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The Clinical Significance of GP73 in Immunologically Mediated Chronic Liver Diseases: Experimental Data and Literature Review

Mingjie Yao[1] · Leijie Wang[1] · Patrick S. C. Leung[2] · Yanmei Li[3] · Shuhong Liu[4] ·
Lu Wang[1] · Xiaodong Guo[4] · Guangde Zhou[4] · Ying Yan[1] · Guiwen Guan[1] ·
Xiangmei Chen[1] · Christopher L. Bowlus[5] · Tianhui Liu[6] · Jidong Jia[6] ·
M. Eric Gershwin[2] · Xiong Ma[3] · Jingmin Zhao[4] · Fengmin Lu[1]

Abstract There is significant void in establishing validated non-invasive surrogate biomarkers of liver fibrosis/cirrhosis in chronic liver diseases (CLD). Golgi protein 73 (GP73) has been suggested as a potential serum marker for the diagnosis of hepatocellular carcinoma (HCC). However, significant background of cirrhosis could have accounted for the elevation of serum GP73 in HCC. In this study, we have taken advantage of a well-defined extensive cohort of 3044 patients with either compensated cirrhosis ($n = 1247$), decompensated cirrhosis ($n = 841$) or pre-cirrhotic CLD ($n = 956$) and our ability to quantify serum GP73 to define the potential of serum GP73 as a biomarker of liver cirrhosis/fibrosis in CLD. The diagnostic value of GP73 was compared with aspartate aminotransferase-to-platelet ratio index (APRI), fibrosis index based on four factors (FIB-4) and liver stiffness measurement (LSM). Immunohistochemical analysis was performed to measure hepatic GP73 expression. Receiver operating characteristic curve analysis demonstrated that serum GP73 had a good diagnostic potential for compensated cirrhosis regardless of etiology. The diagnostic performance of GP73 is better than APRI, FIB-4 and similar with LSM, especially in patients with severe inflammation, steatosis and cholestasis. Notably, in patients of autoimmune liver diseases, non-alcoholic fatty liver disease and viral hepatitis, serum GP73 also exhibited diagnostic value for advanced fibrosis as well as cirrhosis. Furthermore, there is also a gradual increase in GP73

Mingjie Yao, Leijie Wang, Patrick S. C. Leung and Yanmei Li contributed equally to this work.

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✉ Patrick S. C. Leung
   psleung@ucdavais.edu

✉ Xiong Ma
   maxiongmd@hotmail.com

✉ Jingmin Zhao
   jmzhao302@163.com

✉ Fengmin Lu
   lu.fengmin@hsc.pku.edu.cn

1   Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, 100191 Beijing, People's Republic of China

2   Division of Rheumatology/Allergy and Clinical Immunology, School of Medicine, The University of California, Davis, CA 95616, USA

3   State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of Digestive Disease, 145 Middle Shandong Road, 200001 Shanghai, People's Republic of China

4   Department of Pathology and Hepatology, Beijing 302 Hospital, 100039 Beijing, People's Republic of China

5   Division of Gastroenterology and Hepatology, School of Medicine, The University of California, Davis, CA 95616, USA

6   Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing Key Laboratory of Translational Medicine in Liver Cirrhosis & National Clinical Research Center of Digestive Diseases, 100050 Beijing, People's Republic of China

expression with disease progression from mild fibrosis to cirrhosis. In conclusion, GP73 is an effective and reliable serological marker for the diagnosis of advanced fibrosis and prediction of appearance of cirrhosis.

Keywords Fibrosis · Chronic liver disease · Liver stiffness · FIB-4 · APRI

Abbreviations

GP73	Golgi protein 73
HCC	hepatocellular carcinoma
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ALP	alkaline phosphatase
GGT	gamma glutamyl transpeptidase
TBA	total bile acid
ALB	albumin
PA	prealbumin
qPCR	quantitative real-time PCR
PLT	platelet count
IQR	interquartile range
APRI	AST-to-platelet ratio index
FIB-4	fibrosis index based on four factors
LSM	liver stiffness measurement
AILDs	autoimmune liver diseases
NAFLD	non-alcoholic fatty liver disease
CLD	chronic liver diseases
HBV	hepatitis B virus
CHB	chronic hepatitis B
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
CHC	chronic hepatitis C
H & E	hematoxylin and eosin
HRP	horseradish peroxidase
ROC	receiver operating characteristic curve
AUC	the area under the receiver operating characteristic curve
PPV	positive predictive value
NPV	negative predictive value
BMI	Body Mass Index

Introduction

Chronic liver diseases (CLD) pose significant health problems worldwide and account for about 1.5 million deaths annually [1–4]. Chronic hepatitis B (CHB), chronic hepatitis C (CHC) and non-alcoholic fatty liver disease (NAFLD), as well as primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH), the autoimmune-mediated CLD, attribute to the end-stage liver diseases in China and worldwide [5–9]. Despite various means of clinical interventions in CLD, hepatic transplant remains the ultimate effective treatment for patients with end-stage CLD [10].

Liver fibrosis is a significant predictor of disease progression and clinical outcomes in patients with CLD [7, 11]. Since fibrosis or even early cirrhosis may be reversible with early clinical intervention [12–15], reliable methods for evaluating and monitoring the stage of liver fibrosis and cirrhosis are critical for optimal management of CLD [16, 17]. Liver biopsy has long been regarded as the "gold standard" for staging liver fibrosis and compensated cirrhosis [18]. However, sampling error, cost, potential medical complications and inter-observer variability have limited the use of liver biopsy [19–23]. Therefore, novel non-invasive biomarkers for accurate diagnosis and disease staging in CLD patients are necessary [24].

Non-invasive methods including serum markers and imaging techniques offer potential alternatives to liver biopsy. Among them, AST-to-platelet ratio index (APRI), fibrosis index based on four factors (FIB-4) and liver stiffness measurement (LSM) are relatively inexpensive and commonly accessible in most hospitals. However, various factors including necroinflammation, steatosis and cholestasis have affected their specificities [25–30].

GP73 is a resident Golgi trans-membrane glycoprotein primarily expressed in biliary epithelial cells but rarely in hepatocytes in normal livers [31]. Recently, serum GP73 was reported as a potential diagnostic marker for HCC [32–36]. However, serum GP73 can also elevate in patients with liver cirrhosis even at levels higher than HCC [33, 37–39] and has compromised its diagnostic accuracy in HCC because most HCC cases develop from cirrhosis [40–42].

