ml, without radiographic or other evidence of HCC for at least one year of follow-up, were identified. Informed consent was obtained from all patients. The study was approved by the Institution's Ethics Committee.

AFP-L3% and DCP. Total serum AFP was determined by routine immunoassays (mainly Architect, Abbott) at the time of visit or hospitalization. Serum aliquots were collected and stored at -70˚C until further analysis. AFP-L3% and DCP were measured by an automated Liquid Phase Binding Assay System (LiBASys, Wako). A liquid-phase binding reaction between antigen and antibody was used with bound and free forms separated by column chromatography (20,21). Total AFP and the L3 fraction were measured simultaneously. An AFP-L3% assay, using on-chip electrokinetic reaction and separation by affinity electrophoresis (μTAS, Wako i30) was performed when AFP-L3 was undetectable by LiBASys (22). AFP-L3% >10% and DCP >7.5 ng/ml were considered positive.

Statistical analysis. Data entry and statistical analysis were performed with the statistical package IBM SPSS (version 20, SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate statistically significant differences and all reported p-values were based on two-tailed tests. Corrected χ^2 or two-tailed Fisher's exact test and t-test or the Mann-Whitney test were used for qualitative and quantitative data, when appropriate.

Results

Patients. The underlying liver disease for the 60 patients studied with HCC was indicated as chronic viral hepatitis in 43/60 patients (71.5%), as alcohol-related liver disease in 2 patients (3%), and as cryptogenic liver disease, including steatohepatitis, in 15 patients (25%). Their mean AFP was 22 ng/ml (median 8.5 ng/ml, range 1.7-173 ng/ml) and in the vast majority (88.5%) <50 ng/ml. The group of patients without radiographic evidence of HCC consisted of 22 patients with chronic viral infection (40%), 1 (2%) with alcohol-related liver disease and 27 with cryptogenic disease (54%). Their mean AFP was 22 ng/ml (median 15 ng/ml, range 10.15-140 ng/ml). Patient characteristics are shown in Table I.

AFP-L3% and DCP in patients with HCC. AFP-L3% by LiBASys was positive in 12/60 (20%) patients with HCC with mean levels of 48.7% (median 49.8%, range 13.2-89.6%). Total AFP in patients positive for AFP-L3% was 12.4-137.2 ng/ml (mean 38.6, median 22.1 ng/ml) by LIBASys compared to common methods that determined total AFP to be 9-173 ng/ml (mean 52.4, median 41.9 ng/ml). Furthermore, total AFP values by LiBASys were lower in 45/60, equal in 13/60, and minimally higher in 2/60 cases than previously determined, with a mean difference of -60%. In one of the 34 patients with AFP <10 ng/ml, as indicated by routine methodology, AFP levels were found to be >10 ng/ml by LiBASys (14.4 ng/ml). In this patient, both AFP-L3% and DCP were positive. AFP-L3% was not detectable in any of the remaining 33 patients, whereas DCP was positive in 13 patients. DCP was positive in a total of 26/60 (43%) patients with median levels of 115 ng/ml. Eighteen of the 26 DCP positive patients were negative for AFP-L3%. A total of 30 patients (50%) tested positive for either DCP or AFP-L3 by LiBASys. Supplementary AFP-L3% testing on

Table II. DCP and AFP-L3% in patients with HCC and non-diagnostic total AFP.

	AFP-L3%		
DCP	+ (%)	- (%)	Total (%)
+	12 (20)	14 (23)	26 (43)
-	10 (17)	24 (40)	34 (57)
Total	22 (37)	38 (63)	60

DCP, des-γ carboxyprothrombin; AFP-L3%, α-fetoprotein L3%; HCC, hepatocellular carcinoma.

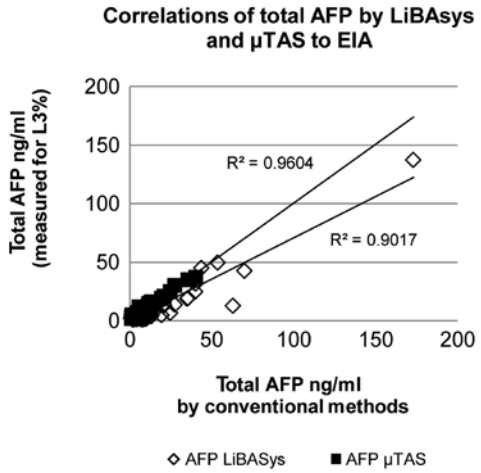

Figure 1. Total AFP results generated by μTAS correlated better (R^2=0.96) with AFP results obtained by other routinely used immunoassays in comparison to total AFP values recorded by LiBASys (R^2=0.90).

