Simultaneous Determination of α -Fetoprotein Immune Complexes and α -Fetoprotein Concentration in Hepatocellular Carcinoma Using Dual-Label Time-Resolved Immunofluorometric Assays

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α -Fetoprotein (AFP) is a commonly used tumor marker in the detection of hepatocellular carcinoma (HCC), and its sensitivity and specificity is insufficient to detect HCC in all patient samples. Recently, the immunocomplexed forms of AFP (AFP-IgM) have been reported to be present in HCC patients. This study was aimed at developing a novel time-resolved immunofluorometric assay (TR-IFMA) for the simultaneous determination of AFP-IgM and AFP concentration in HCC. We constructed a double-label assay by using Sm³⁺-labeled mAb to human IgM antibody, Eu³⁺-labeled mAb to AFP, and immobilized another mAb to AFP on the solid phase. The performances of the assay were all found to be satisfactory. The validity of the

novel assay was confirmed by the good correlation between the results obtained by TR-IFMA and commercial ELISA or electrochemiluminescence immunoassay. AFP-IgM and AFP were increased above the cutoffs in 65.25 and 45.76% of HCC, respectively. ROC analysis yielded the following area under the curve: AFP-IgM 0.774 (CI 95% 0.736–0.809), AFP 0.771 (CI 95% 0.733–0.860). The combined use of AFP-IgM and AFP increased the sensitivity of detection to 72.88% in patients with HCC. These data suggest that the use of a combination of two markers in clinical practice could increase the accuracy of HCC diagnosis. *J. Clin. Lab. Anal.* 23:179–185, 2009.

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Key words: AFP; AFP-IgM; dual-label time-resolved immunofluorometric assay; hepatocellular carcinoma

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Because of the global pandemic of hepatitis B and C viral infections, the incidence of HCC is rapidly increasing in Asian and Western countries (1,2), and this trend is expected to continue for the next 50 years because of the long latency between infection and the development of HCC. The prognosis of advanced HCC remains poor, and novel treatment and diagnosis strategies are urgently needed.

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 (3,4). Recently, it has been reported that AFP can react with the IgM class of immunoglobulins to form the

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immunocomplexes (AFP-IgM, also called AFP-IC), detectable in the serum of HCC patients (5,6). AFP-IgM is a complementary serological marker to AFP and the combination of these biomarkers may be useful in the diagnosis of liver cancer.

The purpose of our study was to develop dual-label time-resolved immunofluorometric assays (dual-label TR-IFMAs) for the simultaneous measurement of the serum concentrations of AFP-IgM together with AFP. The sample volume is small, and the assay procedure is simple and fast. The analytical range of the dual-label TR-IFMA is large and well suited for clinical purposes.

MATERIALS AND METHODS

Chemicals

Monoclonal anti-human AFP antibodies (M19310 and M94115) and anti-human IgM antibody (M19301) were obtained from Fitzgerald Industries International, Inc. DELFIA Eu^{3+} and Sm^{3+} -Labeling kit is commercially available from Wallac Oy (Turku, Finland). Buffers were as described previously (7). Other chemicals used were of analytical grade.

Calibrators

Human purified AFP-IgM were obtained from Xeptagen (Naples, Italy). Calibrators of dual-label TR-IFMAs were prepared by adding AFP (Fitzgerald Industries International, Concord, MA) to the purified AFP-IgM. The levels of AFP-IgM and AFP in the calibrators were measured with Hepa AFP-IC kits (Xeptagen) and electrochemiluminescence immunoassay on the ElecSys 2010 system (Roche Diagnostics Corp., Indianapolis, IN), respectively. One set of calibrators containing both analytes was prepared by diluting with pooled healthy donor serum and stored at -20°C .

Specimens

AFP and AFP-IgM concentrations were measured before treatment in 118 HCC patients and in the reference group, which consisted of 218 healthy persons and 123 patients with benign liver diseases (60 cirrhosis, 63 chronic hepatitis; benign liver diseases (BLD) group). The age and sex distributions in both groups were comparable. The clinical diagnosis of HCC was confirmed each time by a pathological diagnosis of HCC after surgery or with evidence of tumor stain on computed tomography or angiography. Blood specimens were obtained by a standard procedure between 08:00 and 09:00 in the morning. Serum was separated 30 min after collection and stored at -80°C until assays.