In this study, we first examined the potential value of serum GP73 in diagnosing CLD both in cirrhotic and pre-cirrhotic CLD subjects. Then, we compared the diagnostic value between APRI, FIB-4, LSM and serum GP73 in patients with fibrosis and compensated cirrhosis with different etiologies including CHB, CHC, NAFLD as well as autoimmune-mediated liver diseases AIH and PBC. The levels of serum GP73 and GP73 protein expression in liver tissues at different fibrotic stages were also examined. Finally, we investigated IL-6/STAT3 signaling as a possible mechanism for the elevated serum GP73 in patients with fibrosis/cirrhosis.

Methods

Subjects

This retrospective study includes 3044 patients with either pre-cirrhotic CLD ($n = 956$), compensated cirrhosis ($n = 1247$) or decompensated cirrhosis ($n = 841$) in Beijing 302 Hospital and Shanghai Renji Hospital between January 2010 and March 2016 (Fig. 1). The demographics and laboratory

data including levels of GP73 of these patients were collected (Table 1). As control, 121 healthy volunteers without acute or chronic diseases, hyperlipidemia, diabetes, hypertension and BMI higher than 28 were also included, and their levels of serum GP73 were quantified.

The diagnostic criteria for compensated cirrhosis include the following: (a) typical findings of CT or MRI with one of the following observations: irregular liver surface, granular or nodular liver parenchyma with or without splenomegaly (thickness of spleen > 4 cm or > 5 pedicle-rib units); (b) platelet count of <100,000/mm^3 excluding the other possible causes; (c) serum albumin <3.5 g/dL, prolonged prothrombin time or increased international normalized ratios (INR) > 1.3 (anticoagulants or thrombolytic drugs discontinued for >7 days) and (d) no history of decompensated cirrhosis defined as jaundice in the presence of cirrhosis, ascites, bleeding gastric or esophageal varies or encephalopathy.

For pre-cirrhotic CLD patients, the definition of NAFLD requires that (a) there is evidence of hepatic steatosis, either by imaging or by histology and (b) there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steratogenic medication or hereditary disorders [5]. The diagnosis of CHB was based on hepatitis B surface antigen (HBsAg) positive for more than 6 months, with clinical or laboratory signs of chronic hepatitis [43]. The diagnosis of chronic hepatitis C (CHC) was based on the detection of both hepatitis C virus (HCV) antibodies and HCV RNA in the presence of signs of chronic hepatitis [44]. Autoimmune liver diseases (AILDs) including AIH and PBC were diagnosed based on established criteria [45, 46]. This study was approved by the Ethics

Committee of Beijing 302 Hospital and Shanghai Renji Hospital. Informed consent was obtained from all participants. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Liver Histology

The scoring of liver fibrosis and necroinflammation activity was based on histopathological examination of percutaneous liver biopsy specimens. Liver tissue specimens were fixed in buffered formalin, embedded in paraffin and then subjected to hematoxylin and eosin (H & E) and Masson trichrome staining. The slides were reviewed by an experienced pathologist, who was blinded to the patients' clinical information. Necroinflammation activity was identified according to the METAVIR system: A = histological activity (A0 = no activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity) [47]. Liver fibrosis in NAFLD, AIH and PBC patients was staged according to the Brunt staging system [5, 48] with F1 defined as perisinusoidal or periportal fibrosis, F2 as perisinusoidal fibrosis with portal or periportal involvement, F3 as bridging fibrosis and F4 as cirrhosis. In this study, significant fibrosis is defined as a score of ≥ F2 and advanced fibrosis of ≥ F3 [48].

LSM

LSM was performed with FibroScan® (Echosens, Paris, France) within one week of liver biopsy. The measurements

Fig. 1 Patients' flowchart, data provided in absolute numbers
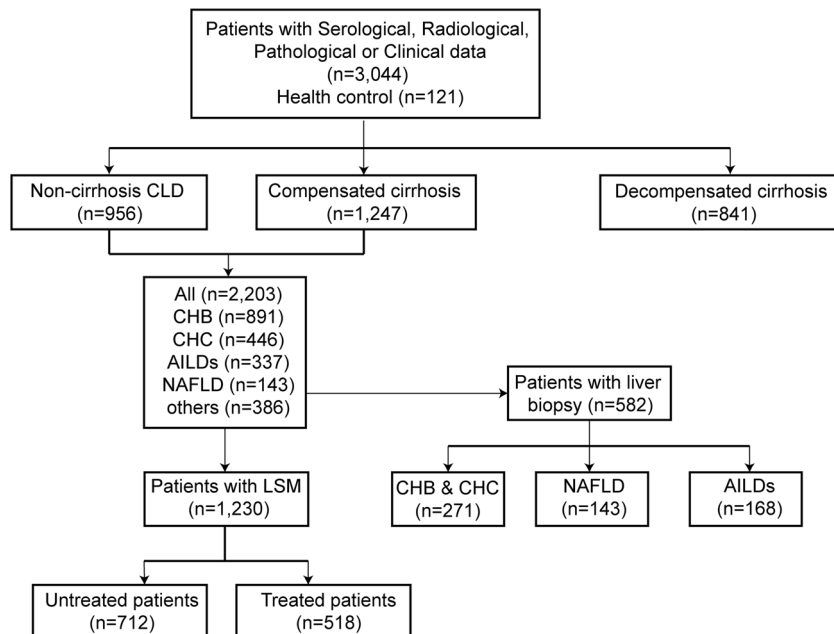
Table 1 Demographic and laboratory characteristics of the 3044 patients

