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ORIGINAL ARTICLE

Serum fucosylated paraoxonase 1 as a potential glycobiomarker for clinical diagnosis of early hepatocellular carcinoma using ELISA Index

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Abstract Serum paraoxonase 1 (PON1) is highly fucosylated in hepatocellular carcinoma (HCC) compared with liver cirrhosis (LC). Herein, lectin ELISA using Aleuria aurantia lectin (AAL) was established, which specifically measured optical density (OD) value of serum fucosylated PON1. PON1 protein ELISA was applied simultaneously. ELISA Index (OD value of fucosylated PON1/OD value of protein PON1) was introduced to indicate PON1 fucosylation level on its protein level (Fuc-PON1). ELISA Index in training group (90 LC and 90 HCC) was measured and area under the ROC curve (AUROC) was 0.803 with 80 % of sensitivity and 64.4 % of specificity in distinguishing early HCC from LC. Within training group, AFP^- HCC (20/90) exhibited better AUROC (0.850), higher sensitivity (90 %) and specificity (75 %) than AFP^+ HCC (70/90). An independent testing set (20 LC and 20 HCC) validated the model and 17 HCC patients were successfully predicted. Meanwhile, serum AFP of 43 LC and 43 HCC had an AUROC of 0.760 with sensitivity of 79.1 % and specificity of 53.5 %. Thus, Fuc-PON1 may serve as a glycan biomarker for distinguishing early HCC from LC patients even with low AFP levels.

Keywords Fucosylated paraoxonase 1 · Hepatocellular carcinoma · Aleuria aurantia lectin · ELISA Index · Glycobiomarker

Introduction

HCC is the major primary liver cancer with high mortality and its overall 5-year survival rate remains less than 5 % [1]. Current methods for the diagnosis of HCC rely on serological markers such as alpha fetoprotein (AFP) and imaging techniques such as CT-ultrasonography and magnetic resonance imaging [2]. However, the diagnosis for HCC is far from satisfactory because of limited sensitivity and specificity [3]. Recently, the LCA-reactive fraction of AFP (AFP-L3) has become a more specific glycan biomarker and the US Food and Drug Administration (FDA) approved it as a diagnostic index for HCC [4, 5]. Glycans play an important role in a variety of biological processes like cell adhesion, molecular trafficking and clearance, receptor activation and signal transduction. Further, alterations in cell surface glycan are associated with various physiological and pathological status including malignant transformation and metastasis [6, 7]. Therefore, better serological markers such as glycobiomarkers, are urgently needed for improving the diagnosis of HCC.

PON1 is a calcium-dependent hydrolase protein synthesized mainly in the liver and secreted into the circulatory system [8]. It contains 355 amino acids residues and three identified glycosylation sites, but the structures and functions of its carbohydrate content remain unknown [9–11]. It was reported that PON1 played an active role in the regulation of oxidative stress, fibrosis and hepatic cell apoptosis in chronic liver diseases [12]. Moreover, PON1 has been discovered to be a novel diagnostic biomarker for microvascular invasion in HCC [13]. In our previous study, PON1 was found to be highly

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fucosylated and sialylated in HCC patients compared with that in LC patients [14].

There are many types of fucosylated modifications such as core fucose, Lewis X, Lewis A, H1 and H2 [15]. Core fucose (Fuc) residues in N-glycan are attached in α1-6 linkage to the inner, asparagines-linked GlcNAc residue. Lewis X, Lewis A, H1 and H2 structure consist of the same numbers of the same kinds of monosaccharides, but the linkage position and linkage type of the Gal residues and the Fuc residue are different. Fucosylation is one of the most important oligosaccharide modifications involved in cancer [16]. For example, increasing in fucosylated haptoglobin levels have been reported in several types of cancer such as HCC and pancreatic cancer. The fucosylated N-glycans at each glycopeptide of haptoglobin were reported to be increased significantly in HCC patients [17, 18]. Noriko Okuyama *et al.* showed that the core fucosylation as well as α1-3/α1-4 fucosylation was increased in haptoglobin of pancreatic cancer [19].

