Clinical Evaluation of Serum Alpha-Fetoprotein–IgM Immune Complexes on the Diagnosis of Primary Hepatocellular Carcinoma

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We evaluated clinical significance of serum alpha-fetoprotein (AFP)-IgM immune complexes, in comparison with free AFP, on the diagnosis of primary hepatocellular carcinoma (HCC). Serum levels of AFP-IgM immune complexes and free AFP were determined by the ELISA method and electrochemiluminescence, respectively, in 103 healthy controls, 74 patients suffering from primary HCC, 27 patients suffering from liver cirrhosis, and 63 patients suffering from chronic hepatitis. The best cut-off value of AFP-IgM and free AFP for diagnosis of primary HCC were 300 AU/mL and 10 µg/L respectively, according to the area under the curve (AUC) in this study. The sensitivity of AFP-IgM and free AFP were 64.9 and 79.7%, and the specificity were 75.6 and 80.3%, respectively, when all cases of primary HCC were analyzed, and the AUC of free AFP was larger than that of AFP-IgM (0.85 vs. 0.72, $Z=3.21$). However, in case of primary HCC at early

stages (stages I and II) were analyzed, the AUC of AFP-IgM was larger than that of free AFP (0.91 vs. 0.82, $Z=1.73$), which demonstrated that the sensitivity of AFP-IgM and free AFP were 94.4 and 72.2%, and the specificity were 81.9 and 79.9%, respectively. When both AFP-IgM and free AFP were positive, the specificity of diagnosis of primary HCC was 89.1%, and the efficacy was 79.0%. It is concluded that either sensitivity or specificity of serum level of AFP-IgM immune complexes was higher than that of free AFP in the diagnosis of primary HCC at early stages. As there was false positive AFP-IgM existed in the patients suffering from cirrhosis and chronic hepatitis, the combination of free AFP and AFP-IgM could significantly increase specificity and decrease false negative and/or false positive in the primary HCC at early stages. *J. Clin. Lab. Anal.* 23:213–218, 2009.

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Key words: hepatocellular carcinoma; tumor marker; AFP-IgM immune complexes; alpha-fetoprotein

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and its incidence is rising in the world (1–4). About 55% of total primary HCC in the world occurred in China, because of high incidence of HBV infection and large population in China, and the mortality of primary HCC in China is about 20.4/100,000 (5,6). The main cause for the primary HCC in China is principally based on the HBV

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infection (7), whereas the HCV infection and alcohol are the major causes of liver cancer in the western countries (8–10). Primary HCC lacks typical clinical manifestations at the early stages, and it has higher malignancy with poor prognosis if diagnosis was delayed (11). However, it is not easy to have an early diagnosis (12,13) although the early diagnosis is of great importance for the further treatment of primary HCC. The diagnosis of primary HCC was obviously improved after the application of determination of serum-free alpha-feto-protein (AFP) that has been considered as a golden tumor marker of primary HCC (14), although certain patients suffering from primary HCC have very low or negative serum-free AFP.

Free AFP is a 70 kD glycoprotein that is mainly synthesized by the fetal yolk sac, fetal liver, and fetal intestines, and the half-life of free AFP is about 5–7 days. In normal adults' serum, in general, free AFP is negative or very low, but it may show positive reaction during the pregnancy (15,16). It has been suggested that serum levels of free AFP could be used as a prognostic indicator of the therapeutic response and survival in the germ cell tumors (17), and it has also been considered as a golden tumor marker for the diagnosis of primary HCC, although both false positive and false negative may exist in certain primary HCC patients (18). To improve the early detection and diagnosis of primary HCC, determination of serum levels of AFP-IgM immune complexes in primary HCC patients was applied. AFP-IgM immune complex is a newly discovered HCC tumor marker that is the complexes of AFP and IgM in the circulation (19), which has been suggested to be used as a sensitive and specific tumor marker on the diagnosis of primary HCC in recent years (20). In this study we investigated clinical significance of serum AFP-IgM complexes, in comparison to serum-free AFP, on the diagnosis of primary HCC in Chinese patients.

