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Original Article

Protein expression and fucosylated glycans of the serum haptoglobin-β subunit in hepatitis B virus-based liver diseases

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Glycosylation, which regulates the configuration and function of glycoproteins, is the most important post-translational modification. The aim of this study was to observe the differential patterns in glycan and protein parts of the serum haptoglobin-β subunit (Hp-β) purified from patients with hepatitis B virus (HBV) infection, liver cirrhosis (LC), or hepatocellular carcinoma (HCC). 2-D gel electrophoresis and multiplexed proteomics staining technique were employed to investigate whether the Hp-β glycan level was proportional to the protein level. Multi-lectin blot, high-performance liquid chromatography (HPLC), and western blot analysis were carried out to identify the glycoform of Hp-β quantitatively. Our experiments showed that the ratio of total serum Hp-β to the glycosylated form of Hp-β varied among the patients with different liver diseases. The total Hp-β protein expression level was much higher in HCC than LC, while an incremental proportion of fucosylated Hp-β was also observed in LC and HCC patients compared with that in HBV and healthy controls. Differential fucosylation was further identified as a Lewis X structure by HPLC and anti-human Sialyl–Lewis X antibody. In conclusion, the aberrant alternation of Hp-β glycan and total protein expression may be a promising biomarker for early hepatocarcinogenesis.

Keywords glycosylation; glycan analysis; liver disease

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Introduction

Protein glycosylation, one of the most important post-translational modifications, is observed in ~50% of proteins in mammalian cells, and it plays a critical role in many biological processes, including transcription, differentiation, apoptosis, signaling, and adhesion [1]. There are five known types of protein glycosylation: N-glycosylation, O-glycosylation, C-manonosylation, phosphoglycation, and glypiation [2]. Protein sequences containing Asn-X-Thr/Ser sequons (where X denotes any amino acid except for Pro) are frequently glycosylated at the asparagine residue through N-glycosylation. Human haptoglobin (Hp) is a liver-secreted serum glycoprotein with four potential N-glycosylation sites on its β chain. Aberrant glycan structures of Hp-β are reported in tumors such as breast cancer [3], pancreatic cancer [4,5], colon cancer [6], and prostate cancer [7]. Besides, changes in the Hp glycan structure are observed in several other diseases. Sarrats *et al*. [5] have detected increased levels of Sialyl–Lewis X (SLe(x)) on Hp in chronic pancreatitis, and Maresca *et al*. [8] have found that the level of distinct fucosylated glycoforms is higher in psoriasis patients than in healthy controls.

Chronic hepatitis B virus (HBV) infection is one of the most common etiologies for liver cirrhosis (LC), and the development of cirrhosis leads to an increased risk of hepatocellular carcinoma (HCC) [9], which is the fifth most common cancer and the third leading cancerous cause of death worldwide [10]. Nakagawa *et al*. [11] have suggested that fucosylation is a possible signal for the secretion of glycoproteins into bile ducts in the liver. The disruption of this system might involve an increase in fucosylated alpha fetoprotein (AFP) in the serum of patients with HCC. Our previous studies have also demonstrated the diagnostic value of serum Hp as a HCC candidate marker that was complementary to AFP [12]. However, the question about whether the proportion of glycosylated Hp-β differs during the developing stages of liver diseases remains further study. Additionally, the questions about the diagnostic value of these proportions for classifying HCC, LC, and HBV patients also need to be addressed.

To investigate the glycan patterns of serum Hp-β, three commonly used techniques for detecting the differential expression of glycans were implemented in this study:

lectin blot, high-performance liquid chromatography (HPLC), and multiplexed proteomics (MP) staining. Lectin blot can be considered to be an extension of western blot that detects carbohydrate epitopes on proteins based on lectins' binding specificities for specific sugar moieties [13]. Oligosaccharides released from glycoproteins can be labeled by fluorescence, subjected to normal-phase HPLC, and then compared with standard glycans for structure identification. MP staining technique, which includes a SYPRO Ruby protein gel stain and Pro-Q Emerald 488 glycoprotein stain, has also been developed to visualize the total protein or glycoproteins, respectively [14].

In this study, 2-D gel electrophoresis (2-DE) followed by the MP technique was used to investigate the changing of the total Hp-β expression and the expression of the protein's glycosylated forms in the sera of patients with HBV, LC, and HCC as well as healthy controls. Afterward, the glycan structure of SLe(x) on Hp-β was identified as a potential serum marker by determining the differential expression in the four groups mentioned above using lectin blots, HPLC, and anti-human SLe(x) antibody staining.