A common method of glycan structure determination is to use plant or animal lectins that bind specifically to certain oligosaccharides. Fucosylated structure could be recognized by several kinds of lectin including AAL, Ulex europaeus agglutinin (UEA), Lens culinaris agglutinin (LCA) and Aspergillus oryzae lectin (AOL). AAL recognizes α1-2/α1-3/α1-4 and α1-6 fucose, UEA recognizes α1-2 fucose linked *N*-acetyllactosamine (type2H), LCA recognizes the native form of α1-6 fucose with a mannose arm and AOL recognizes α1-6 fucose more specifically [16, 20]. Our previous results showed the AAL-reactive fraction of PON1 in HCC increased dramatically [14] and Jung-Mo Ahn *et al.* also used AAL to analyze fucosylated PON1 alterations in small cell lung cancer [21]. Thus, in this study, ELISA Index by AAL affinity binding ability was established to determine Fuc-PON1 for early HCC diagnosis or prediction.

Materials and methods

Preparation of specimens

The serum specimens were obtained from the First Affiliated Hospital of Guangxi Medical University. Informed consent was obtained from each patient and this study was approved by the Research Ethics committee of First Affiliated Hospital of Guangxi Medical University and the Institutional Review Board of the National Cancer Center. The pathological data of the patients were provided in Table 1. All serum samples were collected using standard protocol and stored at −80 °C until use.

Table 1 Characteristics of LC and early HCC patients

Group	LC[c]	HCC[c]
Number	110	110
Sex (F/M)	17/93	11/99
Age (years)	56.7±10.6	57.9±9.7
HBV DNA (copy)[a]	1.7×10^5 (1.8×10^3~6.9×10^5)	2.6×10^4 (1.0×10^3~1.7×10^5)
AFP (ng/ml)[b]	68.5 (0.73~1464.1)	817.3 (0.88~14222.0)
$HbsAg^+$ (%)	100	100
AST (U/L)	70.7 (17.7~505.3)	76.1 (4.7~353.5)
ALT (U/L)	48.4 (11.2~286)	47.2 (13.3~204.2)
TNM[d]	/	I (n=36), II (n=74)

The values supplied in Table 1 were means with SD or range

[a] HBV DNA was detected with fluorescent quantitative PCR (FQ-PCR) and has a detection limit of sensitivity of approximately 1×10^3 genome equivalents *per* mL

[b] AFP (alpha fetoprotein) was determined using standard kits (Abbott Labs) and 20 ng/mL was considered the upper limit of normal

[c] LC and HCC diagnosis was confirmed by ultrasound imaging and biopsy

[d] The sixth edition of TNM classification of the American Joint Committee on cancer was used to select early HCC patients (TNM I, II)

ELISA Index for Fuc-PON1

Each well of 96-well plate 1 (lectin ELISA) was coated with 100 ng PON1 monoclonal antibody (R&D Systems, MN, USA) in a 100 μL volume at 37 °C overnight and blocked with 3%BSA in PBS (pH7.2-7.4) for 1 h at RT. Then, the coated antibodies on the plate were reacted with oxidation buffer (100 mM $NaIO_4$, 50 mM citric acid, pH 4.0) at 4 °C for 1 h. Serum samples were diluted 30-fold with PBS and 50 μL of each diluted serum sample was applied to each well. After 2 h incubation, the plate was rinsed with PBST (0.05%Tween20 in PBS, pH7.2-7.4) 4 times and 1 μg/mL biotinylated AAL (Vector, Burlingame, CA) was added for 2 h at RT. HRP-streptavidin was applied to each well followed by TMB working solution and stop solution. OD value of Fuc-PON1 at 450 nm was measured with NanoQuant infinite M200 (TECAN, Switzerland).

