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ORIGINAL ARTICLE

Multiple lectin assays for detecting glyco-alteration of serum GP73 in liver diseases

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Abstract Serum GP73 is a functional resident Golgi type II membrane protein with three potential N-glycosylation sites. In this study, we used multiple lectin assays to analyze glycan patterns of serum GP73 and evaluated its diagnostic value for distinguishing hepatocellular carcinoma (HCC) from liver cirrhosis (LC). Firstly, Antibody overlay lectin microarray and lectin blot were performed to observe altered glycans of GP73. Fucosylated structures were found to increase significantly in LC compared with HCC patients. Then, AAL ELISA assay using ELISA Index was utilized to measure fucosylation level of GP73 on its protein level (Fuc-GP73). ELISA Indices of 54 LC and 54 HCC patients was obtained and the area under the ROC curve (AUC) was 0.807 with a sensitivity of 85.2 % and a specificity of 63.0 % (cutoff of 3.182). In addition, combining Fuc-GP73 and AFP-L3 greatly improved the diagnostic accuracy (AUC=0.953) and the diagnostic values were 94.4 % sensitivity at 88.9 % specificity. These data indicated that multiple lectin assays could contribute to pre-clinical evaluation of focused glycoprotein and Fuc-GP73 could act as a potential glycobiomarker complementary to AFP-L3 for discrimination of HCC from LC patients.

Keywords GP73 · Lectin · Fucosylation · Liver diseases · Glycobiomarker

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Introduction

HCC is one of most common cancers with leading cause of cancer mortality globally [1, 2]. Cirrhosis is the most important risk factor in HCC development. The diagnosis for HCC is usually at an advanced stage, and this delays the timely therapy which leads to a bad prognosis [3]. α-fetoprotein (AFP) is the currently used clinical biomarker for HCC. Unexpectedly, it increases in patients with chronic hepatitis and/or cirrhosis in the absence of HCC [4]. Hence, one of the AFP isoforms (AFP-L3) which has a high binding affinity to lectin *Lens Culinaris Agglutinin*, is reported to be a more specific glyco-biomarker. It was approved by the US Food and Drug Administration (FDA) as a biomarker for HCC in 2005 [5–8] and has been commonly used in Japan.

Protein glycosylation is an important posttranslational modification that contributes to disease development and progression [9, 10]. More evidences have demonstrated that glycosylation changes of serum proteins are associated with cancer progression [11–13]. β6GlcNAc side chain branching catalyzed by *N*-acetylglucosaminyltransferase V displays prometastatic effect, whereas β4GlcNAc (bisecting GlcNAc) synthesized by *N*-acetylglucosaminyltransferase III is antimetastatic [14]. In our previous study, glycosylation site occupancy of Asn241 of haptoglobin β chian was observed to change significantly in HCC compared with LC and chronic

hepatitis B virus (HBV) patients. Moreover, the fucosylated glycans of this protein at each glycopeptide were increased significantly in LC and HCC patients [15–17]. Thus, relevance between glycans and human disease has been a major focus of researches and potential biomarkers for cancers could be identified based on the glycosylation changes.

GP73 was originally described as a resident Golgi type II transmembrane protein expressed primarily in epithelial cells of many human tissues [18] and it was found to be strongly up-regulated in hepatocytes from patients with viral and non-viral liver disease [19]. Block *et al.* used targeted glycoproteomics technology to identify serum glycoproteins that correlated with liver cancer and found GP73 was elevated and hyperfucosylated in animals with HCC [12]. Drake *et al.* applied lectin capture strategies combining with mass spectrometry analysis for the discovery of serum glycoprotein biomarkers and also showed the altered level of fucosylated GP73 was in the serum of patients with varying HCC disease states [20]. Ma *et al.* established a precise core fucosylation-glycosylation site identification strategy with UHPLC LTQ-Orbitrap Elite under low- and high-normalized collision energy conditions. Using this strategy, they identified 357 unique core fucosylation-glycosylation sites from 209 proteins which also included GP73 [21].