Variables	Pre-cirrhotic CLD ($n = 956$)	Compensated cirrhosis ($n = 1247$)	Decompensated cirrhosis ($n = 841$)	Variables
Sex (male/female)	532/424	706/541	580/261	0.000
Age (years)	45.00 (37.00–52.00)	50.00 (43.00–58.00)	51.00 (44.00–59.00)	0.000
BMI (kg/m^2)	24.49 (22.00–26.55)	24.00 (21.72–26.42)	23.88 (21.52–26.50)	0.154
GP73 (ng/mL)	43.74 (28.24–61.34)	122.00 (82.33–181.28)	149.85 (95.49–215.70)	0.000
ALT (U/L)	27.00 (16.00–49.00)	32.00 (20.25–69.75)	28.00 (20.00–44.00)	0.000
AST (U/L)	25.00 (19.00–37.50)	43.00 (27.00–82.00)	42.00 (29.00–65.00)	0.000
Platelet count (10^9/L)	181.00 (149.00–219.50)	107.00 (70.00–152.00)	65.00 (45.25–107.50)	0.000
APRI	0.38 (0.27–0.66)	1.19 (0.64–2.36)	1.72 (0.85–3.13)	0.000
FIB-4	1.25 (0.87–1.82)	3.62 (2.09–6.81)	6.19 (3.49–10.48)	0.000
LSM ($n = 1230$)	6.70 (5.28–9.13)	16.00 (10.23–26.30)	NA	0.000

All data are presented as median (IQR) or n (%). Comparison between two groups was performed using Mann–Whitney U test or Chi-square test as appropriate. BMI, body mass index; GP73, Golgi protein 73; ALT, alanine aminotransferase; AST, aspartate aminotransferase; APRI, AST-to-platelet ratio index; FIB-4, fibrosis index based on four factors; LSM, liver stiffness measurement

were performed blinded to the results of the biopsy. LSM was determined in the right hepatic lobe through the intercostal space with the patient (at least 2 h after meal) in the supine position and the right arm in maximum abduction. The results were expressed in kilopascals (kPa) corresponding to the median of ten determinations. Only valid measurements (success rate of more than 60% and interquartile range/median ratio < 0.3) were included [49].

Determination of Serum Levels of GP73, ALT, AST and Platelet Count

Quantitative detection of serum GP73 was performed with a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Hotgen Biotech Inc., Beijing, China), according to the manufacturer's protocol. In brief, sample dilution buffer (50 μL) and 20 μL serum were added to wells precoated with monoclonal anti-GP73 and incubated for 1 h at 37 °C. The wells were washed and then incubated with 100 μL of HRP-conjugated anti-human antibody for 30 min at 37 °C. After washing for five times, the plate was developed with tetramethylbenzidine, and OD_{450} was measured with a microplate reader (Thermo Labsystems, Vantaa, Finland). Purified recombinant GP73 was assayed in parallel as a calibration standard. All assays were run in duplicate and repeated twice.

The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic biochemical analyzer (Beckman, CA, USA). Platelet count was measured by an electrical resistance method (Sysmex, Kobe, Japan). APRI was defined as [(AST/ULN)/platelet count (10^9/L)] × 100. FIB-4 was calculated according to the following formula: FIB-4 = (age × AST) / (platelet count [10^9/L] × ALT$^{1/2}$). The upper limit of normal (ULN) for both AST and ALT was 40 U/L.

Immunohistochemistry

Immunohistochemistry for GP73 was performed using paraffin-embedded liver tissues. Firstly, liver tissues were dewaxed at 70 °C for 1 h, microwaved in 10 mM citrate buffer (pH 6.0) and cooled to room temperature. After washing with PBS (phosphate buffered saline, pH 7.6) for three times, tissues were covered with 3% H_2O_2 for 20 min to block endogenous peroxidase activity. Thereafter, the tissues were incubated with 25% goat serum for 45 min, washed and incubated with primary antibody 1:1000 dilution (ab109628; Abcam, UK) for 2 h at 37 °C. After washing, the tissues were incubated with universal horseradish peroxidase conjugated anti-mouse/rabbit antibody (D-3004, Supervision™, China) for 30 min at room temperature then visualized using a 3, 3-diaminobenzidine color kit (DAB-0031, Maixin Biotech, China) and counterstained with hematoxylin (G1080, Solarbio Science & Technology, China).

Cell Culture and Reverse Transcription-Quantitative PCR (RT-qPCR)

Human liver cancer cell line Huh7 was purchased from the American Type Culture Collection (ATCC) and cultured in DMEM (19217008, Sigma-Aldrich, USA) with 8% fetal

bovine serum (r35-076-cv, cellgro, USA) in a 5% CO_2 incubator at 37 °C. Cells were treated with 25 ng/mL IL-6 (200-06-20, PeproTech, USA) for 2 h, and total RNA was extracted with TRIzol (15596018, Invitrogen, USA). cDNA was synthesized from 1 μg RNA using an all-in-one first-strand cDNA synthesis supermix for quantitative PCR (qPCR) (AT341-02, TransGen Biotech, China). qPCR for furin and GP73 were performed with actin as housekeeping gene using FS Universal SYBR Green Master (4913914001, Roche, USA). The primers used for qPCR were listed in supplementary materials (Supplementary Table 1).

Dual-Luciferase Reporter Assay

GP73 promoter was amplified from genomic DNA extracted from Huh7 cells and cloned into pGL3-luc firefly luciferase reporter vector [50]. pGL-furin promoter-luc and pGL3-pim1 promoter-luc were constructed with similar method with specific PCR primers (Supplementary Table 2). pcDNA3.1-STAT3C (a constitutive active form of STAT3) expression plasmid was constructed as described previously [51]. For dual-luciferase reporter assay, Huh7 cells were transfected with either 300 ng pGL3-GP73 promoter-luc, 300 ng pGL3-furin promoter-luc or 300 ng pGL3-pim1 promoter-luc together with 1.2 μg pcDNA3.1-STAT3C and 50 ng pGL3-actin promoter-renilla. Fourty eight hours later, cells were harvested and analyzed by luciferase assay (E1980, Promega, USA). Briefly, cells were washed with PBS twice and lysed with 150 μL 1× passive lysis buffer for 30 min. After centrifuging for 10 min at 12,000 rpm, 20 μL of supernatant was transferred to 96-well plate in triplicate. A total of 25 μL of firefly luciferase reagent was then added and luminescence measured immediately, followed by addition of 25 μL of Renilla luciferase reagent and measurement of luminescence at 2 min. The results are expressed as ratio of firefly to Renilla luciferase activity.