Each well of 96-well plate 2 (protein ELISA) was coated with 200 ng PON1 monoclonal antibody in a 100 μL volume at 37 °C overnight and blocked with blocking buffer (1%BSA, 0.05%NaN_3 in PBS, pH7.2-7.4) for 1 h at RT. Serum samples were diluted 200-fold with solution (1 mM EDTA, 0.5%Triton X-100 in

PBS, pH7.2-7.4) and 100 μL of each diluted serum sample was applied to each well. After 2 h incubation, the plate was rinsed with PBST (0.05 % Tween20 in PBS, pH7.2-7.4) 4 times and 600 ng/mL detection antibody (R&D Systems, MN, USA) was added for 2 h at RT. HRP-streptavidin was applied to each well followed by TMB working solution and stop solution. OD value of protein PON1 at 450 nm was measured with NanoQuant infinite M200 (TECAN, Switzerland).

ELISA Index which was calculated as OD value of fucosylated PON1, divided by OD value of protein PON1 was used to show PON 1 fucosylation level on its protein level.

AFP ELISA assay

The AFP level was also measured in this study. The ELISA kit for AFP was purchased from R&D Systems (MN, USA). ELISA assay was performed according to manufacturer's instructions and the absorbance value was also read at a wavelength of 450 nm.

Statistical analysis

Statistical comparisons were calculated using *t*-test and $p<0.05$ was taken as statistically significant. Receiver operating characteristic (ROC) curves were performed using SPSS 19 and the cutoff was determined as the point in the ROC curve that maximizes the value of sensitivity plus specificity. Diagnostic model was also obtained by binary logistic regression.

Results

Evaluation of AAL ELISA assay

For the lectin ELISA, it was critical to block the binding between lectins and the glycans of PON1 antibodies and other serum glycoproteins. $NaIO_4$ was used to oxidize the glycans of antibodies to prevent their reaction with lectins. The average intensity of AAL ELISA incubated with serum PON1 was more than 10 times stronger than the average intensity of it incubated with PBS, recombinant PON1 and recombinant GP73 after oxidation (Table 2). Serum glycoproteins AFP and AGP were also used to measure PON1 antibody capture efficiency and the average intensity of AAL ELISA incubated with serum PON1 was also more than 10 times stronger than the average intensity of it incubated with AFP and AGP (Table 2). Thus, the reaction between AAL and the glycans of spotted antibodies and the reaction between AAL and other serum glycoproteins were successfully blocked.

To assess the accuracy of this method, three independent AAL ELISA using different pooled sera were measured independently and each pooled sera were measured three times. As shown in Table 3, the coefficient of variance (CV%) were 7.6, 0.4 and 4.9 %, respectively. The average coefficient of variance was 4.3 %, which indicated the reproducibility of the AAL ELISA was good.

Table 3 Three independent measurements of AAL ELISA using different pooled sera sample

Pooled Sera Sample (n=3)[a]	OD_{450} [b]	CV%
1	0.9909±0.075	7.6
2	0.9690±0.004	0.4
3	2.2639±0.112	4.9

[a] Each pooled sera sample was mixed with three different sera

[b] Each pooled sera was measured three times

Measurement of sera samples using ELISA Index

Before sera samples measurement, standard curves were generated for protein ELISA and AAL ELISA separately in order to obtain the concentration range of ELISA Index assay. Figure 1a showed a linear proportionality between OD values of standard recombinant PON1 and their concentrations in protein ELISA. The drawn curve is fitted using an equation of $R^2=1.000$. For this protein ELISA system, the linear detection limit range of protein PON1 was from 0 to 10,000 pg/mL. Figure 1b showed OD values of fucosylated PON1 plotted against concentration for captured sera PON1 for different dilutions of sera samples in AAL ELISA. The linear

Table 2 Measurement of binding between AAL and the glycans of spotted antibodies and as well as other serum glycoproteins after oxidation

Sample	PBS	Recombinant PON1	Recombinant GP73	AFP	AGP	SeraPON1
OD_{450}	0.1462	0.1310	0.1727	0.1261	0.1313	2.1177

