Liver cirrhosis–specific glycoforms of serum proteins in chronic hepatitis B infection: identification by lectin affinity chromatography and quantitative proteomic profiling

TCW Poon *, HLY Chan, HWC Leung, A Lo, RHY Lau, AY Hui, JJY Sung

KEY MESSAGES

1. Carbohydrate side-chains of serum glycoproteins are altered in patients with liver cirrhosis.
2. Sialylated and fucosylated variants of serum glycoproteins are altered in chronic hepatitis B (CHB) patients with liver cirrhosis.
3. Particular sialylated Hp variants are down-regulated in CHB patients with liver cirrhosis, as are in patients having liver cirrhosis secondary to chronic hepatitis C, non-alcoholic fatty hepatitis or autoimmune hepatitis.
4. Development of novel practical assays that can quantify a specific glycosylated variant of a particular serum glycoprotein is needed.

Hong Kong Med J 2015;21(Suppl 4):S22-6
RFCID project number: 04050392

TCW Poon, HLY Chan, HWC Leung, A Lo, RHY Lau, AY Hui, JJY Sung

Department of Medicine and Therapeutics, The Chinese University of Hong Kong

* Principal applicant and corresponding author: tcwpoon@cuhk.edu.hk

Introduction

In Hong Kong and China, chronic hepatitis B (CHB) is the commonest cause of liver cirrhosis, which is also a risk factor for development of hepatocellular carcinoma. In CHB patients, persistent hepatic inflammation leads to progressive liver fibrosis and subsequently cirrhosis. The process of liver cirrhosis is reversible. Accurate diagnosis is crucial for the management of CHB patients.[1] Liver biopsy is the gold standard for diagnosing cirrhosis, but is an uncomfortable and risky procedure. Therefore, serum markers for reliable detection of liver cirrhosis are needed.

Carbohydrate side-chains of serum glycoproteins are altered in patients with liver cirrhosis. Degrees of fucosylation on haptoglobin, alpha1-acid glycoprotein and cholinesterase increase in liver cirrhosis, but not in viral or chronic hepatitis.[2] Hyposialylated variants of haptoglobin, alpha1-antitrypsin, and transferrin are detected in patients with alcoholic cirrhosis.[3]

We developed a method to profile specific types of glycosylation variants of serum proteins by the combined use of lectin affinity chromatography and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).[4] We aimed to identify serum glycoproteins as biomarkers of liver cirrhosis.

Methods

This study was conducted from 1 March 2006 to 31 August 2008. Patients with CHB who underwent liver biopsies from 1998 to 2004 in the Prince of Wales Hospital were included. They were recruited or screened for previous and ongoing therapeutic trials or were suspected to have active liver disease on laboratory or radiological investigations. Serum samples were collected from 80 CHB patients with and without liver cirrhosis, and from 20 normal healthy subjects. In addition, 18 serum samples from patients with liver cirrhosis secondary to chronic hepatitis C (CHC) [n=10], non-alcoholic fatty liver disease (n=6), and autoimmune hepatitis (n=2) were examined. Serum samples were saved in a freezer (-80°C) for future study.

The serum samples were handled individually and subjected to differential lectin affinity chromatography to obtain glycoproteins fractions with altered glycosylation structures (including fucosylated N-glycan and sialylated N-glycan), which have been observed in CHB patients with liver cirrhosis or other chronic liver diseases. Fucosylated glycoproteins and sialylated glycoproteins were obtained by using LCA lectin and SNA lectin, respectively. The procedures were performed as previously described.[4]

The 2D-PAGE technology enabled differentiation of protein variants with various post-translational modifications.[4] Isoelectric focusing (first dimensional separation by charge) of the proteins in the IPG strip was carried out in the PROTEAN IEF cell (Bio-rad). The IPG strip was then loaded on a SDS gel (PROTEAN II Ready Gel, Bio-rad) for second dimensional separation by protein

size. The gel images were subsequently analysed with the image analysis software PDQuest (Bio-rad). The samples were profiled in duplicate. The protein spots were matched and indexed across the 2D-PAGE images. The quantities of the protein spots were measured and normalised.