Statistical Analysis

Descriptive statistics for GP73 in different patient groups were compared using box plot. The difference between groups was tested using the Mann–Whitney U test. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the diagnostic performance. For the identification of significant or advanced liver fibrosis and cirrhosis, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated with an optimal cut-off value that maximized the sum of sensitivity and specificity. All statistical analyses were performed with MedCalc (15.8.1)

software. All tests of significance were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of Patients

A total of 3044 patients who fulfilled the study criteria were enrolled. Details of the patient groups and clinical data are shown in Fig. 1 and Table 1.

Serum Levels of GP73 Were Higher in Cirrhotic than in Pre-Cirrhotic CLD Patients

The serum levels of GP73 were significantly higher in cirrhosis patients when compared with those of the healthy controls and the pre-cirrhotic CLD groups $(p < 0.001)$. Serum levels (median, IQR) of GP73 were 35.07 (24.97–45.37) ng/mL in healthy controls, 43.74 (28.24–61.34) ng/mL in pre-cirrhotic CLD group, 122.00 (82.33–181.28) ng/mL in compensated cirrhotic group and 149.85 (95.49–215.70) ng/mL in decompensated cirrhotic group. They all were significantly higher than that of CLD patients without cirrhosis (Supplementary Fig. 1).

Multivariate analysis was performed to determine if the increased levels of serum GP73 were liver fibrosis/cirrhosis relevant. The results indicated that higher serum GP73 level was independently associated with several fibrosis/cirrhosis relevant markers, including higher collagen type IV (CIV), hyalurona (HA), total bilirubin (Tbil), collagen type III N-terminal peptide (PIIINP), monocytes (MNC), globulin, fibrinogen (FIB), collagen type IV (C IV) and lower platelet count (PLT), respectively (Supplementary Table 3).

Serum Levels of GP73 Exhibited Better Diagnostic Value than APRI and FIB-4 in Patients with Compensated Cirrhosis

Analysis of the ROC values between GP73, APRI and FIB-4 indicated that GP73 had a better diagnostic value for compensated cirrhosis (AUC 0.899, 95% CI 0.886 to 0.911, $p < 0.001$) (Fig. 2a). To further confirm the diagnostic value of GP73 in compensated cirrhotic patients, stratified analysis was conducted based on the different etiologies of those compensated cirrhosis patients. ROC analysis indicated that the AUCs for cirrhosis due to CHB, chronic hepatitis C (CHC), AILDs, NAFLD and other causes were 0.880 (95% CI 0.857 to 0.900), 0.926 (95% CI 0.897 to 0.948), 0.885 (95% CI 0.846 to 0.917), 0.962 (95% CI 0.917 to 0.987) and 0.935 (95% CI 0.906 to 0.958), respectively (Fig. 2b–f).
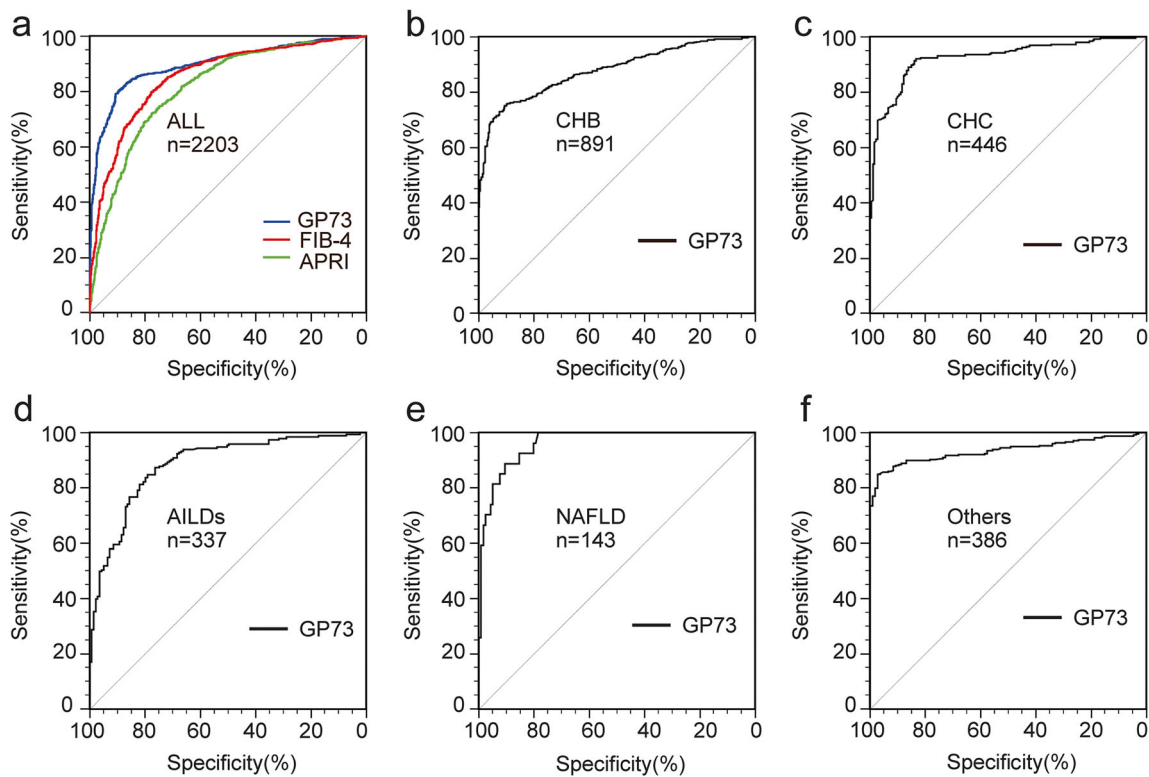
Fig. 2 Diagnostic values of serum GP73 for liver cirrhosis were analyzed using ROC curves. **a** The ROC curve of GP73 for diagnosing compensated cirrhosis regardless of etiologies (case number varies depending on the data available). **b–f** The ROC curve for diagnosing compensated cirrhosis among patients with different etiologies (case number varies depending on data availability. "Others" means causative factor other than HBV, HCV, AILDs or NAFLD)

Serum GP73 Had Better Performance for Diagnosing Cirrhosis in Patients with Active Hepatic Necroinflammation and Steatosis

To compare the performance of serum GP73 and LSM in the diagnosis of cirrhosis, we analyzed GP73 and LSM data within a subgroup of 1230 compensated cirrhotic patients. The AUC was 0.873 (95% CI 0.854 to 0.892) for GP73 and was 0.856 (95% CI 0.835 to 0.875) for LSM. When GP73 and LSM were combined, AUC was increased to 0.923 (95% CI 0.906 to 0.938), sensitivity was increased to 87.14% and specificity was increased to 86.71% (Fig. 3a). Even the negative predictive value (NPV) was also increased (Fig. 3a and Table 2).