In this study, antibody overlay lectin microarray was utilized to identify the glycosylation status of serum GP73 in LC and HCC patients. This system is considered to be an ideal approach to achieve differential glycan analysis of focused glycoprotein [22, 23]. With this method, specific signals corresponding to GP73 glycans represented as lectin affinity profiles were obtained with the aid of specific antibodies in a rapid, reproducible, and high-throughput manner. Then, lectin blot analysis was used to confirm significant glycan alterations of serum GP73 in LC and HCC patients. Finally, lectin-ELISA assay using ELISA Index was applied to measure glycan level of GP73 on its protein level. We focused on the glyco-alteration of serum GP73 using multiple lectin assays. Antibody overlay lectin microarray and lectin blot analysis allowed us to identify the most useful lectin for validation of glycan changes of serum GP73. Lectin-ELISA assay using this most useful lectin enable pre-clinical evaluation of serum GP73 as a glycobiomarker candidate.

Methods

Preparation of specimens

The serum specimens used in this study 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 clinicopathological data of the patients were provided in Table 1. All serum samples were collected using standard protocol and stored at −80 °C until use.

Immunoprecipitation and antibody overlay lectin microarray

GP73 was immunoprecipitated using Pierce Direct IP Kit (Thermo, Rockford, USA) from pooling sera of 10 LC and 10 HCC patients, respectively. The equal amount of purified GP73 were diluted to100 μL with PBS containing 1 % Triton X-100 (PBSTx) and then applied to the lectin microarray containing 6 repeated spots of 50 lectins. After incubation at 20 °C for 12 h, an excess amount of blocking glycoprotein (20 μg nonlabeled human serum polyclonal IgG) was added to the glass slide. After incubation at room temperature for 30 min, the glass slide was washed 3 times with PBSTx. 100 μL biotinylated GP73 antibody solution in PBSTx was applied to the array, then incubated at room temperature for 1 h. After 3 washes with PBSTx, 60 μL Cy5-labeled streptavidin (Invitrogen, MD, USA) solution in PBSTx was added to the array and incubated at room temperature for another 30 min. The glass slide was rinsed with PBSTx and scanned by an evanescent-field fluorescence scanner (Capitalbio, Beijing, China). The data was obtained by the Array Pro Analyzer, version 4.5 (Media Cybernetics). The signal intensity of spot was considered valid when the ratio of spot intensity/background intensity>1.5, then, the intensity of each spot was calculated by subtracting the background intensity from the signal intensity. The intensity value for each lectin was the average intensity of 6 repeated spots.

Lectin blot analysis

Purified serum GP73 from 10 HCC and 10 LC patients individually was separated by 10 % SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, USA) using conventional method. The membrane was blocked with 3 % BSA in TBST (TBS containing 1 % Tween20) at room temperature for 1 h, followed by incubating with biotinylated lectin overnight. After 3 times wash with TBST, the membrane was further incubated with avidin-HRP at room temperature for 1 h, and then washed with TBST 3 times. The same purified GP73 was also applied for western blot. The membrane was blocked with 5 % skim milk in TBST at room temperature for 1 h, followed by incubating with GP73 antibody (Santa Cruz, Texas, USA) overnight. The membrane was further incubated with HRP-conjugated antibody at room temperature for 1 h, and then washed with TBST 3 times. Immunoreactive protein bands were visualized with

Table 1 Characteristics of LC and HCC patients

Group	LC [c]	HCC [c]
Number	74	74
Sex (F/M)	20/54	15/59
Age (years)	59.3±10.9	47.5±11.6
HBV DNA (copy)[a]	1.2×10^5 (1.5×10^3~6.3×10^5)	2.6×10^4 (1.2×10^3~1.6×10^5)
AFP (ng/mL)[b]	94.6 (0.73~1464.1)	6150.0 (1.56~60500)
$HBsAg^+$ (%)	100	100
AST (U/L)	72.5(22.6~274.1)	102.6 (11~1630)
ALT (U/L)	55.0 (11.5~337.7)	76.9 (10~1120)

[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

the lectin blot bands at the same time using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ).