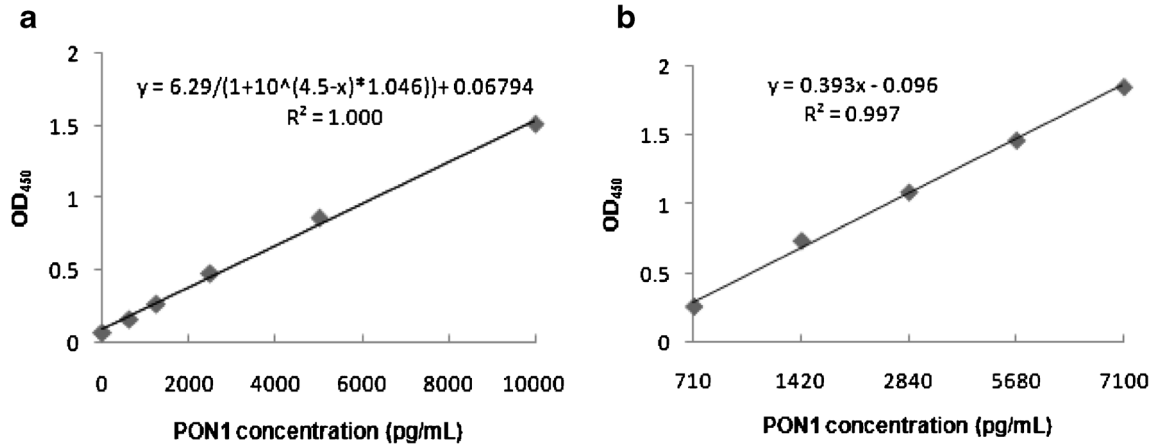

Fig. 1 The detection limit range of ELISA Index. **a** Standard curve for protein PON1 in protein ELISA system, which showed a linear proportionality between OD values of standard recombinant PON1 and their concentration. **b** Standard curve for fucosylated PON1 in AAL ELISA system, which showed OD values of fucosylated PON1 plotted against concentration for captured sera PON1 for different dilutions of sera samples

relation was obtained using an equation of R^2=0.997. For this AAL ELISA system, the linear detection limit range of fucosylated PON1 was from 710 to 7100 pg/mL. Thus, different dilution ratios of AAL ELISA (30-fold dilution) and protein ELISA (200-fold dilution) were chosen respectively. In training group, 90 serum samples from LC patients and 90 serum samples from HCC patients were assayed using AAL ELISA and

