Serum AFP and AFP-L3 in Clinically Distinguished Hepatocellular Carcinoma from Patients with Liver Masses

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***Objective**: To determine the ability of alpha fetoprotein (AFP) and AFP-L3% serum level in discriminating hepatocellular carcinoma (HCC) from other types of liver mass.*
***Material and Method**: This study was performed according to a prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design. A total of 109 HCC patients and 51 patients with other types of liver mass were consecutively selected. The levels of AFP and AFP-L3% in their sera were measured.*
***Results:** AFP levels in serum significantly elevated while AFP-L3% levels significantly decreased in HCC patients (AFP: $p < 0.001$, AFP-L3%: $p < 0.001$). The area under the curve (AUC) of a receiver operating characteristic (ROC) curve analysis for the diagnosis of HCC of AFP and AFP-L3% was 0.71 and 0.67, respectively. In addition, the serum level of AFP-L3% was significantly different between the small (mass occupying lesser than 50% of liver volume) and large (mass occupying more than 50% of liver volume) HCC ($p = 0.040$).*
***Conclusion**: The diagnostic accuracy of serum AFP and AFP-L3% could provide them as candidate biomarkers to discriminate patients with HCC from patients with other types of liver mass. Serum AFP-L3% as a prognostic factor for HCC should be further evaluated in more details.*

***Keywords**: AFP, AFP-L3%, Hepatocellular carcinoma*

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Hepatocellular carcinoma (HCC) is one of the most leading causes of cancer that lead to death in Thailand[1]. The majority of patients with HCC present with liver mass that can be identified due to the widespread use of imaging modalities such as ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI)[2,3]. However, there are many types of liver mass including intrahepatic cholangiocarcinoma, liver metastatic lesions and benign tumors. These tumors present clinical symptoms similar to those of patients with HCC[4]. Because of the difference in the modality treatment for each kind of liver mass, the most important issue is to develop a reliable method to distinguish patients with HCC from other patients having different types of liver masses. In many cases, the imaging findings fail to identify HCC or non HCC lesion. Therefore, tissue biopsy is essential to the diagnosis and treatment of these liver masses. However, the risk of bleeding can be found 0.1-1% after liver biopsy[4,5]. To avoid liver biopsy, identifying tumor markers in the serum would be the alternative in the diagnosis of HCC and differentiate it from other kinds of liver masses.

Alpha fetoprotein (AFP) has been used in the standard serum screening test for the detection of HCC. However, AFP is often elevated in patients with chronic hepatitis infection in the absence of HCC[6,7]. Therefore, identification of the novel serum markers to differentiate HCC from the other liver mass is very important. Recently, the lens culinaris agglutinin (LCA)-reactive alpha-fetoprotein percentage of total AFP concentration [(AFP-L3/total AFP) x 100] or AFP-L3% has been used as a marker for early diagnosis, for assessment of therapeutic effects, and for predicting the prognosis of HCC. The AFP-L3% is reported to be more specific for the diagnosis of HCC than total AFP level[7,8]. However, there are no studies on the serum levels of AFP and

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AFP-L3% in patients presenting with liver mass (including HCC, cholangiocarcinoma, liver metastasis and benign liver mass).

Therefore, the aim of the present study is to determine serum concentrations of AFP and AFP-L3% in these patients. In addition, the diagnostic sensitivity, specificity and area under the receiver operating characteristic (ROC) curves of serum AFP and AFP-L3% for differentiating HCC from non HCC patients presenting with liver mass will be defined. To avoid selection bias, this study was designed as a nested case-control study that involved prospective collection of specimens before outcome ascertainment from a study cohort of patients presenting with liver mass. The serum values of AFP and AFP-L3% were assayed in a blinded fashion in sera from randomly selected case patients (HCC) and control subjects (non HCC patients presenting with liver mass) within the study cohort.

Material and Method

Study design

This study was performed at the Department of Surgery, Rajavithi Hospital. The Rajavithi Hospital ethics committee approved the study protocol. Sample size was determined on the basis of an expected area under the ROC curve of AFP-L3% serum levels for the diagnosis of HCC from other kinds of liver masses. There is no previous report on the accuracy of serum AFP-L3% in differentiating HCC from other types of liver masses. Therefore, we suggested that for the detection of serum AFP-L3% to be clinically helpful, the area under the ROC curve (AUC) of serum AFP-L3% should be higher than 0.70. By using a level of significance at 0.05 (two-sided) and a power of 0.95, we determined that a sample of 50 HCC patients was required for the study[9]. This study was carried out according to a prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design[10]. We prospectively included consecutive patients presenting with liver mass from Dec 2009 to Aug 2010. After diagnosis of these patients was validated, we randomly selected serum from HCC and non-HCC patients and measured the levels of serum AFP and AFP-L3%.