ELISA Index of Fuc-GP73

GP73 protein ELISA kits were obtained from Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

Each well of 96-well plate 1 (AAL ELISA) was blocked with 3 % BSA in PBS (pH7.2–7.4) for 1 h at room temperature. 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. 100 μL non-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 room temperature. HRP-streptavidin was applied to each well followed by TMB working solution and stop solution. OD value of fucosylated GP73 was measured at 450 nm with NanoQuant infinite M200 (TECAN, Switzerland).

Each well of 96-well plate 2 (protein ELISA) was blocked with blocking buffer (1 % BSA, 0.05 % NaN_3 in PBS, pH7.2–7.4) for 1 h at room temperature. Serum samples were diluted 1.4-fold with PBS and 100 μL of 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 detection antibody was added for 2 h at room temperature. HRP-streptavidin was applied to each well followed by TMB working solution and stop solution. OD value of protein GP73 was measured at 450 nm with NanoQuant infinite M200.

Fifty four HCC and 54 LC patients were measured individually by AAL ELISA and protein ELISA at the same time. ELISA Index defined as OD value of fucosylated GP73, divided by OD value of protein GP73 was used to obtain Fuc-GP73.

AFP-L3 ELISA assay

The AFP-L3 level was also measured in this study. The ELISA kit for AFP-L3 was purchased from Abnova (Taipei, Taiwan). 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 maximized the value of sensitivity plus specificity.

Results

Glycan alterations of serum GP73 by antibody overlay lectin microarray and lectin blot

For differential glycan analysis of GP73 in LC and HCC patients, an equal amount of immunoprecipitated GP73 from pooling sera of LC and HCC was subjected to the antibody overlay lectin microarray containing 50 different lectins (Fig. 1a). We obtained lectin affinity glycan profiles with 10 lectins, namely, AAL, ACL, Con A, DSA, ECL, LCA, LEL, NML, PSA and STL (Fig. 1b) and their sugar-binding specificity was shown in Table 2. Among them, the binding affinity capacity of 5 lectins (AAL, ACL, LCA, PSA and STL) were