Materials and Methods

Materials

Peptide *N*-glycosidase F (PNGase F) was obtained from New England Biolabs (Ipswich, USA). Polyclonal rabbit anti-human Hp antibody was purchased from Dako Cytomation (Glostrup, Denmark), and anti-human SLe(x) was obtained from LifeSpan Biosciences (Seattle, USA). Biotinylated lectins (wheat germ agglutinin (WGA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Aleuria aurantia* lectin (AAL), *Lens Culinaris* agglutinin (LCA), *Phaseolus vulgaris* leucoagglutinin (PHA-L), concanavalin A (Con A), and *Datura stramonium* Lectin (DSA)) and horseradish peroxidase avidin D were purchased from Vector Laboratories (Burlingame, USA). The ECL™ plus western blotting detection reagent came from GE Healthcare (Piscataway, USA). The 2-aminopyridine (PA) and fucosylated glycan standards were purchased from Takara (Shiga, Japan).

Patients and specimens

Sera were pooled from 20 patients with HCC, 20 patients with LC, 20 patients with HBV, and 20 healthy controls (N) from the First Affiliated Hospital at Guangxi Medical University (NanNing, China) between February 2007 and March 2009. In addition, the individual serum samples for lectin blot and western blot analyses were derived from the same sample bank and stored at −80°C until analysis. The diagnosis of cirrhosis and hepatitis was based on clinical, laboratory tests (measurement of serum aspartate transaminase, alanine transaminase, HBV-DNA, HBsAg, and AFP), and imaging evidence in hepatic compensation stage. The diagnosis of HCC was made by liver histopathology or by two imaging modalities (ultrasound and computed tomography). The clinicopathological data of the patients are summarized in **Table 1**. This study was approved by the First Affiliated Hospital at Guangxi Medical University Research Ethics committee and the Institutional Review Board of the National Cancer Center.

Two-dimensional gel electrophoresis

Albumin and immunoglobulin (IgG) of the pooled sera were depleted with an Albumin/IgG Removal Kit (Merck KGaA, Darmstadt, Germany). For first-dimension gel electrophoresis, 70 μg of protein was mixed with lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM Tris-base, 65 mM dithiothreitol and 1.0% pharmalyte), and then applied to Immobiline Drystrips (7 cm, pH 3–10 nonlinear; GE Healthcare Bio-Sciences). Isoelectric focusing was carried out in an Ettan IPGphor 3 unit (GE Healthcare Bio-Sciences) for a total 35 kVh at 20°C. A second-dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was run at a constant current of 7.5 mA/gel for 45 min, followed by a run at 15 mA/gel until the bromphenol blue reached the bottom of the gel. Triplicate gels were run for each sample to reduce the gel-to-gel variations.

Multiplex proteome staining

Multiple 2-DE gels were stained serially with Pro-Q Emerald 488 and the ultrasensitive SYPRO Ruby gel stain kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. A gel scan of Pro-Q staining (λ_{ex}= 473 nm, λ_{em}= 520 nm) and Ruby staining (λ_{ex}= 532 nm, λ_{em}= 580 nm) was processed using a laser scanner Fuji FLA-5100 system (Fuji Film, Tokyo, Japan) at a resolution setting of 100 μm. Image analysis was performed with the ImageMaster 2D platinum 6.0 software to calculate the intensity of Hp-β spots (GE Healthcare Bio-Sciences). The intensity for each spot was normalized and averaged for further statistical analysis using the SPSS 12.0 package (SPSS Inc., Chicago, USA).

Purification of serum Hp-β

Approximately 20 μl of the individual serum samples (for lectin blot and western blot analyses) and 500 μl of the pooled sera (for HPLC analysis) were centrifuged and diluted with binding buffer (20 mM sodium phosphate, 0.15 M NaCl, pH 7.4). HiTrap N-hydroxy-succinimide activated Hp (GE Healthcare, Uppsala, Sweden) columns coupled with 200 μl of anti-human Hp antibody were used to purify Hp. The elution was concentrated by acetone precipitation followed by SDS-PAGE for further analysis of the β subunit.