Shanghai Jiao Tong University School of Medicine Ethics Committee approved the project and written informed consent was obtained from all patients and controls before their inclusion.

Conjugation of Antibodies With Eu^{3+} - and Sm^{3+} -Chelates

Before antibodies were labeled with Eu^{3+} or Sm^{3+} , columns prepacked with gel-filtration medium (Sephadex G-25M, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were used for desalting and buffer exchange. Antibodies were labeled using the DELFIA Eu^{3+} or Sm^{3+} -Labeling kit. Briefly, 0.5 mg of antibodies in 50 mmol/L carbonate buffer, pH 9.8, was incubated with a 20-fold molar excess of label reagent in a final volume of 500 μL overnight at 4°C . The mAb to AFP was labeled to contain seven to nine Eu^{3+} per molecule of IgG. The labeling yield for the mAb to hIgM was six to eight Sm^{3+} per IgG molecule.

Coating of Polystyrene Microtiter Strips

Coating of polystyrene microtiter strips (Nunc, Roskilde, Denmark) was performed by a modification of our previously described methods (7). Concentrated monoclonal anti-human AFP antibody (5.0 mg/mL) was diluted to 8 $\mu\text{g/mL}$ in the coating buffer. The coated strips were stored at $4-8^{\circ}\text{C}$ in a dry sealed bag. No loss of immunoreactivity was observed during storage for 4 weeks at 37°C .

Assay Protocol of Dual-Label TR-IFMA

We added 25 μL of standards or serum per coated microtitration strip well, followed by 100 μL of assay buffer. The plates were incubated with continuous shaking for 60 min at room temperature. After a wash step, we added 100 μL of assay buffer containing 100 ng per well of each of the Sm^{3+} -labeled mAb to hIgM and the Eu^{3+} -labeled mAb to AFP and incubated the strips for 30 min under continuous shaking at room temperature. After six washings and addition of 200 μL of enhancement solution per well, the strips were shaken for 5 mm. We measured Eu^{3+} and Sm^{3+} fluorescence at 613 and 643 nm, respectively, for 1 sec each with a time-resolved fluorometer (Arcus 1235; Wallac), using the $\text{Eu}^{3+}/\text{Sm}^{3+}$ dual-label software.

Assessment of Assay Performance

We assessed the performance of TR-IFMA by evaluating its calibration curve, detection limit, analytical recovery, precision, and its dilution linearity.

Statistical Analysis

All data are presented as median value (interquartile range (IQR), Q3–Q1), with nonparametric analyses being employed to assess differences. The Kruskal–Wallis analysis of variance and the rank sum test (Wilcoxon) were used to evaluate differences between multiple groups, unpaired observations, respectively. All analyses with the exception of the ROC analysis were conducted using SAS v8.1 (SAS Institute, Cary, NC). The ROC analysis was conducted with the aid of MedCalc v7.2.0.2 (Med Calc Software, Mariakerke, Belgium). Significance was presumed at $P < 0.05$.

RESULTS

Calibration Curve and Detection Limit

Standards covered a range of 1.21–1210 ng/mL for AFP and 0.5–250 AU/mL for AFP-IgM. The standard curves for AFP and AFP-IgM were linear over the ranges determined (Fig. 1). The lower limit of detection was defined as the concentration corresponding to a signal 3 SD above the mean of 12 replicates of the zero calibrator. The detection limit of the dual-label assay was 0.41 ng/mL for AFP and 0.01 AU/mL for AFP-IgM. The precision profile of the assay was determined from 15 replicates; the coefficients of variation at the concentration standards were all $< 6.0\%$.

Analytical Recovery

The analytical recovery was studied by adding purified AFP or AFP-IgM to five serum samples from different patients. The results are given in Table 1. The average recovery of added analytes was 98.33% for AFP and 98.77% for AFP-IgM.

Fig. 1. Typical standard curve (□) and precision profile (●) for the dual-label TR-IFMA for AFP-IgM (A) and AFP (B). TR-IFMA, time-resolved immunofluorometric assay; AFP, α -fetoprotein.

Precision

Seven samples were assayed 3 times, in 10 runs, on 1 systems ($n = 30$ for each sample), over a period of 5 days. The results are given in Table 2.