Protein identification was performed with some modifications.[4] A protein spot was excised from the gel, destained and subsequently digested with trypsin. The masses of the recovered tryptic peptides were obtained by MALDI TOF/TOF mass spectrometer, and then compared to the masses of a peptide database calculated from the NCBI's nr protein database. LIFT analysis was performed to obtain the amino acid sequence information of one or more tryptic peptides to confirm the protein identity.

Serum level of total Hp was measured by an in-house enzyme-linked immunosorbent assay (ELISA). Serum levels of s-Hp and ns-Hp were measured by combined use of a 96-well microtitre plate coated with SNA lectin, which captured alpha-2,6 sialylated glycoproteins, and a standard ELISA for Hp.[4]

The Mann-Whitney *U* test was used to compare differences between groups. The area under

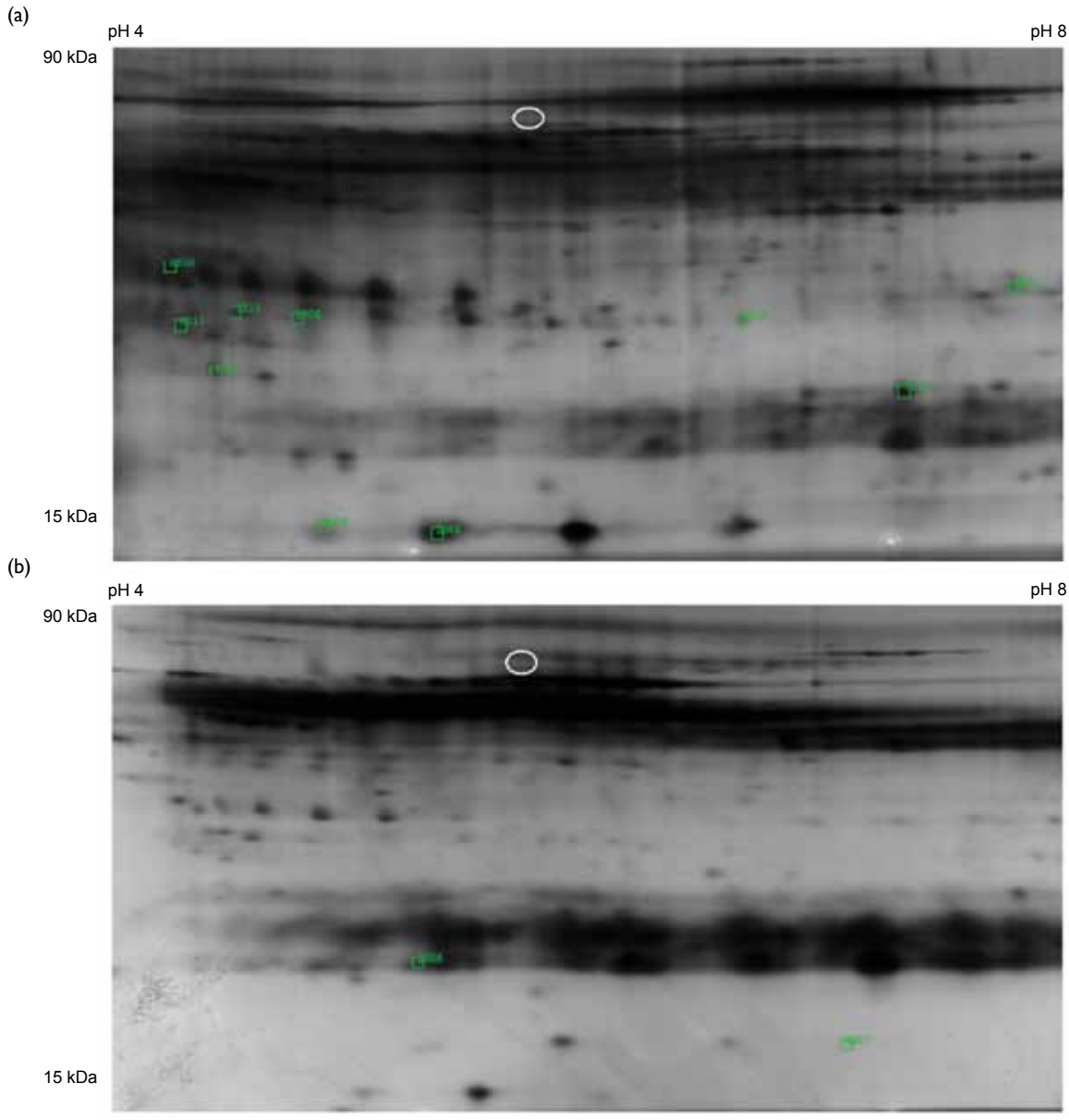

FIG. Representative images of (a) serum sialylated glycoproteins and (b) serum fucosylated glycoproteins purified by lectin affinity chromatography and subsequently resolved by two-dimensional polyacrylamide gel electrophoresis. The glycoprotein spots showing altered levels in chronic hepatitis B patients with liver cirrhosis are highlighted. White circles indicate the expected position (pI 5.8, 66.5 kDa) of albumin if present in the gel

the receiver operating characteristic (ROC) curve of a potential marker in the detection of liver cirrhosis was calculated according to standard formulae.

Results

Serum sialylated and fucosylated glycoproteins associated with liver cirrhosis

In CHB patients with liver cirrhosis, eight glycoprotein spots were down-regulated and two were up-regulated, compared to CHB patients without liver cirrhosis. All the 10 differential glycoprotein spots showed a similar dysregulation trend in CHC patients (Fig a). Only two differential glycoprotein spots (SSP215 and SSP310) showed similar down-regulation patterns in patients with liver