μTAS revealed 10 more positive patients. The sensitivity of AFP-L3% increased significantly, reaching 37% (assuming all samples positive by LiBASys would be positive by μTAS), although 14 of the 26 DCP positive patients remained AFP-L3 negative (Table II). Total AFP by μTAS correlated better with values obtained by routine immunoassays (mean difference 0.7 ng/ml) (Fig. 1) and differed from LiBASys (mean difference 4 ng/ml). Overall, 36/60 (60%) patients with HCC were positive for either AFP-L3% or DCP (sensitivity 60%). In patients with AFP <10 ng/ml, the sensitivity of combined testing was 56% (19/34). The total AFP sensitivity (as HCC marker at levels >200 ng/ml) of combined testing in the whole HCC cohort was 84%. No correlation between AFP-L3% or DCP and number of lesions, stage of underlying fibrosis or etiology of HCC was found.

AFP-L3% and DCP in patients with high total AFP and no radiographic evidence of HCC. Among the 50 patients with no evidence of HCC and persistent AFP >10 ng/ml (mean 22 ng/ml), total AFP by LiBASys was elevated (mean 26.8 ng/ml, range 11-120 ng/ml) in 14 (28%) and in normal range (mean 4.1 ng/ml) in 36 (72%) patients. The mean AFP difference between enzyme immunoassay (EIA) and LiBASys was 9 ng/ml (median 6.5 ng/ml, range 1.5-36 ng/ml). DCP was

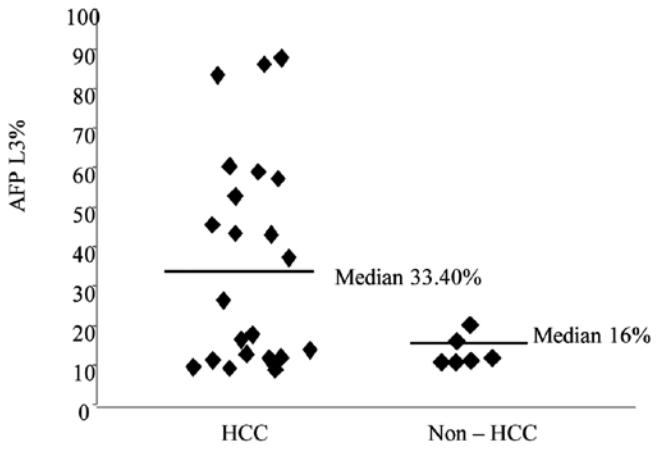

Figure 2. AFP-L3% distribution in positive cases of the studied population demonstrates significant differences between patients with and without radiographic evidence of HCC.

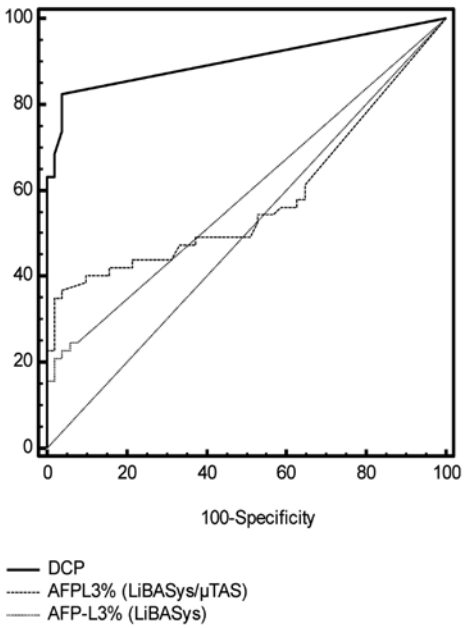

Figure 3. Comparative ROC curves for the tested populations. DCP demonstrates greater sensitivity and specificity compared to AFP-L3%.

negative in all patients and AFP-L3% in 49/50 patients. The patient mean follow-up was 3 years (range 1-4 years). One patient with chronic hepatitis C and AFP 140 ng/ml who tested negative for both AFP-L3% and DCP, was recently diagnosed with HCC. This patient was not used in the calculations of sensitivity. In another patient, with AFP 13 ng/ml, AFP-L3% was found positive by LiBASys (11.7/16%) in the two different samples that were collected at a time interval of 20 days (only the first was used for calculations). This patient has not developed HCC during follow-up. Supplementary AFP-L3% testing on μTAS was positive in 5 more patients. AFP-L3% levels in positive cases were higher in HCC (mean 38.07%) than in non-HCC patients (mean 16%) (Fig. 2). The specificity in the tested population was 100% for DCP and 78.6% for AFP-L3%. Receiver operating characteristic (ROC) curves are shown in Fig. 3.