Dilution

Table 3 gives the results of our evaluation of the dilution linearity of dual-label TR-IFMAs when we used samples serially diluted with assay buffer. Expected values were derived from initial concentrations of analytes in the undiluted samples. Correlating the results obtained from dual-label TR-IFMAs with the expected concentrations, we found that the dilution curves were linear over the whole range of concentrations. Expected and measured values were well correlated.

Comparison of Methods

Serum samples from all patients with 118 HCC were concurrently tested with dual-label TR-IFMA, ELISA (AFP-IgM), and ElecSys 2010 system (AFP). The correlation between the values obtained by these methods was studied. The linear regression equation was as follows: dual-label TR-IFMA result = 1.05 (Hepa AFP-IC ELISA kits) – 0.07, $r = 0.9687$; dual-label TR-IFMA result = 0.97 (ElecSys 2010 system) + 0.12, $r = 0.9354$.

Distribution of AFP-IgM and AFP Concentrations

Serum AFP-IgM and AFP levels of each patient are plotted in Figure 2. Serum AFP-IgM levels were detectable in healthy persons. The median level of AFP-IgM was 72.79 AU/mL (IQR 95.07–43.97) in healthy persons, 75.70 AU/mL (IQR 118.0–27.45) in cirrhosis persons, and 74.20 AU/mL (IQR 109.05–47.65) in chronic hepatitis persons. There was no significant

TABLE 1. Analytical Recovery of AFP and AFP-IgM Added to Serum Samples

AFP (ng/mL)				AFP-IgM (AU/mL)			
Added	Expected	Observed	Recovery	Added	Expected	Observed	Recovery
11.89				10.67			
	27.81	26.62	95.71		10.594	10.93	103.17
	54.50	54.28	99.60		22.507	20.93	92.99
	86.97	83.66	96.20		56.232	50.67	90.11
	147.43	142.86	96.90		111.142	111.48	100.30
Mean	285.29	287.86	100.90		264.111	256.98	97.30
97.86				96.78			
51.06				58.81			
	29.7705	30.13	101.21		13.001	12.96	99.68
	56.456	57.87	102.50		24.914	24.24	97.29
	88.927	89.64	100.80		58.639	55.88	95.29
	149.385	148.94	99.70		113.549	114	100.40
Mean	287.249	298.16	103.80		266.518	264.65	99.30
101.60				98.39			
110.33				226.03			
	32.734	29.5	90.12		21.36	21.15	99.00
	59.4195	56.27	94.70		33.28	33.51	100.70
	91.8905	89.69	97.61		67.00	64.72	96.60
	152.3485	146.25	96.00		121.91	133.49	109.50
Mean	290.2125	287.89	99.20		274.88	275.98	100.40
95.52				101.24			

Mean $\pm$ SD = 98.33% $\pm$ 3.51, n = 15 (AFP). Mean $\pm$ SD = 98.77% $\pm$ 4.46, n = 15 (AFP-IgM). AFP, α -fetoprotein.

TABLE 2. Precision of Dual-Label TR-IFMA for AFP and AFP-IgM

AFP				AFP-IgM			
Mean	Within-run%CV	Run-to-run%CV	Total%CV	Mean	Within-run%CV	Run-to-run%CV	Total%CV
16.78	4.54	1.09	4.94	19.08	4.2	1.09	5.1
20.57	3.61	1.89	4.07	50.56	2.97	2.66	3.11
30.41	2.48	2.9	3.06	63.83	3.9	2.96	4.06
50.6	2.81	1.05	3.05	90.56	3.78	1.05	3.55
94.75	4.73	0.64	4.96	136.73	4.06	2.08	4.95
186.8	4.09	2.79	4.88	350.67	4.36	3.06	4.87
389.41	4.71	2.05	5	462.17	4.07	3.05	4.82

TR-IFMA, time-resolved immunofluorometric assay; AFP, α -fetoprotein.

difference in AFP-IgM levels between healthy persons and benign liver disease groups ($P = 0.898$). The median level of AFP-IgM was 152.55 AU/mL (IQR 233.68–77.90) in HCC patients. We observed significant differences for AFP-IgM between HCC patients compared with healthy persons ($P < 0.001$) as well as benign liver diseases group ($P < 0.001$). No correlation was found between AFP-IgM concentration and AFP levels in our study ($r = -0.17$; $P = 0.321$).

To assess the diagnostic utility of tumor markers in HCC, the cutoff values chosen were the 95% percentiles of healthy persons. The cutoff is 7.23 ng/mL for AFP and 127.52 AU/mL for AFP-IgM. Figure 3 shows the ROC curve in the HCC patient group.