cirrhosis secondary to alcoholic cirrhosis or autoimmune hepatitis (Table 1). Of two glycoprotein spots (SSP2006 and SSP8005) up-regulated in the CHB patients with liver cirrhosis (Fig b), only one (SSP8005) showed the same up-regulation pattern as in patients with liver cirrhosis secondary to chronic hepatitis C infection, alcoholic cirrhosis or autoimmune hepatitis (Table 1).

TABLE 1. Serum sialylated and fucosylated glycoprotein spots that were significantly altered in chronic hepatitis B (CHB) patients with liver cirrhosis

Spot No.	Protein identity obtained by mass spectrometry		Mean (SD) normalised gel spot intensity					P value (2 tails)*
	Uniprot ID	Protein name	CHB patients without liver cirrhosis (n=40)	Patients with liver cirrhosis caused by				
				CHB (n=40)	Chronic hepatitis C (n=10)	Non-alcoholic fatty liver disease (n=6)	Auto-immune hepatitis (n=2)	
Sialylated glycoprotein spots								
SSP215	P10909	Clusterin, alpha chain	1264 (570)	773 (364)	685 (213)	1024 (714)	619 (8)	0.028
SSP310	P00738	Haptoglobin, beta chain	1061 (644)	698 (545)	802 (528)	886 (670)	464 (449)	0.041
SSP1101	-	-	797 (241)	559 (118)	593 (205)	923 (200)	684 (115)	0.023
SSP1313	P05156	Complement factor I	1535 (629)	958 (312)	1115 (474)	1408 (681)	833 (304)	0.009
SSP2008	P00738	Haptoglobin, alpha chain	7141 (8742)	2807 (1566)	5429 (4509)	6250 (3212)	8683 (6965)	0.029
SSP2009	-	-	1139 (1936)	259 (242)	692 (558)	553 (203)	2661 (716)	0.029
SSP2306	P00738	Haptoglobin, beta chain	1308 (709)	763 (283)	710 (553)	1322 (1003)	1317 (513)	0.043
SSP5206	-	-	456 (306)	196 (98)	216 (159)	600 (458)	196 (27)	0.029
SSP7112	-	-	2196 (566)	2828 (671)	2478 (565)	2621 (561)	2408 (86)	0.035
SSP8205	-	-	316 (122)	547 (172)	661 (216)	407 (42)	246 (179)	0.015
Fucosylated glycoprotein spots								
SSP2006	P01834	Ig kappa chain C region	1321 (237)	1621 (304)	1427 (500)	1137 (628)	815 (919)	0.016
SSP8005	P01620	Ig kappa chain V-III region	2814 (872)	5573 (4859)	4978 (3971)	4053 (1846)	4177 (1801)	0.016