Serum collection and the measurement of serum biochemistry

After receiving informed consent from the patients, 5 ml of fasting peripheral venous blood was collected and the serum was separated and stored at -78°C within 2 hours. Assays for serum levels of albumin, globulin, AST, ALT, total and direct bilirubin and alkaline phosphatase (ALP) were conducted using routine automated methods in Rajavithi Hospital Pathological Laboratory.

Measurement of serum AFP and AFP-L3% levels

The serum levels of AFP and AFP-L3 were measured using an enzyme-linked immunosorbent assay (ELISA) kit (standards AFP and AFP-L3 were purchased from R&D Systems, Minneapolis, MN and Uscn life science Inc, Wuhan, China, respectively). The diluted serum samples were added in duplicate to 96-well plates coated with AFP or AFP-L3 antibody. After incubation at room temperature for two hours, the conjugated secondary antibody was added. The substrate solution was then added to the plates and incubated for one hour. Following termination of the reaction with the stop solution (1 M sulfuric acid), the optical density was measured at 450 nm using a spectrophotometric microplate reader. The concentration of AFP and AFP-L3 in each sample was calculated from a standard curve. In addition, the AFP-L3% was calculated according to the formula [(AFP-L3/total AFP) x 100]. The scientist examining these serum specimens was blinded to the patient's diagnosis.

Statistical analysis

Data are presented as mean $\pm$ SD. Comparisons between the quantitative variables were performed using Mann-Whitney U test or Student's t-test, as appropriate. Qualitative variables were reported as counts, comparisons between independent groups were performed using Pearson Chi-squared tests and p-values < 0.05 were considered statistically significant. An ROC curve was generated by plotting the sensitivity against 1-specificity, and the area under the curve with 95% confidence intervals (95% CI) was calculated. The optimal cut-off points for AFP and AFP-L3% were selected based on the ROC curve analysis. Sensitivity, specificity, positive predictive value and negative predictive values were calculated using a 2 x 2 table of the collected data.

Results

Patient characteristics

A total of 160 patients presenting with liver masses (from CT scan or MRI) were enrolled. Then, 109 HCC and 51 control (non HCC) cases were selected after the results of pathological biopsies were determined. The non HCC cases included cholangio-

carcinoma (20 cases), metastatic liver cancers (14 cases) and benign liver masses (17 cases). As shown in Table 1, no statistically significant differences in gender, age, serum bilirubin, albumin and globulin levels were found comparing between HCC and non HCC patients. However, the levels of serum AST and ALT in HCC patients were significantly higher than those in non HCC patients.

Serum levels of AFP and AFP-L3% in HCC and non HCC patients

Serum levels of AFP and AFP-L3% were compared between the two disease groups. The median values of serum AFP and AFP-L3% levels in HCC group were 119.4 ng/ml (range: 0.48-50,000 ng/ml) and 0.43% (range: 0-85.02%), respectively. The median values of serum AFP and AFP-L3% levels in non HCC group were 2.91 ng/ml (range: 0.21-15,000 ng/ml) and 7.23% (range: 0-95.84%), respectively. As shown in Fig. 1A and 1B, serum levels of AFP and AFP-L3% in HCC patients were significantly different from those in non-HCC patients (AFP and AFP-L3%: Mann-Whitney U test; $p < 0.001$).

Moreover, we also classified HCC patients into two groups: small (tumor occupies < 50% of liver volume) and large (tumor occupy > 50% of liver volume) HCC. The data shown in Fig. 1C demonstrate that serum AFP levels tended to increase while AFP-L3% values tended to decrease according to the progression of HCC. Although the serum AFP values in the small HCC were significantly higher than in control (Kruskal-Wallis test; $p = 0.001$), the values were not significantly different between small and large HCC (Kruskal-Wallis test; $p = 0.408$). On the other hand, AFP-L3% values were significantly different between small and large HCC (Kruskal-Wallis test; $p = 0.048$) (Fig. 1D).