As can be observed, the area under the curve (AUC) corresponding to AFP-IgM ($0.81 \pm_{\text{SEM}} 0.03$) is larger than that of AFP ($0.76 \pm_{\text{SEM}} 0.03$), which do not differ significantly from each other ($P = 0.155$). In terms of sensitivity, AFP-IgM was detected above the cutoff level in 77 out of 118 HCC patients (65.25%). AFP was elevated only in 45.76% (54/118). AFP levels are above the cutoff level in 20% (12/60) and 7.93% (5/63) of cirrhosis and chronic hepatitis, respectively, compared with 23.33% (14/60) and 14.29% (9/63) of positive patients for AFP-IC in the same groups. Co-determination of both markers improves the indexes of diagnostic accuracy for liver cancer diagnosis. As reported in Table 4, 72.88% (41/50) of HCC patients are positive

TABLE 3. Dilution Linearity of Samples With High AFP and AFP-IgM Levels

Sample	Dilution	AFP (ng/mL)			AFP-IgM (AU/mL)		
		Expected	Observed	Recovery %	Observed	Expected	Recovery %
1	NA		76.1		50.6		
	1:2	38.0	39.9	104.8	28.2	25.3	111.3
	1:4	19.0	19.3	101.2	15.2	12.7	119.7
	1:8	9.5	8.8	92.2	7.7	6.3	122.3
	1:16	4.8	4.4	92.6	4.0	3.2	125.3
	Mean			97.7	Mean		123.3
2	NA		117.9		272.6		
	1:2	58.9	60.1	102.0	137.4	136.3	101
	1:4	29.5	30.0	102.0	67.8	68.2	100
	1:8	14.7	15.1	102.0	32.9	34.1	96
	1:16	7.4	6.9	94	15.7	17.0	92
	Mean			100.0	Mean		97.3
3	NA		245.1		383.1		
	1:2	122.6	125.8	103	198.9	191.6	103.8
	1:4	61.3	62.0	101	97.2	95.8	101.4
	1:8	30.6	30.8	100	49.9	47.9	104.2
	1:16	15.3	14.4	94	22.7	23.9	94.6
	Mean			99.5	Mean		101.0

NA, not applicable; AFP, α -fetoprotein.

for at least one marker, and detection of both markers in parallel significantly increases the diagnostic efficiency for HCC (combination 82.35 vs. 80.80% and 75.90%, AFP-IgM and AFP, respectively).

DISCUSSION

In agreement with reports in the literature (3,8–10), the “gold standard” for the detection of HCC as reported by many groups varies around a value of approximately 40–65%. In our study the sensitivity of the marker AFP is only 45.76% with a low diagnostic efficiency (75.90%). This means that 54.24% of examined HCC patients may be considered false negatives. Therefore, a number of new biomarkers such as des- γ -carboxy prothrombin (DCP) and glypican-3 (GPC3) have been studied for their diagnostic accuracy and potential clinical applications. Elevated levels of DCP have been found in 35–53% of patients with HCC (11). Although some investigators have reported the usefulness of this marker compared with AFP (12), others found no improvement over AFP determination (13). GPC3 mRNA overexpression has been found in the liver in 75% of HCC but in only 3.2% of normal livers (14). The circulating GPC3 protein has been detected in 40–53% of patients with HCC (3,15).

Diagnosis of malignant hepatocellular liver tumors can be a challenge for pathologists and points out the need to develop relevant sensitive markers of malignant nodules. In this study, we showed that AFP-IgM, a immunocomplex of AFP, is a sensitive and specific serum marker

of HCC. Serum AFP-IgM levels were detectable in healthy persons, cirrhosis, hepatitis, and HCC patients. We observed significant differences for AFP-IgM between HCC patients compared with healthy persons as well as benign liver diseases group. There was no significant difference in AFP-IgM levels between healthy persons and benign liver disease groups.

To assess the diagnostic utility of tumor markers in HCC, the cutoff values chosen were the 95% percentiles of healthy persons. The diagnostic sensitivity of AFP-IgM was higher than that of AFP. AUC were higher for AFP-IgM than for AFP, which did not differ significantly from each other. Because of no correlation between AFP-IgM concentration and AFP levels in our study, co-determination of both markers may improve the indexes of diagnostic accuracy for liver cancer diagnosis. The combined use of AFP-IgM and AFP increased the sensitivity of detection to 72.88% without compromising specificity. The diagnostic efficiency of the combination was significantly higher than that for one marker only.