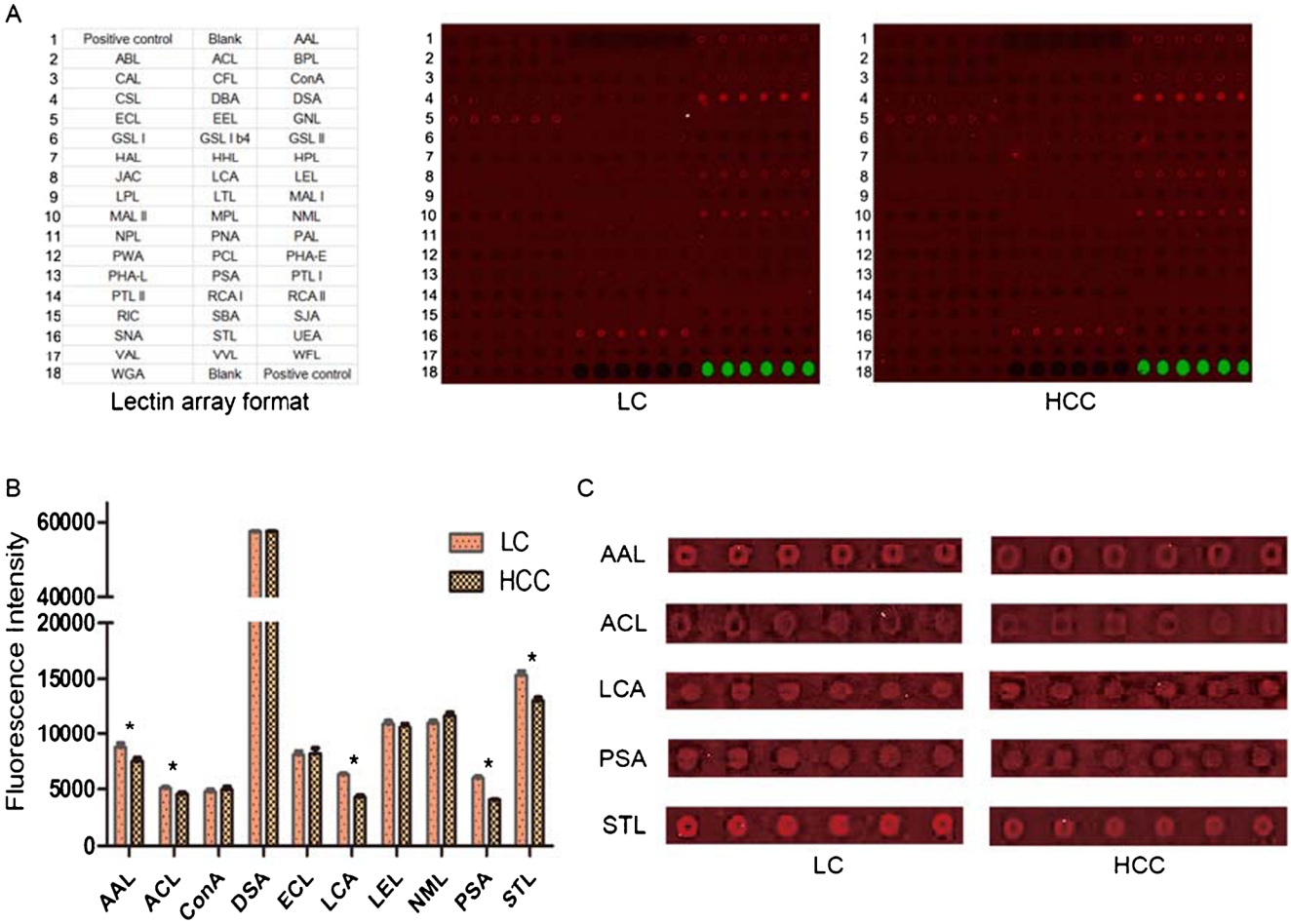

Fig. 1 The glycan pattern of GP73 detected by antibody overlay lectin microarray. **a** The lectin microarray contains 50 lectin spots with different binding specificities (*left*). Typical glycan profiles of GP73 from LC and HCC patients were shown on the right. **b** Signals of ten lectins were detected from GP73 glycan in both LC and HCC patients. Differences ($p<0.05$) between two groups in the signal patterns of 5 lectins were indicated (*). **c** Enlarged spots of the 5 statistically different lectins (AAL, ACL, LCA, PSA and STL) in LC and HCC patients

reduced in HCC patients with significant difference as $p<0.05$ (Fig. 1c).

Then, lectin blot for GP73 of individual serum were performed to confirm the signal pattern of lectin microarray correlated with HCC and LC patients. Lectin/GP73 ratio which was calculated as band intensity in lectin blot, divided by band intensity in western blot was used to represent the glycan level on the protein level. Figure 2 showed AAL, LCA and PSA preferentially bound to the GP73 of LC compared with HCC patients,

Table 2 Ten lectins reacted with GP73 using antibody overlay lectin microarray and their sugar-binding specificities

Lectins	Monosaccharide specificity	Preferred glycan structure (terminal epitope)
DSA (Datura stramonium agglutinin)	GlcNAc	$(GlcNAc\beta4)_n$, triantennary, tetraantennary N-glycans
ACL (Amaranthus caudatus lectin)	Gal	Galβ3GalNAc
ECL (Erythrina cristagalli lectin)	Gal	Galβ4GlcNAc
STL (Solanum tuberosum lectin)	GlcNAc	$(GlcNAc\beta)_n$, $(GlcNAc\beta4MurNAc)_n$ (peptidoglycan backbone)
LEL (Lycopersicon esculentum lectin)	GlcNAc	$(GlcNAc\beta4)_n$, $(Gal\beta4GlcNAc)_n$ (polylactosamine)
NML (Naja mossambica lectin)	Man	High-Man including Manα6(Manα3)Man, exopolysacchride
PSA (Pisum sativum agglutinin)	Fuc/Man	Fucα6GlcNAc, High-Man
Con A (Concanavalin A)	Man	High-Man including Manα6(Manα3)Man
LCA (Lens culinaris agglutinin)	Fuc/Man	Fucα6GlcNAc, High–Man
AAL (Aleuria aurantia lectin)	Fuc	Fucα6GlcNAc (core Fuc), Fucα3(Galβ4)GlcNAc (Le^x)