MATERIALS AND METHODS

Study Design and Blood Sample

Seventy-four patients suffering from primary HCC (60 men and 14 women, aged 29–83 years, the median age is 56 years), 27 patients suffering from moderate liver cirrhosis (20 men and 7 women, aged 38–69 years, the median age is 52 years), and 63 patients suffering from chronic hepatitis (51 men and 12 women, aged 19–72 years, the median age is 39 years) were subjected in this study. And 103 healthy subjects (76 men and 27 women, aged 20–73 years, the median age is 42 years) were included as controls. All primary HCC patients were diagnosed according to the UICC standards (21,22) and were confirmed pathohistologically. Five milliliter fasting venous blood were obtained from

patients and controls, and the serum were collected after centrifugation and were stored at -70°C until further examinations.

Determinations of Serum AFP-IgM Immune Complexes and Free AFP

Serum levels of AFP-IgM immune complexes were determined by the ELISA method by using the AFP-IgM kit that was purchased from XEPTAGEN Company, Italy (distributed by the Changzhou Xindi Commerce Company). Serum levels of free AFP were determined by using the electrochemiluminescence kit (Roche Company, Switzerland) on the Elecsys 2010 electrochemiluminescence instrument (Roche Company).

Statistical Analysis

Statistical analysis was performed with SPSS 13.0 software. All data was translated by the ranks cases step, the differences of serum levels of AFP-IgM immune complexes between patients and controls were analyzed by the one-way ANOVA followed the Newman–Keuls multiple comparison tests. A *P*-value less than 0.05 was considered as significant. The receiver operating characteristic (ROC) curve was applied for determining the diagnostic value and cut-off value of AFP-IgM and free AFP for the diagnosis of primary HCC. When serum levels of AFP-IgM or free AFP in the primary HCC patients were larger than the cut-off value, it could be considered as true positive (TP), and positive of other liver diseases was considered as false positive (FP), and negative of other liver diseases was considered as true negative (TN). Diagnostic efficacy evaluation was represented as sensitivity and specificity ($\text{Sensitivity} = \text{TP}/(\text{TP} + \text{FN}) \times 100\%$; $\text{Specificity} = \text{TN}/(\text{TN} + \text{FP}) \times 100\%$; $\text{Total effective rate} = \text{TP} + \text{TN}/(\text{TP} + \text{FP} + \text{TN} + \text{FN}) \times 100\%$).

RESULTS

Serum Levels of AFP-IgM Immune Complexes in the Primary HCC Patients and the Comparison of AFP-IgM and Free AFP in the Patients and in Normal Subjects

As shown in Table 1, the serum levels of AFP-IgM immune complexes in the primary HCC patients, relation to the gender, ages, tumor quantity etc., were analyzed. There was no statistical significant difference on patients' characters, except there were obvious correlations between serum levels of AFP-IgM immune complexes and tumor diameter and tumor stages. Serum levels of AFP-IgM and free AFP in patients suffering from primary HCC, cirrhosis or chronic hepatitis, and in healthy subjects were shown in Table 2. The serum

levels of AFP-IgM were statistically significant between primary HCC patients and healthy subjects ($Z = 8.35$, $P < 0.01$); however, there was no statistical significance between primary HCC patients and the patients suffering from cirrhosis or chronic hepatitis (P values were 0.504 and 0.074, respectively), which indicates that there is a false positive of AFP-IgM occurred in the patients suffering from cirrhosis and chronic hepatitis. As shown

TABLE 1. Serum Levels of AFP-IgM (AU/mL) in HCC Patients

Index	Cases	Median (min value–max value)	Z	P
Sex				
Men	60	408.53 (20.66–3,417.45)	0.70	0.482
Women	14	212.46 (115.30–2,942.37)		
Age (yr)				
≤ 50	21	378.34 (33.24–2,942.37)	0.15	0.882
> 50	53	394.75 (20.66–3,417.45)		
Tumor quantity				
1	42	370.92 (20.66–3,417.45)	1.18	0.239
> 1	32	413.00 (37.36–3,210.66)		
Tumor diameter (cm)				
≤ 3	10	1,049.46 (348.24–2,304.14)	3.15	0.002
> 3	64	332.14 (20.66–3,417.45)		
Clinical stage				
I–II	18	843.84 (348.24–2,304.14) ^{a,b}	11.02	0.000
III	31	270.59 (33.24–2,942.37) ^c		0.044
IV	25	332.14 (20.66–3,417.45)		0.008

^a $P = 0.000$, compared to stage IV.

^b $P = 0.008$, compared to stage III.

^c $P = 0.044$, compared to stage IV.