It has been suggested that the application of LSM in clinical practice had some limitations. Therefore, additional evaluation of GP73 as a diagnostic marker was conducted. Noticeably, compared with LSM, serum GP73 had a better diagnostic value in patients with hepatic inflammation score of A ≥ 2 (AUC 0.888 vs 0.809), increased ALT (≥ 2 ULN, AUC 0.922 vs 0.858), increased fatty liver (AUC 0.959 vs 0.867), increased TBIL (≥ 1.5 ULN, AUC 0.919 vs 0.835) or increased TBA (≥ 2 ULN, AUC 0.962 vs 0.818) (Fig. 3b–f).

A Stepwise and Significant Increase of Serum GP73 Levels Was Observed with Fibrosis Disease Progression Among CLD Patients with Virus Hepatitis, AILDs or NAFLD

In order to investigate whether GP73 has potential value in predicting liver fibrosis in patients with viral hepatitis, AILDs or NAFLD, the levels (median, IQR) of serum GP73 of patients with different fibrotic stages were analyzed in several subgroups of patients with CHB and/or CHC (n = 271), AILDs (n = 168) and NAFLD (n = 143). The serum GP73 levels in CHB and/or CHC, AILDs and NAFLD correlated with the stage of fibrosis. The serum GP73 levels (median, IQR) in virus hepatitis in each stage were 38.48 (22.71–55.65) ng/mL (F0–1), 45.03 (28.67–58.01) ng/mL (F2), 65.35 (43.92–90.64) ng/mL (F3) and 129.50 (90.59–188.83) ng/mL (F4), respectively. A similar tendency was also observed in AILDs and NAFLD patients where serum GP73 levels (median, IQR) were 36.29 (27.67–47.11) ng/mL (F0–1), 53.37 (36.53–72.25) ng/mL (F2), 81.83 (59.34–129.40) ng/mL (F3) and 136.78 (111.72–221.50) ng/mL (F4) (for AILDs), and 32.54 (22.91–48.68) ng/mL (F0–1), 54.85 (45.72–77.12) ng/mL (F2), 69.52 (53.19–94.88) ng/mL (F3) and 127.60 (98.43–211.40) ng/mL (F4) (for NAFLD) (Fig. 4a–c).
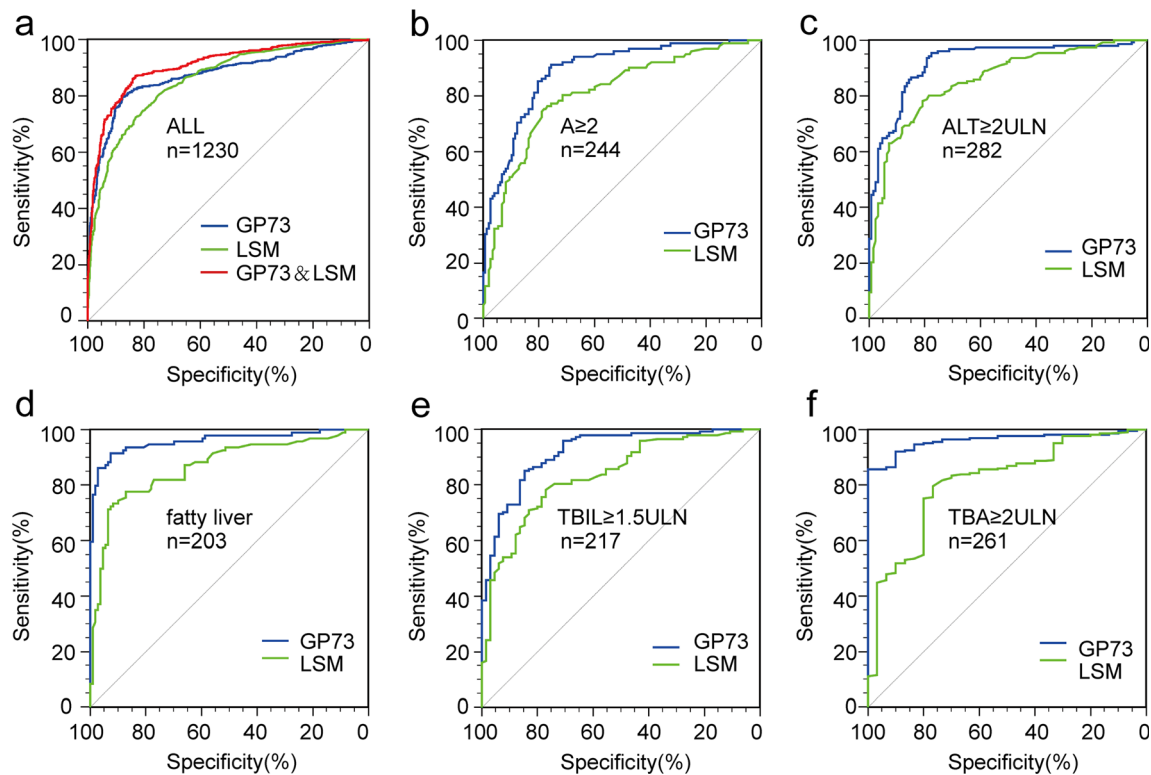
Fig. 3 **a** The ROC curve of GP73 and LSM for diagnosing cirrhosis regardless of etiologies. **b–f** The ROC curve of GP73 and LSM for diagnosing cirrhosis in all and subgroups of patients with active hepatic necroinflammation, fatty liver or cholestasis (case number varies depending on data availability)

To further validate the correlation between levels of GP73 expression in liver tissue and the fibrosis stage, the expression of GP73 protein in liver tissues with different fibrotic scores from patients of viral hepatitis, AILDs and NAFLD was analyzed by immunohistochemistry. Notably, a stepwise increase of GP73 expression in the liver tissue was observed with the increasing severity of liver fibrosis (Fig. 4d–f, Supplementary Fig. 2), and Spearman correlation analysis revealed that the expression of GP73 in situ was well correlated with the severity of fibrosis in viral hepatitis patients ($r = 0.854$, $p < 0.001$), in AILDs ($r = 0.704$, $p < 0.001$) and also in NAFLD ($r = 0.671$, $p < 0.001$).