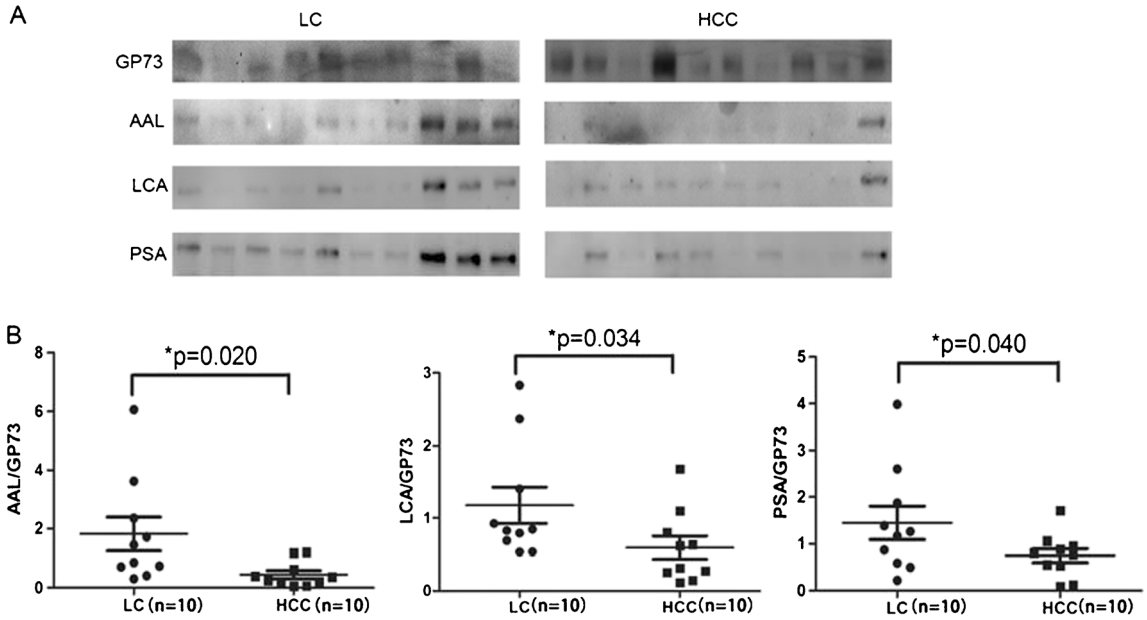

Fig. 2 Confirmation of glycan alterations of purified GP73 in LC and HCC patients. **a** Immunoprecipitated GP73 of 10 LC and 10 HCC patients were analyzed by lectin blot and western blot. **b** Scatter plot of lectin/GP73 (band intensity in lectin blot/band intensity in western blot)

and these three lectins all bind to α-linked fucose related structures.

Measurement of Fuc-GP73 using AAL ELISA and protein ELISA

It was reported that AAL had a superior performance with the lectin-ELISA assay [24]. AAL was selected as the best lectin for pre-clinical evaluation of fucosylated GP73. For AAL ELISA, $NaIO_4$ was used to oxidize the glycans of antibodies to block the AAL binding to the antibodies. For protein ELISA, routine experimental procedures were performed. Before sera samples measurement, standard curves were generated for AAL ELISA and protein ELISA to evaluate the detective limitation (data not shown). Considering the low concentration of GP73 in serum samples, 1.4-fold diluted serum and non-diluted serum were chosen for protein ELISA and AAL ELISA, respectively. Additionally, for assessing the accuracy of AAL ELISA, three independent measurements using different pooled sera were carried out independently and each pooled sera were measured three times. The average coefficient of variance (CV%) was 4.6 % and it indicated the reproducibility of AAL ELISA was good (Table 3).