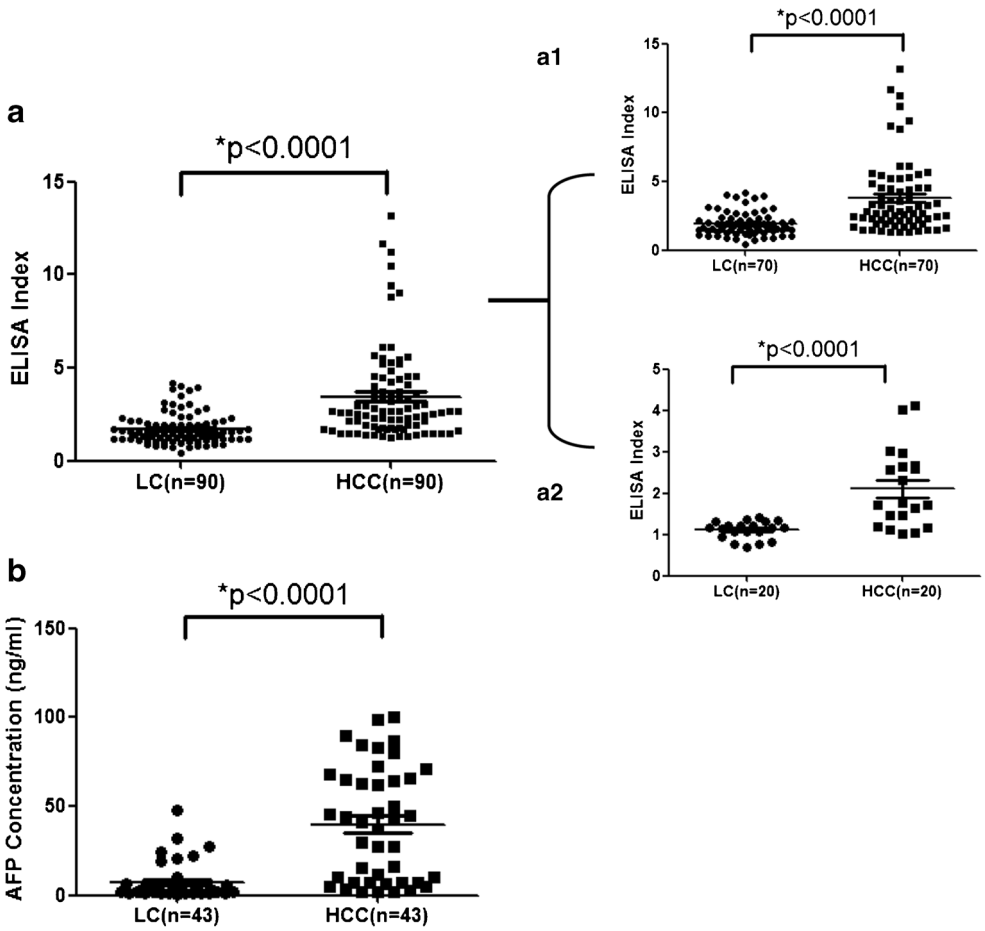

Fig. 2 Scatter plot graphs of Fuc-PON1 intensity and AFP concentration. **a** Scatter plot of Fuc-PON1 intensity (training group, 90 LC and 90 HCC sera samples) using ELISA Index. (a1) Scatter plot of Fuc-PON1 intensity (AFP+ sera samples in training group, 70 LC and 70 HCC sera samples) using ELISA Index. (a2) Scatter plot of Fuc-PON1 intensity (AFP- sera samples in training group, 20 LC and 20 HCC sera samples) using ELISA Index. **b** Scatter plot of AFP concentration using R&D Systems ELISA kit