Diagnostic accuracy of serum AFP and AFP-L3%

The diagnostic accuracy of serum AFP and AFP-L3% levels for differentiating HCC from non HCC patients was tested using an ROC curve analysis. The AUC of the ROC curve for serum AFP and AFP-L3% was 0.709 (95% CI; 0.618-0.801) and 0.68 (95% CI; 0.568-0.765), respectively (Fig. 2). The sensitivity and specificity and positive and negative predictive values for selected cut-off points of AFP and AFP-L3% are presented in Table 2.

Discussion

Hepatocellular carcinoma (HCC) is a common malignancy in Thailand. In clinical diagnosis of HCC, a mass lesion in the liver is detected by imaging modalities such as dynamic CT or MRI with contrast media. Typical HCC shows hypervascularity in the arterial phase and washout of contrast media in the portal-venous phase[2]. Cholangiocarcinoma and liver metastasis diseases can be presented with liver mass similar to HCC, but the treatment and prognostic of these patients are quite different from HCC patients. Other than imaging techniques, therefore, the method to differentiate HCC patients from others is still necessary. Identification of tumor markers in the serum is an alternative and success in this approach would be beneficial in the clinical management of these diseases.

Alpha fetoprotein (AFP) has been recognized as a serum marker for HCC since 1970s, when most patients with HCC were diagnosed at an advanced stage with clinical symptoms[7]. Previous studies indicated that AFP levels are elevated both in patients with HCC and in those with chronic liver diseases, and the AFP levels widely overlapped between the two

Table 1. Clinical characteristics of patients with control and HCC

	Control (n = 51)	HCC (n = 109)	p-value
Age (yr)	52.0 ± 11	54.0 ± 10	0.417
Sex (male: female)	31: 20	87: 22	0.021*
Albumin (mg/dL)	4.0 ± 0.68	4.1 ± 3.05	0.814
Globulin (mg/dL)	3.8 ± 0.69	4.1 ± 0.94	0.098
Direct bilirubin (mg/dL)	3.2 ± 7.56	2.1 ± 5.49	0.350
AST (U/L)	80.7 ± 77.83	140.8 ± 102.19	0.001*
ALT (U/L)	49.7 ± 41.96	67.54 ± 49.51	0.036*
ALP (U/L)	271.9 ± 320.21	261.8 ± 259.23	0.841

Values are represented as means ± SD, * Significant at $p < 0.05$, HCC = Hepatocellular carcinoma, AST = Aspartate Aminotransferase, ALP = alkaline phosphatase, AFP = Alpha fetoprotein