* CHB patients with liver cirrhosis vs CHB patients without liver cirrhosis

TABLE 2. Serum levels of total Hp, ns-Hp, s-Hp, and s-Hp% in chronic hepatitis B (CHB) patients with or without liver cirrhosis

Marker	Healthy normal subjects (n=19)	CHB patients without liver cirrhosis (n=40)	Patients with liver cirrhosis caused by			Patients without liver cirrhosis (n=59)	Patients with liver cirrhosis (n=53)	P value (2 tails)*	
			CHB (n=40)	Chronic hepatitis C (n=9)	Non-alcoholic fatty liver disease (n=4)			Patients with CHB only	All patients
Mean (SD) total Hp (mg/mL)	1.01 (0.48)	1.46 (1.61)	0.75 (1.03)	1.52 (0.96)	2.79 (2.06)	1.23 (1.18)	1.04 (1.25)	0.038	0.073
Mean (SD) ns-Hp (mg/mL)	0.05 (0.04)	0.15 (0.15)	0.12 (0.17)	0.96 (1.44)	0.68 (1.19)	0.10 (0.12)	0.30 (0.74)	0.459	0.167
Mean (SD) s-Hp (mg/mL)	0.96 (0.50)	1.31 (1.56)	0.64 (1.02)	0.67 (0.56)	2.12 (2.41)	1.13 (1.16)	0.76 (1.17)	0.031	0.004
Mean (SD) s-Hp%†	87 (26)	77 (33)	64 (40)	53 (33)	51 (55)	82 (30)	61 (40)	0.253	0.006

* Patients with liver cirrhosis vs patients without liver cirrhosis

† s-Hp%=100% – ns-Hp%, where ns-Hp%=serum ns-Hp level/serum total Hp level ×100%

Identities of the liver cirrhosis–associated serum glycoproteins

Among the 10 differential sialylated glycoprotein spots, protein identities of five down-regulated spots were obtained (Table 1). All identified glycoproteins were produced by the liver and secreted into the blood stream. The glycoprotein spots SS215 and SSP310 showing similar down-regulation patterns in all patients with liver cirrhosis (regardless of the pathogenesis) were clusterin and beta-chain of haptoglobin (Hp), respectively. For the two differential fucosylated glycoprotein spots (SSP2006 and SSP8005), they were in the immunoglobulin kappa chain C and V-III regions, respectively (Table 1). As immunoglobulin kappa chains did not carry any glycosylation, these two spots reflected up-regulation of fucosylated immunoglobulin variants in patients with liver cirrhosis.

Diagnostic value of sialylated haptoglobin in liver cirrhosis

In the 2D-PAGE image, four sialylated glycosylation variants of the alpha-chain of clusterin and 18 sialylated glycosylation variants of the beta-chain of Hp were found in patients with CHB. Among the 10 differential sialylated spots, two were the beta-chain of Hp, one was the alpha-chain of Hp, and one was the alpha-chain of clusterin. Therefore, sialylated Hp was more likely to be a useful marker for diagnosing liver cirrhosis. Subsequently, serum levels of total haptoglobin (total-Hp) [ie all haptoglobin variants regardless of glycosylation patterns], serum levels of sialylated Hp variants (s-Hp), and non-sialylated variants (ns-Hp) were measured. When comparing cases with and without liver cirrhosis, s-Hp (P=0.004) and s-Hp% (P=0.006) were significantly lower in the liver cirrhosis group (Table 2). When considering only the CHB patients, total-Hp (P=0.038) and s-Hp (P=0.031) levels were significantly lower in patients with liver cirrhosis (Table 2). This confirmed the results of the 2D-PAGE experiments, in which a sialylated variant of Hp was down-regulated in patients with liver cirrhosis, regardless of cause. Regrettably, ROC curve analyses showed that none of these markers were useful in differentiating cases with and without liver cirrhosis (p>0.3 in all instances).