Diagnostic value of Fuc-GP73 complementary to AFP-L3

Fifty four serum samples from LC and 54 serum samples from HCC patients were assayed using ELISA Index which was calculated as OD value of fucosylated GP73, divided by OD value of protein GP73. ELISA Indices of Fuc-GP73 in 54 LC patients were significantly higher than 54 HCC patients (Table 4) and the ROC curve for the diagnosis of HCC patients was presented in Fig. 3 whose AUC was 0.807. The sensitivity and specificity of Fuc-GP73 (cutoff of 3.182) for the diagnosis of HCC were 85.2 and 63.0 %, respectively.

Because it was a negative biomarker in distinguishing HCC from LC, we also measured AFP-L3 values of the same specimens using commercial AFP-L3 ELISA kit. As shown in Fig. 3, the combination of Fuc-GP73 and AFP-L3 significantly increased the diagnostic sensitivity and specificity of HCC. Combined measurement of Fuc-GP73 and AFP-L3 increased the AUC to 0.953, with a high sensitivity of 94.4 % and specificity of 88.9 %.

Discussion

GP73 is a resident Golgi-specific membrane protein and few studies focused on its glycosylation alterations in LC and HCC patients, especially on discrimination of HCC from LC patients. For GP73 protein level, it was reported to be a

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

Pooled sera sample (n=3)[a]	OD450[b]	CV%
1	0.7573±0.0659	8.70
2	1.1115±0.0189	1.70
3	0.8361±0.0294	3.51

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

[b] Each pooled sera sample was measured three times

Table 4 Comparison of Fuc-GP73 in 54 LC and 54 HCC patients

Group	Cases	Fuc-GP73 (Mean±SD)
LC	54	6.89±5.54
HCC	54	2.57±1.35
t-test		$p<0.0001$

reliable biomarker for early HCC diagnosis because of high expression in HCC [25–27]. However, some studies found that serum GP73 levels in patients with LC were significantly higher than those in HCC [28, 29]. Thus, the protein expression of serum GP73 may be unstable and the relevance between GP73 glycosylation and liver disease has attracted our attention.

Glycosylation changes of serum glycoproteins were often associated with the development of cancers. In the present study, glycosylation status of GP73 was assessed in LC and HCC patients by antibody overlay lectin microarray and the result showed increased AAL, ACL, LCA, PSA and STL reactive fraction of GP73 in LC patients. Then, lectin blot analysis was used to validate the result of antibody overlay lectin microarray and the changes of fucosylated structures were significant. Thus, ELISA Index based on AAL ELISA and protein ELISA was used to measure Fuc-GP73 in LC and HCC patients. Moreover, AFP-L3 was obtained and the combination of Fuc-GP73 and AFP-L3 were assumed to generate better sensitivity and specificity for diagnosis of HCC, which was proven in our study.

In our previous studies, fucosylated glycan of haptoglobin was much higher in samples from patients with LC and HCC than in those from patients with HBV and healthy controls