As europium chelates as labels were developed in 1980s (16,17), a TR-IFMA has been reported as an ideal immunoassay technique. This technique exhibits high sensitivity than those obtained with tritiated tracers and enzyme conjugated tracers, and offers an alternative to challenge the immunoassays using iodine-125 labels with the advantages of a stable, nonradioactive tracer (18). The fluorescence of the europium (Eu^{3+}) is long-lived and allows differentiation of the short-lived background fluorescence of biological material, plastics, and optics (19).

Fig. 2. Significantly elevated serum levels of AFP-IgM and AFP in control participants ($n = 218$ controls), patients with chronic hepatitis (CH) ($n = 63$ patients), patients with cirrhosis ($n = 60$ patients), and patients with hepatocellular carcinoma (HCC) ($n = 118$ patients) as detected by dual-label TR-IFMA. Data are presented as upper and lower quartiles (boxes), median value (horizontal line in box), and 10th and 90th percentiles (whisker lines). TR-IFMA, time-resolved immunofluorometric assay; AFP, α -fetoprotein.

The high specific activity of the label enables immunoassays with a more sensitive detection, while minimizing the nonspecific binding of the labeled bioaffinity molecule (20). Other favorable features of the Eu^{3+} complexes have been reviewed (21–23).

The fluorescent lanthanides (Eu^{3+} , Th^{3+} , Sm^{3+} , and Dy^{3+}) are ideal candidates for double-label or even multiple parameter assays, owing to their characteristic narrow banded emission lines. Their emissions are clearly distinguishable from each other with respect to both wavelengths and lifetimes, and this can be exploited in time-resolved fluorometry. $\text{Eu}^{3+}/\text{Sm}^{3+}$ pair has received more attention for several reasons: (a) Sm^{3+} and Eu^{3+} are excitable at the same wavelength (about 340 nm) in polystyrene wells; (b) they are extracted and chelated in the same medium; (c) their emission maxima are separated by 30 nm in the long wavelength region of

Fig. 3. ROC curves comparing the distribution of serum levels above the cutoff for AFP-IgM (cutoff = 127.25 AU/mL) and AFP (cutoff = 20 ng/mL) in HCC patients vs. those with healthy persons and benign liver disease. AFP, α -fetoprotein; HCC, hepatocellular carcinoma.

TABLE 4. Sensitivity, Specificity, and Positive and Negative Predictive Values of AFP/AFP-IgM in HCC Patients Depending on Control Group^a

Tumor marker	Sensitivity	Specificity	Efficiency	Pv+ ^b	Pv- ^b
AFP	45.76	92.42	75.90	63.50	85.50
AFP-IgM	63.56	90.22	80.80	65.50	89.60
AFP and AFP-IgM	72.88	85.090	82.35	58.50	91.58

^aThe cutoff limits are 7.23 ng/mL (AFP), 127.52 AU/mL (AFP-IgM), and 95% percentile for healthy persons ($n = 218$).

^bPv+, positive predictive value; Pv-, negative predictive value.

the spectrum; (d) their emission lifetimes differ significantly. This study demonstrates, for the first time, that simultaneous assay of AFP-IgM and AFP can be performed with a double-label assay with the use of Eu^{3+} and Sm^{3+} labels together with time-resolved fluorometry. The validity of dual-label TR-IFMA for AFP and AFP-IgM was confirmed by a good correlation between the results obtained by TR-IFMA and those by Hepa AFP-IgM ELISA and ElecSys 2010 system, respectively. Analytical recovery studies, parallelism (dilution of serum samples), and precision data for dual-label TR-IFMA were found to be satisfactory. The assay reagents were used in an instrument so that the assay could be performed on an automated platform. This novel assay also has many advantages of good precision at lower analyte concentrations, and a wider assay range compared with other methods. The wide measuring range would ensure accuracy of results and eliminate the need for any sample dilution.

In conclusion, we have developed a novel and reliable dual-label TR-IFMA for the measurement of AFP-IgM and AFP. We suggest that the use of a combination of

two markers in clinical practice could increase the accuracy of HCC diagnosis.

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