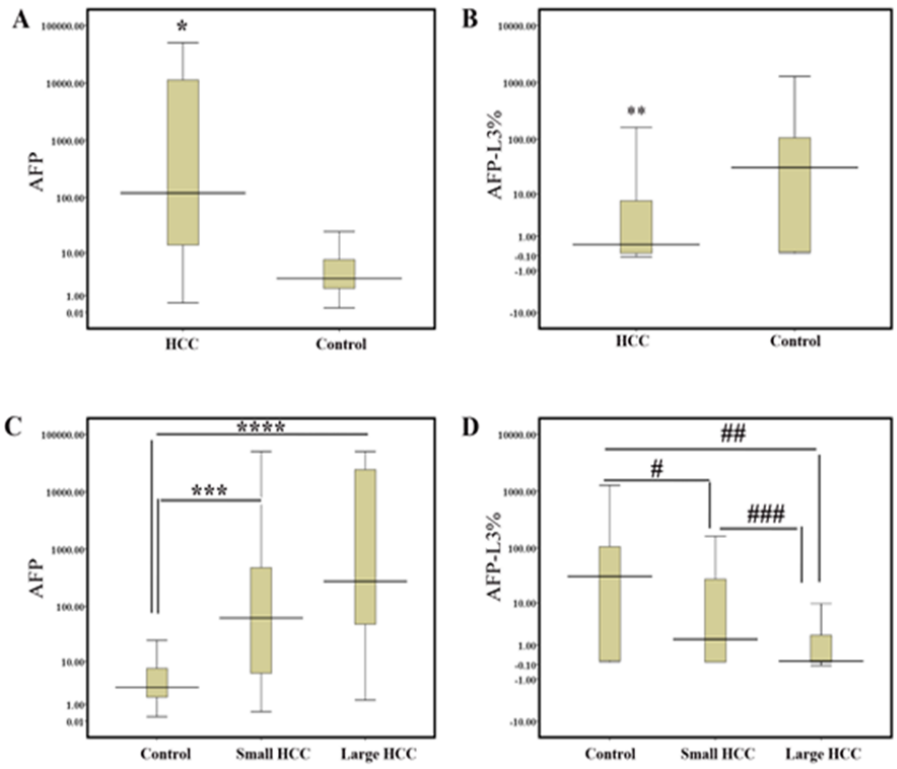

Fig. 1 (A) Box plots comparing levels of AFP and (B) AFP-L3% between HCC and control (other kinds of liver mass patients) are illustrated. (C) Box plots comparing levels of AFP and (D) AFP-L3 between small and large HCC and control are illustrated. Levels of AFP and AFP-L3% are presented with the log data to accommodate the wide range. (*; Mann-Whitney U; $p < 0.001$ compared to control, **; Mann-Whitney U; $p < 0.001$ compared to control, ***; Kruskal-Wallis test; $p < 0.001$ compared to control, ****; Kruskal-Wallis test; $p < 0.001$ compared to control, #,##; Kruskal-Wallis test; $p < 0.001$ compared to control, ###; Kruskal-Wallis test; $p = 0.04$ compared between small and large HCC)

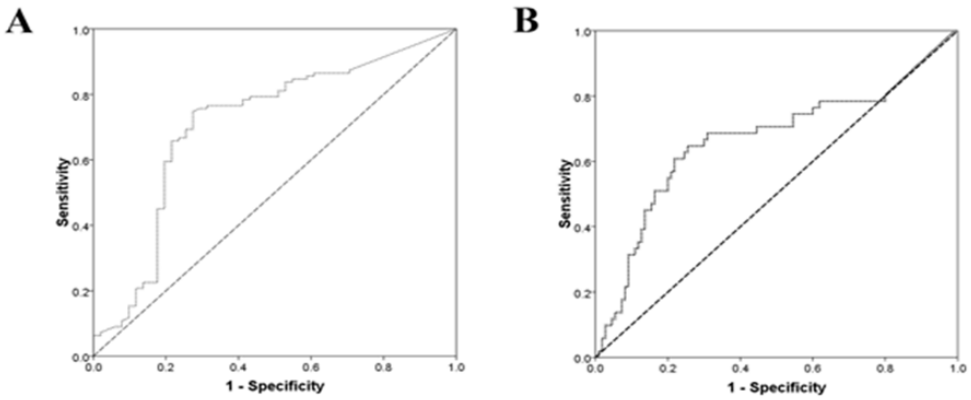

Fig. 2 (A) ROC curve analyses of AFP for the diagnosis of HCC from non HCC liver mass patients. (B) ROC curve analyses of AFP-L3% for the diagnosis of non HCC liver mass patients from HCC. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, is presented by receiver operating characteristic (ROC) curve analysis.

Table 2. Performance of the biomarkers for the diagnosis of HCC

Tumor Markers (cut-off value)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	LR+ (%) (95% CI)	LR- (%) (95% CI)
AFP (50 ng/ml)	61 (51-70)	82 (72-93)	3.43 (1.88-6.33)	0.48 (0.37-0.62)
AFP (100 ng/ml)	51 (42-61)	84 (74-94)	3.28 (1.69-6.35)	0.58 (0.46-0.72)
AFP-L3% (<10%)	78 (70-86)	59 (45-72)	1.90 (1.35-2.67)	0.37 (0.24-0.57)
AFP-L3% (<30%)	84 (77-91)	51 (37-65)	1.71 (1.27-2.28)	0.32 (0.19-0.53)

LR+; positive likelihood ratio, LR-; negative likelihood ratio, CI; confidence interval, AFP = Alpha fetoprotein

groups[11,12]. To date, AFP-L3, an isoform of AFP, has been proposed as a complement or substitute for AFP in diagnosis of HCC. AFP can be fractionated by affinity electrophoresis into 3 glycoforms: L1, L2 and L3 based on the reactivity with the lectin *Lens culinaris* agglutinin (LCA). AFP-L3 binds strongly to LCA via an additional α 1-6 fucose residue attached at the reducing terminus of N-acetylglucosamine[7,8]. To the best of our knowledge, this is the first study to assess serum AFP and AFP-L3% levels in patients presenting with liver mass including HCC, cholangiocarcinoma, liver metastasis and benign liver mass. The strength of the present study is the implementation of PRoBE designs to avoid the problems of bias that may affect statistics of diagnostic test[10]. We collected serum from all patients presenting with liver mass. This procedure ensured that biases related to differences in sample collection and handling would be avoided. Here, we demonstrated the statistically significant difference of serum AFP and AFP-L3% levels comparing between HCC patients and non HCC patients. When comparing the AUC of the ROC curve for AFP and AFP-L3% with a chance value equal to 0.5 (the worst value of AUC of ROC), both the AUC of the ROC for AFP and AFP-L3% were significantly higher than 0.5. These findings are consistent with the previous studies that reported the levels of AFP and AFP-L3% were significantly different between patients with HCC and those without HCC (chronic hepatitis patients)[7,13,14].