Table 1 Clinicopathological characteristics of healthy individuals and patients with HBV-based liver diseases

Group	*n*	HBV	LC[a]	HCC[a]
Number	20	20	20	20
Sex (F/M)	4/16	9/11	2/18	3/17
Age (years)	46.6 ± 8.2	32.4 ± 12.6	42.8 ± 7.9	47.6 ± 6.1
HBV DNA (copy)[b]	NA	NA	4.5×10^5 (1.2×10^3–1.7×10^6)	1.1×10^6 (1.0×10^3–5.7×10^6)
AFP (ng/ml)[c]	NA	4.15 (0.8–5.0)	7.11 (2.8–14.3)	37952.05 (3.1–35350)
$HBsAg^+$(%)	0	100	100	100
AST (U/L)	20.1 (13–35)	46.5 (16–31)	84.71 (28–203)	102.13 (25–278)
ALT (U/L)	22.0 (10–36)	22.1 (13–40)	51.24 (29–99)	49.06 (15–105)

[a]LC and HCC diagnosis was confirmed by ultrasound imaging and biopsy.
[b]HBV DNA was detected with fluorescent quantitative PCR (FQ-PCR). The detection limit was $\sim 1 \times 10^3$ genome equivalents per ml.
[c]AFP was determined using standard kits (Abbott Labs, Abbott Park, USA), and 20 ng/ml was considered the upper limit of normal.

Lectin blot analysis

Purified Hp from the serum samples of HCC, LC, HBV, and control cohorts (three individuals per group) was electrophoresed on 12% polyacrylamide gel, and transferred to poly(vinylidene difluoride) (PVDF) membrane under semi-dry conditions with Trans-blot (Bio-Rad, Hercules, USA). The membranes were incubated with 3% bovine serum albumin in Tris-buffered saline-T (TBS-T) for 1 h and then incubated with 0.8 μg/ml biotinylated lectins for 0.5 h with a high-throughput incubation apparatus (Abmart, Shanghai, China). After washing the membrane three times with TBS-T, the membrane was incubated with 0.3 μg/ml horseradish peroxidase avidin D for 0.5 h, followed by washing three times with TBS-T, and visualized with ECL™plus western blotting detection reagents.

Glycan analysis of Hp-β with HPLC

The purified Hp-β subunit from the pooled sera of 20 individuals was digested with PNGase F, and the released N-glycans were treated with 2 M acetic acid for 2 h at 80°C to remove sialic acids. The sialic acid-free N-glycans were derivatized with PA [15]. The PA-labeled glycans of Hp-β and five kinds of commercial PA-fucosylated glycans were subjected to HPLC (Agilent 1100, Agilent Technologies, USA). The Asahipak NH2P-50 4E column (Showa denko, Kawasaki, Japan) was attached to a fluorescence detector, which was set at Ex 320 nm and Em 400 nm [16]. The flow rate was 1.0 ml/min, and the column was equilibrated with 35% solvent B (5% acetic acid in water containing 3% triethylamine) plus 65% solvent A (2% acetic acid in acetonitrile) for 2 min. Solvent A was then gradually decreased to 57% over 40 min at 50°C.

Western blot analysis

Purified Hp of six samples from each group were electrophoresed on 12% SDS-PAGE and transferred onto the PVDF membrane. The membranes were incubated with 5% skim milk powder in TBS-T, for 2 h, then incubated with anti-human SLe(x) (1:1000) or anti-human Hp antibody (1:1000) for 1 h. After washing with TBS-T for 15 min three times, the membrane was incubated with peroxidase-conjugated human anti-mouse IgG and anti-rabbit IgG (1:10,000, AMS Biotechnology, Abingdon, Oxfordshire, UK) antibodies for 1 h at room temperature. ECL™ western blotting detection reagent (Pierce Chemical, Rockford, USA) was used for detection.

Results

Comparison of Hp-β protein and glycan level on 2-DE gels

Figure 1A showed the enlarged images of five Hp-β spots in 2-DE profiles for the pooled sera staining with Pro-Q Emerald 488 and SYPRO Ruby. The overlaid profile was generated using the Multi Gauge V3.0, Fuji Film Corporation analysis program. The matched and unmatched spots were given a visual inspection after any two images were re-displayed as a single pseudo-colored map. The overlapped spots with similar intensities appear gold-like in color, while spots with different levels appear red or green. According to Image Master 6.0 software, GE Healthcare Bio-Sciences, protein levels of the Hp-β chain varied in different liver disease patients, and there was significantly decreased expression in the LC group [**Fig. 1(B)**]. In terms of Hp-β glycan levels, the intensity ratio of Pro-Q Emerald 488 staining to SYPRO Ruby staining was calculated. This value was much higher in LC and HCC patients than in HBV patients [**Fig. 1(C)**]. In conclusion, the change of the glycosylated Hp-β level in the four different groups was independent of the expression of Hp-β total protein. For example, the LC group had the lowest Hp-β protein level, but the LC groups had the highest glycan level.