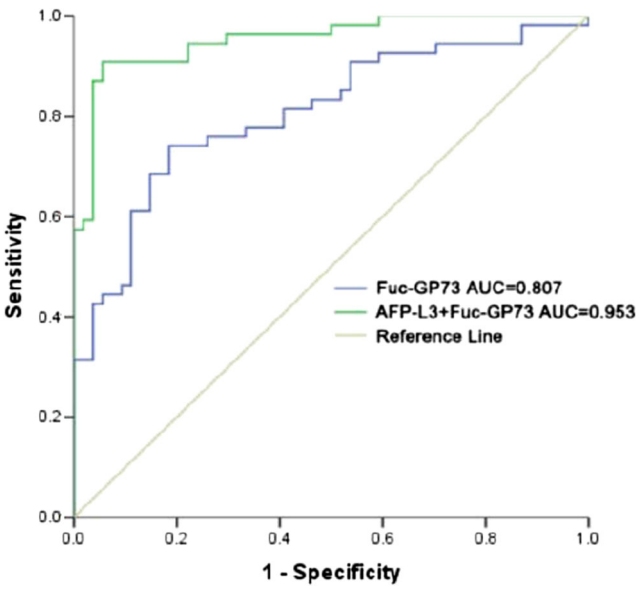

Fig. 3 ROC curves of Fuc-GP73 and combination of Fuc-GP73 and AFP-L3 for HCC diagnosis. The AUC for the combination of Fuc-GP73 and AFP-L3 in HCC diagnosis was 0.953 (95 % CI 0.916 to 0.991)

[16]. Asazawa *et al.* also found that patients with LC and HCC showed significantly increased fucosylated haptoglobin in comparison to chronic hepatitis (CH) patients or healthy volunteers [30]. These results indicate fucosylation is also altered in LC which is a chronic disease of the liver where the normal liver architecture is replaced by fibrotic scar tissue, and is associated with an eventual decline of liver function.

LC patients instead of healthy people were recruited as the controls for the discovery of HCC glycobiomarkers in this study. However, Fuc-GP73 was also measured in 20 healthy controls and the mean value was 1.75. Fucosylated structures seemed to be increased in LC and HCC patients compared with normal controls, and significantly higher in LC than those in HCC patients. This phenomenon may be related with biological function of GP73 in liver disease especially LC. However, many functions of GP73 are still unknown and it was reported that the secretion pathway for GP73 would be different from those for other glycoproteins in liver [6]. Thus, more deep research of its glycobiology is needed.

The antibody overlay lectin microarray is a high-throughput system to analyze the glycan pattern of target glycoprotein. For low abundance of GP73, this method could screen out the differential glycosylation pattern of GP73 between LC and HCC patients. Norton *et al.* detected that at least two of three potential sites of N-linked glycosylation were occupied on most molecules of GP73 secreted from cultured hepatoma cells. Furthermore, this work mentioned the oligosaccharides added to secreted GP73 were mostly bi-antennary with core fucose, with a smaller fraction of tri- and tetra-antennary structures [31]. Fucosylation is commonly used as a glycobiomarker as previously reported in haptoglobin [32]. In our study, AAL, ACL, Con A, DSA, ECL, LCA, LEL, NML, PSA and STL reactive fractions of GP73 were found in LC and HCC serum. Especially, the abundance of DSA fraction which binds (GlcNAcβ4)n, tri-antennary and tetra-antennary N-glycans structure was the highest. Thus, there may be changes of glycan levels of GP73 especially tri- and tetra-antennary structures between serum and cultured hepatoma cells.

Some common clinical utilized serological biomarkers for cancer diagnosis included PSA (prostate cancer), CA125 (ovarian cancer), CEA (colon cancer) are glycoprotein. Only their protein levels are clinically detected and different glycoforms may increase the diagnostic potential of these molecules. For example, AFP-L3 has been shown to improve the specificity for HCC compared with total AFP levels, as AFP could be elevated in pregnancy, hepatitis, and LC [33]. Combination of ELISA method and lectin could be used for clinical application of glycobiomarkers in large scale and the use of lectin ELISA kit in detecting haptoglobin fucosylation was reported [34]. It was also used to analyze serum glycosylation changes of other target proteins [24, 35].

In conclusion, Fuc-GP73 was elevated significantly in LC compared with HCC patients using multiple lectin assays, which were useful tools to detect the glycosylation changes of target glycoproteins. Combination of Fuc-GP73 and AFP-L3 greatly increased the sensitivity and specificity for HCC diagnosis, and this could be used as a practical means for distinguishing HCC from LC patients.

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Author contributions Y.L. K.G. and S.Z. conceived the idea. K.J., S.Z., S.S. and W.L performed the experiments, X.Q. supplied the sera specimens, K.J. and S.Z. analyzed the data, S.Z. wrote the manuscript.

Competing financial interests The authors declare no competing financial interests.

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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