Table 2 Diagnostic values of GP73 and LSM for compensated cirrhosis in patients

Variable	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	95% CI	p value
Total ($n = 1230$)								
GP73	72.53 ng/mL	79.90	87.38	85.92	81.76	0.873	0.854–0.892	< 0.0001
LSM	10.8 kPa	72.35	82.91	80.34	75.65	0.856	0.835–0.875	< 0.0001
GP73 and LSM	–	87.14	86.71	88.18	85.55	0.923	0.906–0.938	< 0.0001
Treatment naive patients ($n = 712$)								
GP73	72.78 ng/mL	86.79	92.34	92.93	85.76	0.930	0.906–0.949	< 0.0001
LSM	13.6 kPa	73.27	90.88	90.32	74.55	0.900	0.873–0.923	< 0.0001
GP73 and LSM	–	87.42	92.70	93.29	86.39	0.949	0.928–0.965	< 0.0001
Treated patients ($n = 518$)								
GP73	68.80 ng/mL	73.63	88.16	87.39	75.00	0.849	0.815–0.879	< 0.0001
LSM	9.0 kPa	71.79	80.00	80.00	71.79	0.830	0.795–0.861	< 0.0001
GP73 and LSM	–	80.22	89.39	89.39	80.22	0.898	0.869–0.923	< 0.0001

GP73, Golgi protein 73; LSM, liver stiffness measurement; AUC, the area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value
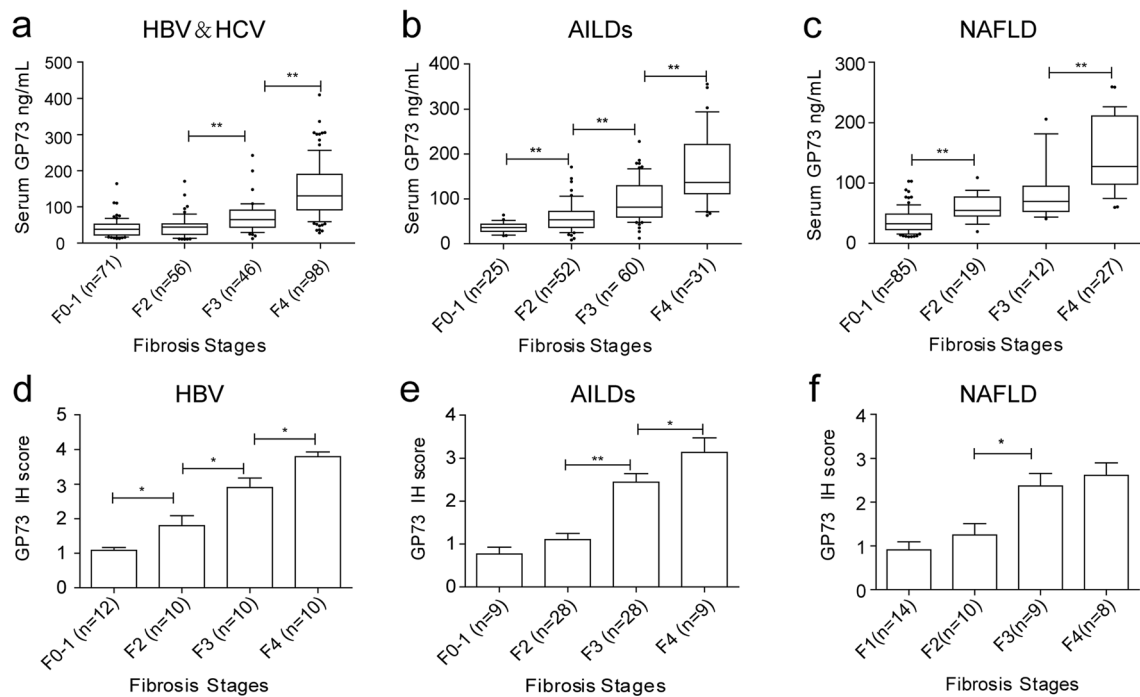
Fig. 4 Serum GP73 and hepatic GP73 were increased with liver fibrosis stage regardless of etiologies. Correlation between serum GP73 and liver fibrosis stages F0–F4 in virus hepatitis (**a**), AILDs (**b**) and NAFLD (**c**) patients; immunohistochemistry of GP73 protein in different degrees of fibrotic liver tissues, CHB (**d**), AILDs (**e**) and NAFLD (**f**) patients. *p value < 0.05, **p value < 0.01 based on differences between medians with the Mann–Whitney U test

Validation of the Diagnostic Value of Serum GP73 for Significant and Advanced Fibrosis As Well As Cirrhosis in Patients with Viral Hepatitis, AILDs or NAFLD

The AUC indicated that serum GP73 exhibited a potential to differentiate patients at different fibrotic stages in virus hepatitis, AILDs or NAFLD. The AUC for GP73 to predict significant fibrosis ($\geq$ F2), severe fibrosis ($\geq$ F3) and cirrhosis (F4) was 0.772, 0.854 and 0.890, respectively, for virus hepatitis, 0.855, 0.852 and 0.880, respectively, for AILDs, and 0.897, 0.935 and 0.960, respectively, for NAFLD (Table 3).

Validation of Serum GP73 Levels and Liver Expression in a Sub-Cohort of Patients with AIH and PBC

The levels (median, IQR) of serum GP73 were 35.18 (26.52–52.57) ng/mL (F0–1), 62.46 (36.41–86.37) ng/mL (F2), 92.10 (58.65–141.25) ng/mL (F3) and 150.48 (114.73–193.30) ng/mL (F4) in patients with AIH. A similar tendency was also observed in PBC patients where serum GP73 levels (median, IQR) were 36.43 (27.59–44.98) ng/mL (F0–1), 45.95 (36.41–60.87) ng/mL (F2), 77.18 (61.09–124.63) ng/mL (F3) and 125.90 (91.63–258.28) ng/mL (F4) (Fig. 5). The serum GP73 level in healthy controls was 35.07 (24.97–45.37) ng/mL, similar with those in fibrosis stage 0–1 patients. To

determine whether increased serum GP73 is derived from the liver, immunohistochemical staining for GP73 was performed in 38 AIH patients and 36 PBC patients. As shown in Fig. 5, serum levels of GP73 were positively correlated with liver GP73 IH score for both AIH ($r = 0.788$, $p < 0.0001$) and PBC ($r = 0.716$, $p < 0.0001$), respectively.