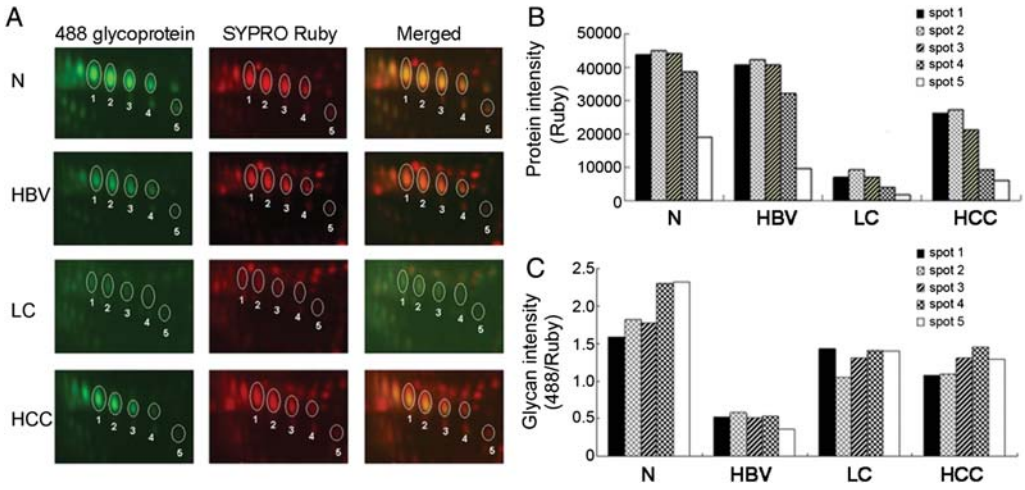

Figure 1 Comparison of Hp-β protein and glycan level on 2-DE gels (A) Enlarged images of Hp-β in 2-DE with Emerald 488 or SYPRO Ruby staining. The differential display maps were generated using the Multi Gauge V3.0 analysis program by overlaying the two maps. The overlapped spots with similar intensities have a gold-like color, while spots with different expression levels appear red or green. (B) A comparison of Hp-β protein expression in liver diseases and the spot intensity from SYPRO Ruby total protein staining. (C) A comparison of Hp-β glycan levels in liver disease. The glycan intensity (488/Ruby) was calculated as the ratio of spot intensity from the Pro-Q Emerald 488 glycoprotein stain to spot intensity from the SYPRO Ruby total protein stain.

Analysis of Hp-β glycoforms by lectin blot

To identify the glycoforms in Hp-β, 20 μl sera of the 12 individuals from four groups (three individual sera per group) was purified, followed by lectin blotting. Seven lectins, WGA, PHA-E, AAL, LCA, PHA-L, Con A, and DSA were chosen to compare the glycoforms of Hp-β from HBV, LC, HCC patients, and healthy controls [**Fig. 2(A)**]. The oligosaccharide-binding specificities of those lectins are summarized in **Table 2**. Significant differences ($P < 0.05$) in the glycan levels between LC, HCC patients, and healthy controls were found with a blotting index (AAL/Hp-β). As shown in **Fig. 2(A)**, Hp-β purified from serum showed much higher affinity to AAL and Con A lectins in HBV, and HCC compared with healthy controls. **Figure 2(B, C)** showed higher AAL binding of Hp-β in LC and HCC patients compared with HBV patients and healthy controls.

Identification of fucosylated glycan structure of Hp-β

The PA labeled glycans of the Hp-β chain from 500 μl of pooled sera were analyzed with normal phase HPLC. Five types of commercial PA-fucosylated glycans [**Fig. 3(A)**] were used as standards, and the chromatography results of fluorescently labeled N-glycans of Hp-β chains are shown in **Fig. 3(B)**. According to the glycan standards, Peak 3 represents the Lewis X-type fucose (α-1,3 fucose) containing glycan in Hp-β chain. The proportion of Peak 3 to the areas of all peaks increased significantly in the LC and HCC samples [**Fig. 3(C)**], which indicated a higher amount of Lewis X-containing fucosylated structures.

Confirmation of Lewis X-type fucosylated glycan in Hp-β

To further verify the Lewis X-type fucosylated glycan of Hp-β chains in sera, immunoblot analysis was used to measure the levels of Lewis X and Hp-β protein in individual sera (six samples from healthy controls, HBV patients, LC patients, and HCC patients). Purified Hp from each sample was subjected to western blotting using anti-human SLe(x) antibody and anti-Hp antibody, respectively. The glycoprotein intensity detected by the anti-human SLe(x) antibody was divided by total protein intensity detected by the anti-Hp antibody, and the quotient was used to quantify the Lewis X-type fucosylation level of Hp-β. The results revealed that Lewis X-type fucosylation in the LC and HCC patients was higher than healthy controls ($P < 0.05$, **Fig. 4**), while there was no significant difference between the LC and HCC groups.