TABLE 2. Serum Levels of Free AFP and AFP-IgM Immune Complexes

Patients	Cases	Median	
		AFP-IgM (AU/mL)	AFP (μg/L)
HCC	74	401.59	384.66
Cirrhosis	27	424.57	6.04
Chronic hepatitis	63	273.55	13.82
Healthy	103	108.34	2.90

TABLE 3. Statistic Analyses of AFP-IgM and Free AFP between HCC Patients, Other Liver Diseases, and Controls

	Group	Cirrhosis		Chronic hepatitis		Healthy	
		Z	P	Z	P	Z	P
AFP-IgM	HCC	0.668	0.504	1.784	0.074	8.35	7.11×10^{-17}
	Cirrhosis			2.311	0.021	6.84	7.70×10^{-12}
	Chronic hepatitis					7.90	2.75×10^{-15}
AFP	HCC	4.121	3.78×10^{-5}	5.934	2.95×10^{-9}	9.46	3.23×10^{-21}
	Cirrhosis			1.180	0.238	5.27	1.47×10^{-7}
	Chronic hepatitis					4.70	2.63×10^{-6}

in Table 2, the serum levels of free AFP in primary HCC patients, cirrhosis patients, chronic hepatitis patients, and healthy subjects were compared, and the differences were all statistical significant (Table 3).

Diagnosis of Primary HCC According to the ROC Curves

According to the analysis of ROC curves, the best cut-off value for the AFP-IgM and free AFP for the diagnosis of primary HCC was set at 300 AU/mL and 10 μg/L, respectively (Fig. 1A and Table 4), in this study. In case of all primary HCC patients were analyzed, the area under ROC curve of free AFP was larger than that of AFP-IgM (0.85 vs. 0.72). And it was relevant between the areas on these two tumor markers for the diagnosis of primary HCC by the ROC curves ($r = 0.12$) and the effectiveness of free AFP was higher than that of AFP-IgM. However, when diagnostic analysis by ROC curves of AFP-IgM and free AFP for the primary HCC patients at early stages (stages I and II), the area under the ROC curve of AFP-IgM was 0.91 ± 0.021 (mean $\pm$ SD) and the area under the ROC curve of free AFP was 0.82 ± 0.047 (mean $\pm$ SD), which indicated that the validity of AFP-IgM was obviously higher than that of free AFP for the diagnosis of primary HCC at early stages (Fig. 1B). Calculating by the Pearson product-moment according to the report of Hanley and McNeil (23) showed that $Z = 1.73$, $P > 0.05$, which indicates that there was no correlation between the areas under ROC curves in these two tumor markers, showing that combination of the two markers was very complementary for the early diagnosis of primary HCC (Fig. 1B and Table 5).

Combination of AFP-IgM and AFP for the Diagnosis of Primary HCC

The sensitivity of AFP-IgM alone or AFP alone for the diagnosis of primary HCC was 64.9 and 79.7%, respectively. And the specificity was 75.6 and 80.3%, respectively. However, when combined AFP-IgM and

Fig. 1. ROC curves of AFP-IgM immune complexes and free AFP for the diagnosis of primary HCC. The figure represents the ROC curves of AFP-IgM immune complexes and free AFP on the diagnosis of primary HCC. Panel **A**, all cases of HCC patients were included; Panel **B**, only HCC patients at stages I and II were applied.

AFP examinations, if both are positive, the sensitivity and specificity were 52.7 and 89.1%, and the positive predictive value was 65.0% (Table 4). Although the sensitivity decreased, the specificity increased.

DISCUSSION

Since the clinical application of the determination of serum-free AFP, the accuracy and effectiveness of diagnosis of clinical primary HCC have been greatly improved (20,24). However, for the diagnosis of subclinical primary HCC, i.e., primary HCC at early stages (stage I and/or stage II), false negative could be up to more than 40% (25), and the occurrence of false negative of free AFP at the progressive liver cancer might also be around 15–20% (26). To decrease false positive or false negative, it is focused to find new specific and sensitive makers for the diagnosis of early primary HCC. An ELISA assay to examine serum circulating complexes of AFP and IgM has been clinically applied and has been suggested to be as a new sensitive and specific tumor marker for the diagnosis of primary HCC recently (19). In brief, the ELISA plate were precoated with rabbit anti-human free AFP antibody that could catch both free AFP and AFP-IgM complexes, and then peroxidase prelabelled goat anti-human IgM antibody was added to detect the AFP-IgM immune complexes. It has been suggested that the sensitivity and specificity for the diagnosis of primary HCC maybe improved by the determinations of serum AFP-IgM complexes together with serum-free AFP (19).