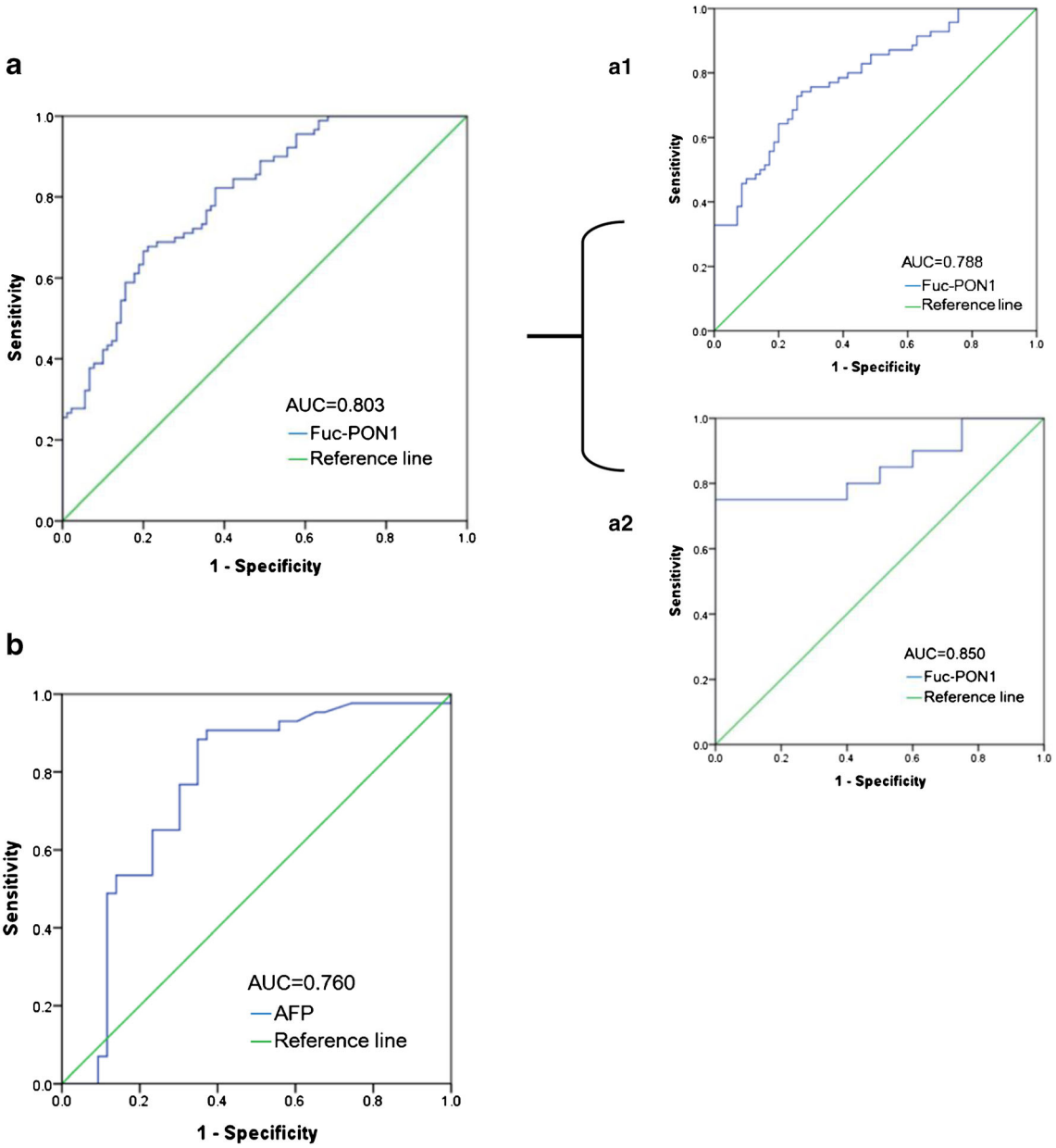

Fig. 3 ROC curves of Fuc-PON1 and AFP. **a** ROC curves of Fuc-PON1 (training group, 90 LC and 90 HCC sera samples) using ELISA Index. (a1) ROC curves of Fuc-PON1 intensity (AFP+ sera samples in training group, 70 LC and 70 HCC sera samples) using ELISA Index. (a2) ROC curves of Fuc-PON1 intensity (AFP- sera samples in training group, 20 LC and 20 HCC sera samples) using ELISA Index. **b** ROC curves of AFP, as the detection of 43 HCC with respect to 43 LC patients

protein ELISA and then ELISA Index was calculated as OD value of fucosylated PON1, divided by OD value of protein PON1 without absolute concentrations of fucosylated PON1 and protein PON1.

Table 4 Diagnosis values of AFP and Fuc-PON1 in differentiating early HCC from LC patients

Biomarker	Sample numbers	AUROC	Sensitivity	Specificity	Accuracy
AFP	43LC;43HCC	0.760	79.1 %	53.5 %	66.3 %
Fuc-PON1	90LC;90HCC	0.803	80.0 %	64.4 %	72.2 %
Fuc-PON1 (AFP+)	70LC;70HCC	0.788	80.0 %	61.4 %	70.7 %
Fuc-PON1 (AFP-)	20LC;20HCC	0.850	90.0 %	75.0 %	82.5 %

Diagnosis value of Fuc-PON1 in distinguishing HCC from LC patients

As shown in Fig. 2a, ELISA Indices of 90 HCC patients were significantly higher than 90 LC patients (3.43±2.48 *vs.* 1.72±0.82; $p<0.0001$) and the ROC curve for the diagnosis of HCC patients was presented in Fig. 3a whose AUROC was 0.803. In this training group, the sensitivity and specificity of Fuc-PON1 (cut-off of 1.69) for the diagnosis of HCC were 80 and 64.4 %, respectively (Table 4). The predictive model of Fuc-PON1 was as follows:

$$P = \exp(2.353 + 1.046^{*}\ \text{Fuc-PON1}) \Big/ [1 + \exp(2.353 + 1.046^{*}\ \text{Fuc-PON1})]$$

An independent testing group of 40 patients (20 HCC and 20 LC patients) was used to validate the differential diagnostic performance of ELISA Index and 17 HCC patients were successfully predicted.