Discussion

Hp is mainly produced by hepatocytes, and displays extensive polymorphism through disulfide bonds [17]. Aberrant glycosylation of Hp in liver diseases has attracted intensive attention, because a series of lectin-related assays and high pH anion exchange chromatography-pulsed amperometric detection have demonstrated that increased concentrations of fucose, *N*-acetyl glucosamine (GlcNAc), and highly branched structures of Hp are predominant in alcoholic liver diseases [18–20]. In Ang *et al.*'s [21] study, lectin affinity purification and 2-D gel electrophoresis were employed, and unique Hp glycoforms with altered

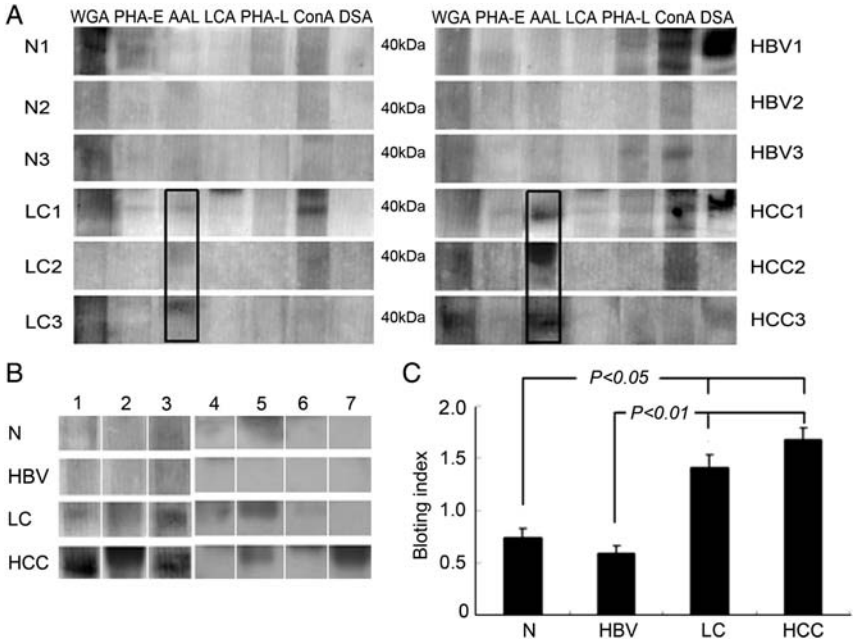

Figure 2 Comparison of glycoforms of serum HP-β from healthy people, HBV, LC, and HCC using lectin blot analysis (A) Purified Hp-β from equal amount of individual sera was run on a lectin blot using seven different lectins. The black frame indicated that the AAL-binding Hp-β was increased in LC and HCC patients compared with HBV patients and normal controls. (B) Lectin blot analyses on Hp-β purified from serum were performed. AAL binds to terminal fucose residue (1–7 represent seven samples). (C) Comparison of the glycan levels by lectin blotting using AAL between LC, HCC patients, and healthy controls ($P < 0.05$). Blotting index was calculated by AAL-binding bands and anti-Hp antibody (seven different bio-samples).

Table 2 Seven lectins used in this study and their carbohydrates-binding specificities

Lectins	Sugar-binding specificity
Wheat germ agglutinin	(GlcNAc)n and multivalent Sia
Aleuria aurantia lectin	Terminal αFuc and ± Sia-Lex
Lens culinaris agglutinin	Fucα1–6GlcNAc and α-Man, α-Glc
Concanavalin A	α-Man (inhibited by presence of bisecting GlcNAc)
Phaseolus vulgaris leucoagglutinin	Tetraantennary, complex oligosaccharides
Phaseolus vulgaris erythroagglutinin	NA2 and bisecting GlcNAc
Datura stramonium lectin	>Biantennary, (GlcNAc)n, polyLacNAc and LacNAc (NA3, NA4)

sialylation and fucosylation that were specific to HCC were found. This finding implies that glycan analysis provides valuable clues to find out which organ secrete Hp, as Hp generated by different organs shows specific glycan structures due to organ-specific fucosyltransferase [16].