GP73 Can Be Induced by IL-6 Through the STAT3 Signal Pathway

To investigate the mechanism of GP73 expression in fibrosis, we focused on IL-6/STAT3 signaling. Online prediction software (http://alggen.lsi.upc.es/, http://jaspar.genereg.net/) indicated that the STAT3 binding site is within the GP73 promoter region. Hence, we applied RT-qPCR and dual-luciferase reporter assay to detect whether GP73 could be up-regulated by IL-6/STAT3 signaling. After treatment with recombinant human IL-6 (rhIL-6), the mRNA level of GP73 in huh7 cell lysis increased about 0.5-fold. Interestingly, furin, which could slice GP73 from the Golgi membrane [52], was also increased in parallel with GP73 upon IL-6 simulation. Consistently, overexpression of STAT3C in Huh7 cells also increased the expression of GP73 as compared to control. Because a STAT3 binding site is found within the GP73 promoter, we performed the dual-luciferase reporter assay to detect the transcription activation of IL-6/STAT3 signaling on GP73 and

Table 3 Diagnosis accuracy of GP73 in predicting fibrosis in virus hepatitis, AILDs or NAFLD

Stage	Cut-off (ng/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	95% CI	p value
Virus hepatitis								
≥ F2	68.84	57.50	88.73	93.5	42.6	0.772	0.718–0.821	< 0.0001
≥ F3	68.84	73.61	86.61	86.2	74.3	0.854	0.806–0.893	< 0.0001
F4	77.61	82.65	84.39	75.0	89.6	0.890	0.847–0.925	< 0.0001
AILDs								
≥ F2	54.33	74.13	92.00	98.1	38.3	0.855	0.792–0.904	< 0.0001
≥ F3	58.42	84.62	71.43	77.8	79.7	0.852	0.789–0.902	< 0.0001
F4	103.66	80.65	81.02	49.0	94.9	0.880	0.825–0.928	< 0.0001
NAFLD								
≥ F2	59.17	76.27	88.10	81.8	84.1	0.897	0.835–0.941	< 0.0001
≥ F3	59.17	92.50	82.52	67.3	96.6	0.935	0.881–0.969	< 0.0001
F4	77.12	89.29	90.43	69.4	97.2	0.960	0.914–0.986	< 0.0001

AUC, the area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value; AILDs, autoimmune liver diseases; NAFLD, non-alcoholic fatty liver disease

furin promoter. The result showed that both IL-6 treatment and STAT3C overexpression could increase transcription of GP73 and furin (Supplementary Fig. 3). Taken together, the data here suggested that the increase of serum GP73 in liver fibrosis may be associated with transcriptional activation of GP73 and furin genes by IL-6/STAT3 signaling.

Discussion

The human GP73 is a 73-kDa protein of the cis-Golgi membrane. It is highly conserved in other species and normally expressed in epithelial tissues [53]. In normal liver, expression of GP73 is limited to biliary epithelial cells [54, 55]. Elevation
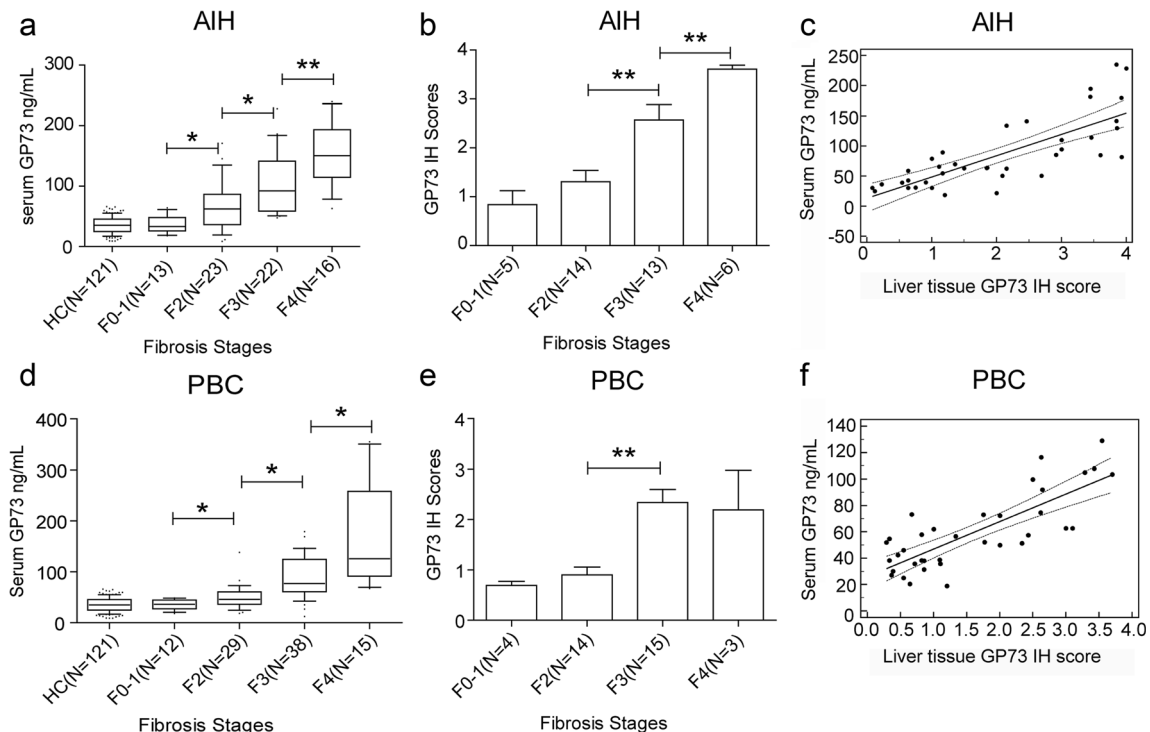
Fig. 5 Serum GP73 was increased with hepatic GP73 as liver fibrosis stage in AIH and PBC. **a**, **d** Serological GP73 levels of AIH and PBC patients at different fibrotic stages. **b**, **c**, **e**, **f** Analysis of immunohistochemical staining score of GP73 in liver tissues of patients with AIH and PBC and correlation between serum GP73 and liver fibrosis stages in AIH and PBC patients. *p value < 0.05, **p value < 0.01 based on differences between medians with the Mann–Whitney U test