Discussion

To the best of our knowledge, this is the first study providing concerted evidence that particular sialylated Hp variants were down-regulated in CHB patients with liver cirrhosis, as were in patients with liver cirrhosis secondary to CHC, non-alcoholic fatty liver disease or autoimmune hepatitis. The total-Hp level decreased (but not significantly) in patients with liver cirrhosis, when all liver cirrhosis cases were considered. This was consistent with findings in a study in which the total-Hp level was not significantly decreased in chronic alcohol abusers.[5]

Differential lectin affinity chromatography and quantitative proteomic profiling were too complicated to be routine tests in chemical pathology laboratories. Thus, we examined the values of serum ns-Hp level, s-Hp level, and s-Hp% for diagnosing liver cirrhosis. Hyposialylated variants of haptoglobin were detected in patients with alcoholic cirrhosis.[3] This was consistent with our finding that the ns-Hp% (which was equal to 100% – s-Hp%), was significantly increased in patients with liver cirrhosis. Regrettably, ROC curve analyses showed that they were not useful markers. This was not unexpected, because only 2 of the 18 sialylated variants of the Hp beta-chain were down-regulated in patients with liver cirrhosis. The s-Hp level or s-Hp% were too crude to reflect the changes in these two variants.

Development of novel practical assays that can quantify a specific glycosylated variant of a particular serum glycoprotein is needed. We have developed a new method to quantify the N-glycans carried on Hp by the combined use of immunoprecipitation and linear MALDI-TOF mass spectrometry (unpublished data). We are developing a multiplex assay that can quantify the sialylated and/or fucosylated N-glycans on Hp, clusterin and immunoglobulin at the same time. With this novel assay, specific sialylated N-glycans and fucosylated N-glycans can be specific biomarkers for liver cirrhosis. Combined analysis of all the differential glycoforms or glycans of haptoglobin, complement factor I, and clusterin may help to improve the diagnosis of liver cirrhosis in CHB patients.

Conclusions

Using differential lectin affinity chromatography and quantitative proteomic profiling, altered levels of specific glycosylation variants of serum glycoproteins were identified in CHB patients with liver cirrhosis. Some of glycoproteins were produced by the liver and secreted into the blood stream. A sialylated Hp variant and a sialylated clusterin variant were down-regulated in CHB patients with liver cirrhosis, whereas fucosylated variants of immunoglobulin were up-regulated. Similar dysregulation trends were observed in patients with liver cirrhosis caused by CHC, alcoholic cirrhosis or autoimmune hepatitis. Decreased serum levels of s-Hp in patients with liver cirrhosis confirmed the proteomic profiling results. However, both serum s-Hp level and s-Hp% were not useful in the diagnosis of liver cirrhosis. Development of novel practical assays that can quantify a specific glycosylated variant of a particular serum glycoprotein is needed.

Acknowledgements

This study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#04050392). We thank Li Ka Shing Institute of Health Sciences for providing support on research facilities.

References

1. Friedman SL. Liver fibrosis—from bench to bedside. J Hepatol 2003;38(Suppl 1):S38-53.
2. Ryden I, Pahlsson P, Lindgren S. Diagnostic accuracy of alpha(1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy. Clin Chem 2002;48:2195-201.
3. Gravel P, Walzer C, Aubry C, et al. New alterations of serum glycoproteins in alcoholic and cirrhotic patients revealed by high resolution two-dimensional gel electrophoresis. Biochem Biophys Res Commun 1996;220:78-85.
4. Ang IL, Poon TC, Lai PB, et al. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glycoproteomic approach. J Proteome Res 2006;5:2691-700.
5. Chrostek L, Cylwik B, Krawiec A, Korcz W, Szmitkowski M. Relationship between serum sialic acid and sialylated glycoproteins in alcoholics. Alcohol Alcohol 2007;42:588-92.