In our study, seven kinds of lectin blots, HPLC analysis, and western blots were used to identify Hp-β glycoforms in the sera of patients with HBV, LC, and HCC. The results clearly indicated that purified Hp-β from LC and HCC patients displayed higher AAL affinity compared with healthy subjects and HBV patients. Because AAL binds α-fucosyl residue in various ways, HPLC was used to screen the exact fucosylated glycan structures in Hp-β of LC and HCC patients, in which the Lewis X-type fucose containing glycan was confirmed. This result was further confirmed by western blotting with anti-human SLe(x) antibody. The best possible explanation of this phenomenon is the ubiquitous increase of fucosyltransferase activity in liver disorder and the consequential increase of SLe(x)-type glycans, which might facilitate the inflammation and tumorigenesis [22].

Besides AAL affinity, the binding to Con A was also higher in HBV and HCC than in control (**Fig. 2**). Con A binds to high mannose-type, biantennary complex or hybrid type of asparagine-linked oligosaccharide with high affinity. The increase in Con A is also important in liver diseases [23]. One of our further studies in this area is to investigate Hp glycans with Con A-specific binding affinity from different stages of liver disease patients

Recently, glycosylation changes in glycoproteins have been investigated for their potential use as biomarkers and therapeutic targets. Taking into consideration the fact that glycans and proteins are the two parts of glycoprotein, it remains to be determined whether glycan expression tends to follow protein expression. Zhou *et al*. [24] have reported that protein levels of Hp have no significant differences between healthy control and climatic droplet keratopathy samples, but the glycosylated form of Hp is elevated in

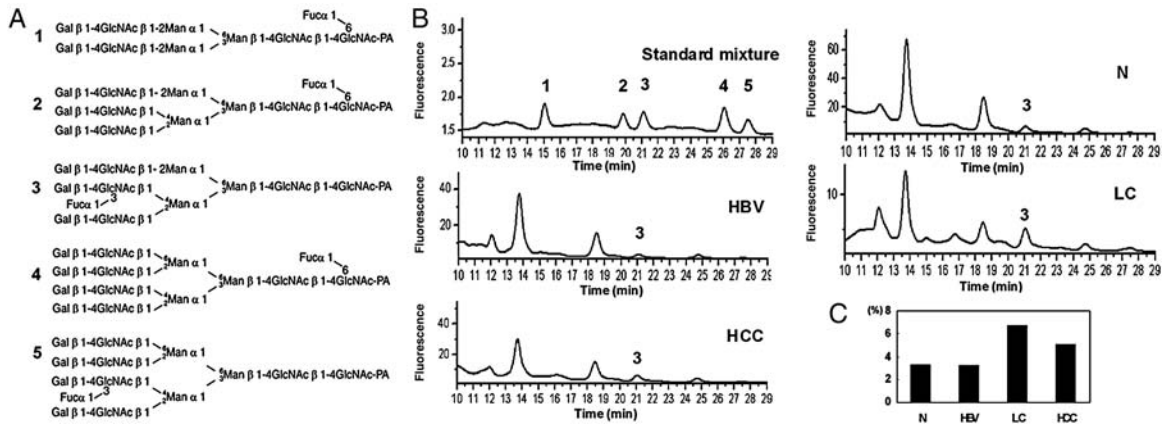

Figure 3 Fucosylated Hp-β glycan structure analysis with HPLC (A) The detailed structures of five commercial PA-fucosylated glycans and their peak numbers in chromatogram. (B) HPLC analysis of a standard mixture (five kinds of commercial PA-fucosylated glycans, 50 pmol each) and PA-labeled glycans of Hp-β purified from the pooled sera. (C) The percentages of Peak 3 derived from the Hp-β subunit amounts in the pooled sera of healthy controls ($n = 20$), HBV patients ($n = 20$), LC patients ($n = 20$), and HCC patients ($n = 20$), respectively.