Discussion

In the present study, we examined the combined use of serum HCC markers in two patient populations with chronic liver disease in which total AFP was inconsistent with radiological findings. Patients with HCC often demonstrate low levels of serum AFP compared to patients with benign liver disease which can present high AFP values, resulting in extensive and repeated radiological examinations (23). Several new serum markers have been investigated for HCC, including proteins, enzymes, cytokines, autoantibodies and molecular markers. Their combined use appears to increase the accuracy and sensitivity of serological diagnosis of HCC (24-30).

AFP-L3% and DCP are HCC markers that can be measured on the same platform, making testing more friendly to clinical laboratories. AFP-L3 is an isoform of AFP derived only by cancer cells, and is, thus, one of the most promising new HCC serum markers. This test is already being used in clinical practices and routine screening mainly in Asia (31,32). Previous reports have indicated the effectiveness of the combined use of AFP-L3% and DCP in HCC surveillance (33,34), however, their implementation in Western Europe remains limited. Our results in a cohort of 150 HCC patients showed that combined measurement of AFP-L3% and DCP by LiBASys, in patients with low total AFP, increases the sensitivity of serological diagnosis by 20%. In the tested population, DCP demonstrated greater sensitivity (43 vs. 20%) and specificity (100 vs. 98%) compared to AFP-L3%. On the other hand, the LiBASys analyzer persistently yielded lower total serum AFP compared to routine methods.

The implementation of the μTAS on-chip immunoassay (35) increased the sensitivity of combined testing by 10%. In previous studies, this sensitive AFP-L3% assay was applied in patients with total AFP <20 ng/ml with promising results (36-38). The cut-off level that should be used for optimal results with this new assay has yet to be fully clarified. In one study, AFP-L3% was >10 in 14.8%, >7 in 26.7% and >5 in 41.5% of patients with HCC (36). Other investigators demonstrated sensitivity of 44.6 and specificity of 71.2% using a 5% limit, in patients with low AFP (37). The cut-off level of 6-7% was suggested in a study involving HCC-treated patients with low AFP (38). High sensitive AFP-L3 was found useful in predicting recurrence of HCC after treatment with a cut-off of 5% (39). In our study, sera from patients with HCC, with low total AFP (mean 9.4 ng/ml) and undetectable AFP-L3% by LiBASys, were tested with μTAS. This resulted in improvement of AFP-L3% sensitivity since it was found positive in 10 additional sera. We used the same cut-off level of 10% as in the LiBASys assay. At lower cut-off values, the sensitivity would further increase, adding 5 more HCC patients at levels >7% (sensitivity 45%) and 10 more with a >5% fraction, (sensitivity 53%), but with a significant concomitant decrease in specificity at levels of 44-72%. AFP-L3 at levels between 5 and 10% by μTAS was observed in 27 (54%) patients without any radiographic evidence of HCC.

In patients with increased AFP of non-neoplastic origin, as determined by thorough radiographic studies, in a relatively long period of follow-up, the combined DCP/AFP-L3% testing displayed high specificity (88%). In 49 of 50 individuals with mean follow-up of 3 years and persistently increased AFP with no evidence of HCC, only 1 patient was found positive for AFP-L3% by LiBASys and none for DCP. With the implementation of a more sensitive assay for AFP-L3%, a total of 6 patients with no radiographic evidence of HCC were found to be positive at levels >10%. These patients are under close follow-up with no radiographic evidence of HCC as yet.

The values of total AFP with μTAS were higher than LiBASys and much closer to routine immunoassays. This may contribute to the increased sensitivity of the assay and could make possible the complete replacement of solitary AFP measurements to combination testing with AFP-L3% and DCP on one instrument.

With combined testing, 60% of patients with HCC and non-diagnostic total AFP had at least one positive tumor marker, AFP-L3% and/or DCP. This approach seems to offer a more sensitive laboratory diagnostic procedure than total AFP and may contribute to earlier detection of HCC and improved survival (40). It has also been suggested that a relationship exists between these serum tumor markers and the stage or outcome of the disease (41,42). However, in our study, AFP-L3% and DCP were not associated with the cause, number of lesions or the outcome of HCC. This could be due to the study population, which, in the great majority, consisted of hospitalized patients with symptoms of advanced liver disease, and the cross-sectional type of the study.

In conclusion, combined testing of DCP and AFP-L3% was found to be of major benefit for the diagnosis of HCC. The higher specificity of these markers, as compared to total AFP, may aid in supporting the benign nature of elevated AFP as a result of hepatocyte regeneration in some patients with chronic liver disease.

Acknowledgements

We thank Professor S.J. Hadziyannis for his contribution and Wako Inc. and local distributors for the provision of reagents.

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