In addition, we also demonstrated that AFP-L3% was significantly different between large HCC (tumor mass occupy > 50% liver volume) and small HCC patients (tumor mass occupy < 50% liver volume). This finding is inconsistent with the previous study of Marrero et al which reported that AFP-L3% level was not different between early and advanced HCC[8]. We suggested that the inconsistent finding is from the difference in the definition of the size of HCC. In our study, we used Okuda staging systems[15,16] which defined the size of HCC as lesser or greater than 50% of liver volume while the study of Marrero et al defined the size of HCC as smaller or bigger than 3 cm.

In conclusion, this study demonstrated that the serum values of AFP and AFP-L3% may be used as a biomarker to discriminate HCC from non HCC disease patients. In addition, further study should be performed to evaluate the role of serum AFP-L3% as a prognostic factor for HCC.

Potential conflicts of interest

Rajavithi reserch fund.

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การใช้ ซีรัม AFP และ AFP-L3 ช่วยในการจำแนกผู้ป่วยโรคมะเร็งตับชนิด hepatocellular carcinoma ออกจากผู้ป่วยที่มีก้อนที่ตับซึ่งไม่ได้เกิดจาก hepatocelluar carcinoma

สมบูรณ์ ทรัพย์วงศ์เจริญ, กวิญ ลีละวัฒน์, สอาด ตรีพงษ์กรุณา, ศิริลักษณ์ นารอง

วัตถุประสงค์*: การศึกษานี้จัดทำเพื่อศึกษาระดับ AFP และ AFP-L3% ในการใช้แยกโรคมะเร็งตับชนิด hepatocellular carcinoma (HCC) ออกจากผู้ป่วยที่ตรวจพบว่ามีก้อนที่ตับซึ่งไม่ได้เกิดจาก HCC*

วัสดุและวิธีการ*: คณะผู้นิพนธ์ทำการเก็บซีรัมจากผู้ป่วยซึ่งตรวจก้อนเนื้อที่ตับ CT-SCAN หรือ MRI ซึ่งภายหลังได้รับการวินิจฉัยว่าเป็นมะเร็งตับชนิด HCC จำนวน 109 ราย และ ก้อนที่ตับชนิดอื่นที่ไม่ใช่ HCC จำนวน 51 ราย ที่มาตรวจที่กลุ่มงานศัลยศาสตร์ โรงพยาบาลราชวิถี ตั้งแต่เดือนธันวาคม พ.ศ. 2552 ถึงเดือนสิงหาคม พ.ศ. 2553 จากนั้นทำการวัดปริมาณ AFP และ AFP-L3 % จากซีรัมผู้ป่วยดังกล่าว*

ผลการศึกษา*: ผลการศึกษาระดับซีรัม AFP ในผู้ป่วยโรฆมะเร็งตับชนิด HCC และระดับซีรัม AFP-L3 ในผู้ป่วยโรคมะเร็งตับชนิด HCC มีปริมาณต่ำกว่าในผู้ป่วยที่มีก้อนในตับซึ่งไม่ได้เกิดจาก HCC อย่างมีนัยสำคัญทางสถิติ นอกจากนั้นยังพบว่าระดับซีรัม AFP-L3 ในผู้ป่วยมะเร็งตับชนิด HCC ที่มีขนาดเล็ก (น้อยกว่าร้อยละ 50 ของปริมาตรตับ) มีความแตกต่างจากผู้ป่วยมะเร็งตับชนิด HCC ที่มีขนาดใหญ่ (มากกว่าร้อยละ 50 ของปริมาตรตับ) อย่างมีนัยสำคัญทางสถิติ*

สรุป*: การใช้ AFP และ AFP-L3% สามารถใช้ช่วยแยกผู้ป่วยโรคมะเร็งตับชนิด HCC จากผู้ป่วยที่มีก้อนตับที่ไม่ใช่ HCC ได้ และระดับ AFP-L3 อาจใช้เป็นตัวทำนายโรคในผู้ป่วยโรคมะเร็งตับ HCC ได้*