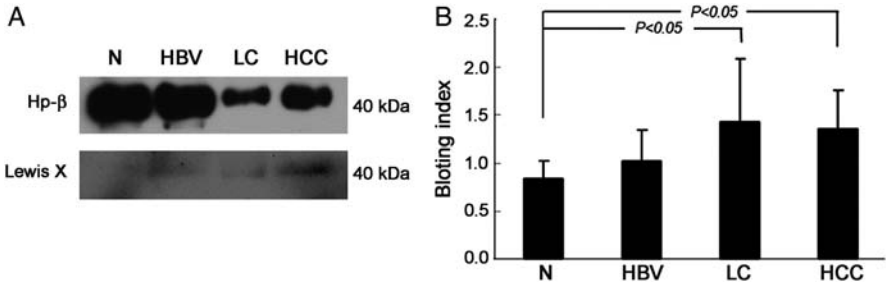

Figure 4 Western blot analysis for Lewis X-type fucosylation and Hp-β level (A) The representative pictures showing Lewis X-type fucosylation and Hp-β expression in liver disease patients and healthy people with western blotting. Purified Hp-β from equal amounts of individual sera from six healthy controls, six HBV patients, six LC patients, and six HCC was run on a western blot using SLe(x) antibody or Hp-β antibody. (B) The level of Lewis X-type fucosylation and Hp-β expression in the Hp-β chain of liver disease patients. Significant differences ($P < 0.05$) in the glycan levels between LC, HCC patients, and healthy controls were found with a blotting index (the quotient of anti-human SLe(x) bands and anti-Hp antibody).

climatic droplet keratopathy patients. Our previous studies have indicated that the Hp-β protein is differentially expressed in the sera of patients with HBV, LC, and HCC [25]. Moreover, we have also shown in this study that there was an increase of Lewis X-type fucosylation in Hp-β that could distinguish HCC and LC patients from healthy people. As demonstrated in this study, there were glycan changes and protein expression changes of Hp-β in liver disease patients. 2-DE analysis followed by the MP technique could be used to investigate the relationship between Hp-β glycan levels and protein levels in the sera of patients with HBV, LC, and HCC. The results revealed that the changing trend of Hp-β glycan level did not parallel with the changing trend of total protein expression. Notably, the protein expression of Hp-β was the lowest in the sera of LC patients. In contrast, Hp-β glycan levels in LC patients were almost as high as the levels in HCC patients.

HBV-based LC is a chronic disease of the liver where the normal liver architecture is replaced by fibrotic scar tissue. This disease is associated with an eventual decline in liver function [26]. Thus, it is extremely important to diagnose LC patients from the high-risk population as early as possible to implement clinical monitoring. AFP is an important marker for the progression of liver disease to liver cancer [27]. However, this marker cannot be used to distinguish chronic liver diseases, and there is no other serum indicator that is specific for LC patients. Our study found that decreased protein expression of serum Hp-β in combination with increased Lewis X-type fucosylation could be a promising diagnostic marker for LC detection. Increase in Hp Lewis X-type fucosylation has been suggested to impair the immune response, inhibit gelatinases in the extracellular matrix, promote angiogenesis, and may give an advantage to the tumor [8]. These findings also suggest that this marker could be employed in a course-monitoring strategy for the clinical management of patients with liver diseases.

In conclusion, increased Lewis X-type fucosylation in Hp-β was specific to LC and HCC patients. The trend of glycoyslated Hp-β expression is not in agreement with the trend of total Hp-β expression in different liver diseases. Instead, to check both the glycoyslated Hp-β and total

Hp-β protein expression in patients' sera can distinguish four groups of HCC, LC, HBV, and healthy individuals. A combined examination of Hp-β protein expression and its glycan level could potentially be utilized to diagnose HBV-based LC.