Considering different AFP levels, the AFP concentrations of training group were split into two groups: AFP^{+} (those with > 20 ng/mL, 70 LC and 70 HCC serum samples) and AFP^{-} (those with <20 ng/mL, 20 LC and 20 HCC serum samples). The AUROC, sensitivity, specificity and accuracy of AFP^{-} were 0.850, 90, 75 and 82.5 %, respectively, which indicated Fuc-PON1 may be a glycan biomarker in the diagnosis of early HCC even with low AFP levels (Table 4).

For comparison, AFP in 43 LC and 43 HCC patients was also measured using R&D Systems ELISA kit. Concentrations of AFP of 43 HCC patients were significantly higher than 43 LC patients (39.78±31.63 *vs.* 7.12±10.40; $p<0.0001$; Fig. 2b) and the AUROC was 0.760 (Fig. 3b) with sensitivity of 79.1 % and specificity of 53.5 % (Table 4).

Discussion

In this study, ELISA Index of Fuc-PON1 was evaluated for observing its applicability to clinical diagnosis of early HCC patients. AAL ELISA and protein ELISA were assayed using the same serum specimens simultaneously, and then ELISA Index was calculated as OD value of fucosylated PON1, divided by OD value of protein PON1 to obtain Fuc-PON1.

At first, AAL ELISA system was evaluated because of the binding between AAL and the glycans of antibodies and the binding between AAL and other serum glycoproteins. Oxidation condition and antibody capture efficiency were confirmed and the repeated measurements indicated the accuracy and specificity of AAL ELISA system. Protein ELISA was often used in many previous reports, thus, the optimized conditions were shown directly without detailed explanations.

Next, the linearity of AAL ELISA and protein ELISA were obtained at different dilutions. The results suggested that a 30-fold serum dilution yielded suitable OD values in AAL ELISA while 200-fold serum dilution yielded suitable OD values in protein ELISA. Thus, the sera specimens were diluted separately and investigated by AAL ELISA and protein ELISA, respectively. In this study, ELISA Index (OD value of fucosylated PON1/OD value of protein PON1) was used in diagnosis of HCC patients, because we found the changes of protein PON1 level itself or the changes of its AAL-reactive fraction itself were not stable. However, the ratios of fucosylated PON1 to the total serum PON1 were always significantly higher in HCC patients than those in LC patients.

Commercial AFP ELISA kit was also employed to reevaluate ELISA Index of Fuc-PON1 system. The diagnostic data demonstrated that ELISA Index of Fuc-PON1 for HCC could rival AFP, even better in AUROC. Moreover, the AUROC, sensitivity, specificity and accuracy of AFP^{-} were 0.850, 90, 75 and 82.5 %, respectively, which suggested Fuc-PON1 may be a glycan biomarker in the diagnosis of early HCC even with low AFP levels. It was reported that the AUROC for fucosylated PON1 between the healthy people and limited disease stage of small cell lung cancer samples was 0.773 and that between the healthy people and extensive disease stage of small cell lung cancer samples was 0.910 [21]. Clinic application of lectin-antibody ELISA kit was also reported for measuring fucosylated haptoglobin in pancreatic cancer [22]. ELISA method could improve high-throughput analysis and lectin could help quantify glycoforms of protein precisely. Combination of lectin and ELISA method has application prospect for validating marker candidates that were produced by glycomic techniques. Additionally, it was reported that AAL used for lectin ELISA had superior performance compared to LCA and UEA [23].

Thus, the results of the present study provided evidences for clinic application of ELISA Index of Fuc-PON1. In addition, the evaluation of this assay demonstrated its superior diagnosis of early HCC patients that proved serum Fuc-PON1 was a potential glycobiomarker for distinguishing early HCC from LC patients, even for HCC patients with low AFP levels.

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Conflict of interest The authors have declared no conflict of interest.

Compliance with Ethical Standards The serum specimens were obtained from the First Affiliated Hospital of Guangxi Medical University. This study was approved by the Research Ethics committee of First Affiliated Hospital of Guangxi Medical University and the Institutional Review Board of the National Cancer Center. Informed consent was obtained from each patient.

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