In this study, we systemically measured serum AFP-IgM immune complexes, in comparison with serum-free

TABLE 5. Comparison of AFP-IgM and Free AFP on the Diagnosis of Primary HCC Patients at Early Stages (Cases at Stages I and II Were Applied) (%)

Item	Sensitivity	Specificity	Accuracy
AFP-IgM	94.4	81.9	82.9
AFP	72.2	79.9	76.1

TABLE 4. Evaluation of AFP-IgM or AFP Alone and the Combination of AFP-IgM and Free AFP on the Diagnosis of Primary HCC (Total Cases Were Applied)

Evaluation index	AFP-IgM	AFP	AFP-IgM+AFP ^a	AFP-IgM+AFP ^b
Sensitivity	64.9 (%) (48/74)	79.7 (%) (59/74)	91.9 (%) (68/74)	52.7 (%) (39/74)
Specificity	75.6 (%) (146/193)	80.3 (%) (155/193)	66.8 (%) (129/193)	89.1 (%) (172/193)
Positive predictive value	50.5 (%) (48/95)	60.8 (%) (59/97)	51.5 (%) (68/132)	65.0 (%) (39/60)
Negative predictive value	84.9 (%) (146/172)	91.2 (%) (155/170)	95.6 (%) (129/135)	83.1 (%) (172/207)
Accuracy	72.7 (%) (194/267)	80.1 (%) (214/267)	73.8 (%) (197/267)	79.0 (%) (211/267)

^aDiagnosis of primary HCC was considered if either AFP-IgM or AFP was positive.

^bDiagnosis of primary HCC considered when both AFP-IgM and AFP were positive.

AFP, in the primary HCC patients, patients suffering from other liver diseases, and in normal subjects. The serum levels of AFP-IgM complexes in the non-HCC patients and in normal subjects were applied as controls. It is demonstrated that the best cut-off value of AFP-IgM and free AFP for the diagnosis of primary HCC were 300 AU/mL and 10 µg/L respectively, according to the ROC curve. Under the cut-off value, the sensitivity of AFP-IgM and free AFP for primary HCC were 64.9 and 79.7%, and the specificity were 75.6 and 80.3%, respectively. Whereas, for the diagnosis of primary HCC at early stages (stages I and II), the sensitivity of AFP-IgM and free AFP were 94.4 and 72.2%, and the specificity were 81.9 and 79.9%, respectively. This indicates that AFP-IgM is better than free AFP in the diagnosis of early primary HCC, although there is false positive reaction of AFP-IgM in patients suffering from cirrhosis and chronic hepatitis. It is best to determine both serum-free AFP and AFP-IgM, which may decrease the occurrence of false positive. In this study we demonstrated that serum levels of AFP-IgM could be related to tumor size. When tumor diameter was less than 3 cm, serum AFP-IgM levels were obviously higher than that of tumor diameter larger than 3 cm. The precise mechanism is unknown yet. It may be related to the cellular differentiation of earlier period of small HCC. This may also be considered as the reason why false positive AFP-IgM occurred in the patients suffering from cirrhosis and chronic hepatitis. As there was no correlation between the areas under ROC curves by measuring serum-free AFP and AFP-IgM, it is suggested that combined determination of these two markers has complementary for the diagnosis of HCC at early stages.

In this study we demonstrated that when examination of serum-free AFP alone for the diagnosis of primary HCC, the specificity was about 80.3%, and when combining AFP-IgM and free AFP, the specificity could be up to 89.1%, which indicates that the combination of free AFP and AFP-IgM complexes can improve the diagnosis of primary HCC. It has been reported that the differentiation of cells (27), tumor types (28), tumor position (29), and cell necrosis or degeneration (30), fibrolamellar HCC (31), and small HCC ≤3 cm (25) may cause false-negative of serum-free AFP. At stage I, the differentiation of small hepatoma carcinoma cells was close to normal cells (32), and these cells do not or only produce a little AFP, which leads to undetectable serum-free AFP. Low or negative serum-free AFP may easily cause misdiagnosis of primary HCC in clinic. Our study suggests that clinical application of measurement of serum AFP-IgM complexes may compensate the misdiagnosis, although there is false positive reaction in the patients suffering from cirrhosis and

chronic hepatitis. The combination of determination of both serum-free AFP and AFP-IgM may improve the sensitivity and specificity for the early diagnosis of primary HCC.

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