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References

1 Ohtsubo K and Marth JD. Glycosylation in cellular mechanisms of health and disease. Cell 2006, 126: 855–867.
2 Calvano CD, Zambonin CG and Jensen ON. Assessment of lectin and HILIC based enrichment protocols for characterization of serum glycoproteins by mass spectrometry. J Proteomics 2008, 71: 304–317.
3 Abbott KL, Aoki K, Lim JM, Porterfield M, Johnson R, O'Regan RM and Wells L, *et al*. Targeted glycoproteomic identification of biomarkers for human breast carcinoma. J Proteome Res 2008, 7: 1470–1480.
4 Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K and Murata K, *et al*. Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer 2006, 118: 2803–2808.
5 Sarrats A, Saldova R, Pla E, Fort E, Harvey DJ, Struwe WB and de Llorens R, *et al*. Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis. Proteomics Clin Appl 2010, 4: 432–448.
6 Park SY, Yoon SJ, Jeong YT, Kim JM, Kim JY, Bernert B and Ullman T, *et al*. N-glycosylation status of beta-haptoglobin in sera of patients with colon cancer, chronic inflammatory diseases and normal subjects. Int J Cancer 2010, 126: 142–155.
7 Yoon SJ, Park SY, Pang PC, Gallagher J, Gottesman JE, Dell A and Kim JH, *et al*. N-glycosylation status of beta-haptoglobin in sera of patients with prostate cancer vs. benign prostate diseases. Int J Oncol 2010, 36: 193–203.
8 Maresca B, Cigliano L, Corsaro MM, Pieretti G, Natale M, Bucci EM and Dal Piaz F, *et al*. Quantitative determination of haptoglobin glycoform variants in psoriasis. Biol Chem 2010, 391: 1429–1439.
9 Anand BS. Cirrhosis of liver. West J Med 1999, 171: 110–115.
10 Block TM, Mehta AS, Fimmel CJ and Jordan R. Molecular viral oncology of hepatocellular carcinoma. Oncogene 2003, 22: 5093–5107.
11 Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T and Gu J, *et al*. Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. J Biol Chem 2006, 281: 29797–29806.
12 Shu H, Kang X, Guo K, Li S, Li M, Sun L and Gan L, *et al*. Diagnostic value of serum haptoglobin protein as hepatocellular carcinoma candidate marker complementary to alpha fetoprotein. Oncol Rep 2010, 24: 1271–1276.
13 Kaji H, Yamauchi Y, Takahashi N and Isobe T. Mass spectrometric identification of N-linked glycopeptides using lectin-mediated affinity capture and glycosylation site-specific stable isotope tagging. Nat Protoc 2006, 1: 3019–3027.
14 Wu J, Lenchik NJ, Pabst MJ, Solomon SS, Shull J and Gerling IC. Functional characterization of two-dimensional gel-separated proteins using sequential staining. Electrophoresis 2005, 26: 225–237.
15 Kondo A, Suzuki J, Kuraya N, Hase S, Kato I and Ikenaka T. Improved method for fluorescence labeling of sugar chains with sialic-acid residues. Agric Biol Chem 1990, 54: 2169–2170.
16 Nakano M, Nakagawa T, Ito T, Kitada T, Hijioka T, Kasahara A and Tajiri M, *et al*. Site-specific analysis of N-glycans on haptoglobin in sera of patients with pancreatic cancer: a novel approach for the development of tumor markers. Int J Cancer 2008, 122: 2301–2309.
17 Polticelli F, Bocedi A, Minervini G and Ascenzi P. Human haptoglobin structure and function − a molecular modelling study. FEBS J 2008, 275: 5648–5656.
18 Chambers W, Thompson S, Skillen AW, Record CO and Turner GA. Abnormally fucosylated haptoglobin as a marker for alcoholic liver-disease but not excessive alcohol-consumption or nonalcoholic liver-disease. Clin Chim Acta 1993, 219: 177–182.
19 Goodarzi MT and Turner GA. Reproducible and sensitive determination of charged oligosaccharides from haptoglobin by PNGase F digestion and HPAEC/PAD analysis: glycan composition varies with disease. Glycoconj J 1998, 15: 469–475.
20 Mann AC, Record CO, Self CH and Turner GA. Monosaccharide composition of haptoglobin in liver-diseases and alcohol-abuse − large changes in glycosylation associated with alcoholic liver-disease. Clini Chim Acta 1994, 227: 69–78.
21 Ang IL, Poon TCW, Lai PBS, Chan ATC, Ngai SM, Hui AY and Johnson PJ, *et al*. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glycoproteomic approach. J Proteome Res 2006, 5: 2691–2700.
22 Niittymaki J, Mattila P and Renkonen R. Differential gene expression of GDP-L-fucose-synthesizing enzymes, GDP-fucose transporter and fucosyltransferase VII. APMIS 2006, 114: 539–548.
23 Sun Q, Liu Y, Lu W, Cheng G, Zhou H, Zhou X and Wei L, *et al*. Con A affinity glycoproteomics of normal human liver tissue. Sci China C Life Sci 2007, 50: 403–411.
24 Zhou L, Beuerman RW, Chew AP, Koh SK, Cafaro TA, Urrets-Zavalia EA and Urrets-Zavalia JA, *et al*. Quantitative analysis of N-linked glycoproteins in tear fluid of climatic droplet keratopathy by glycopeptide capture and iTRAQ. J Proteome Res 2009, 8: 1992–2003.
25 Shu H, Kang XN, Li M, Guo K, Sun L, Li S and Xie L, *et al*. Comparison of the serum proteomes of pathological stages during hepatocarcinogenesis. Zhonghua Gan Zang Bing Za Zhi 2009, 17: 520–525.
26 Mehta A and Block TM. Fucosylated glycoproteins as markers of liver disease. Dis Markers 2008, 25: 259–265.
27 Ruoslaht E, Salaspur M, Pihko H, Andersso L and Seppälä M. Serum alpha-fetoprotein - diagnostic significance in liver-disease. Br Med J 1974, 2: 527–529.