of GP73 protein has also been reported in patients with giant-cell hepatitis and viral infections [53, 56], but the majority of data are from studies of HCC patients, which suggest GP37 as a serum marker for HCC. However, despite extensive data linking GP73 to cell proliferation in HCC [57–61], there is a paucity of evidence indicating that aberrant expression of GP73 is a driver of hepato-carcinogenesis. In contrast, our group and others have reported that GP73 is not necessarily relevant to the pathogenesis of HCC but rather increased in fibrosis and cirrhosis [62].

In this study, we demonstrated that the levels of serum GP73 are elevated in patients with fibrosis/cirrhosis and correlated well with its protein levels in fibrotic/cirrhotic liver tissues, which implicated the fibrosis/cirrhosis diagnosing value of serum GP73. Our data demonstrated that serum GP73 had a higher AUC in the diagnosis of compensated cirrhosis when compared with FIB-4 and APRI, the two most widely validated non-invasive modalities for cirrhosis [63, 64]. This observation is similar to previous study on patients with chronic HBV infection-related cirrhosis [65]. Nevertheless, our data indicated GP73 is a reliable CLD biomarker for predicting fibrosis in viral hepatitis, AILDs or NAFLD regardless of their etiologies (Table 3).

LSM is one of the validated non-invasive methods in evaluating liver cirrhosis and monitoring disease progression. However, one limiting issue is that optimal cut-off values vary for different etiologies [66]. In addition, several other factors including hepatic necroinflammation, steatosis and cholestasis can also affect the diagnostic accuracy of LSM [26–29]. Notably, our data showed that serum GP73 had relative good diagnostic performance in patients with fatty liver, significant liver necroinflammation ($A \geq 2$ and/or $ALT \geq 2$ ULN) and cholestasis ($TBIL \geq 1.5$ ULN and/or $TBA \geq 2$ ULN). It should be emphasized that, for the diagnosis of compensate cirrhosis, combination of GP73 and LSM increased the NPV, which reflects the reduction misdiagnosis rate.

AILDs progress rapidly and can lead to serious complications with high mortality [9, 67]. Although alkaline phosphatase is accepted in PBC as a surrogate marker of transplant-free survival, there is a significant void in predicting the development of fibrosis, not only in PBC but also in AIH [68, 69]. In the study herein, we evaluated the diagnostic potential of serum GP73 for liver fibrosis in AIH and PBC patients. AUCs analysis demonstrated that GP73 performed as well as widely used serum index APRI and FIB-4 in predicting AIH and PBC-related fibrosis stage. Furthermore, our data also demonstrated that GP73 measurements exhibit similar accuracy in predicting fibrosis and the diagnosis of cirrhosis in AIH and PBC patients to LSM. As a serum marker, GP73 measuring is easy and relatively inexpensive. Moreover, it avoids the sample errors and reduces the cost of transient elastography and discomfort of liver biopsy. Considering

that AIH and PBC and other CLD require long-term follow-up, serum GP73 is potentially useful for serial monitoring of patients undergoing treatment and monitoring efficacy of new drugs.

Our previous results had shown that GP73 is not necessarily relevant to the pathogenesis of HCC, but rather it increased in fibrosis and cirrhosis [62], which suggested the involvement of GP73 in the disease progression of fibrosis and cirrhosis. Imbalance between liver necroinflammation and regeneration during persistent liver injury is the key factor that leads to hepatic fibrosis and cirrhosis regardless of etiology. Because plenty of evidences have linked GP73 to cell proliferation [57–61], it is reasonable to speculate that GP73 may play a positive role in liver regeneration and that enhanced GP73 expression may reflect the proliferative potential for hepatic parenchymal cells. In line with this, it has been reported that overexpression of GP73 could assist EGFR/RTK recycling, resulting in prolonged activation of the downstream signal pathway and proliferation of a variety of HCC cell lines [70]. IL-6 is considered one of the most significant factors for liver regeneration [71–74]. Importantly, previous studies have shown that GP73 expression can be affected by IL-6 [59]. In HepG2 cells, IL-6 could transcriptionally induce GP73 expression [75], implicating a role of GP73 in IL-6-mediated liver inflammation and regeneration. STAT3 is one of the most important downstream mediators of IL-6 signaling. Enhanced STAT3 binding and SNPs in *STAT3* gene are associated with various multi-organ autoimmunities [76, 77]. By using online prediction software, we identified potential STAT3 binding site within the GP73 promoter region, as well as in the promoter region of the furin gene. By luciferase reporter assay, we demonstrated that STAT3c, the active form of STAT3 slightly increased the promoter activity of both GP73 and furin genes (Supplementary Fig. 3). Since the release of GP73 into serum is furin proteinase enzyme activity-dependent [52], our data further suggested the multiple roles of IL-6 which contribute to the aberrant elevation of serum GP73 seen in cirrhotic CLD patients.

In summary, we report that serum GP73 is a reliable marker for the diagnosis of compensated cirrhosis, with noticeably improved performance when compared with FIB-4 and APRI. In addition to diagnosing cirrhosis, serum GP73 could also differentiate significant and advanced fibrosis in AILDs or NAFLD patients. In viral hepatitis, serum GP73 could perform well in diagnosing advanced fibrosis. This study has significant clinical applications that may help to fill the void of reliable surrogate markers for fibrosis and cirrhosis in CLD. Future study is needed to define the molecular basis of the binding site of STAT3 and the GP73 promoter in the pathogenesis of CLD. Future multicenter study with prospective design is needed to validate the potential use of GP73 as a surrogate biomarker of liver cirrhosis/fibrosis in chronic liver disease.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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