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Original Article

Quantitative analysis of site-specific *N*-glycans on sera haptoglobin β chain in liver diseases

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The site-specific characterization of *N*-glycans in glycoproteins with the potential of clinical application is important. In our previous report, the overall *N*-glycans of sera haptoglobin (Hp) β chain were found to be different in liver diseases. Hp β chain contains four potential sites of *N*-glycosylation. In this study, we investigated the potential change of *N*-glycans on Hp β chain in a site-specific fashion. Sera Hp β chain in healthy individuals as well as patients with hepatitis B virus (HBV), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) were purified, digested and subjected to liquid chromatography-electrospray ionization-higher energy collision dissociation mass spectrometry, which allowed identification and structure determination of the glycopeptide, as well as the relative quantification of glycans present on each glycopeptide. The quantitative results revealed that the sialylation of NLFLN^{207}HSEN^{211}ATAK and the fucosylated structure at all glycopeptides increased significantly in LC and HCC patients compared with those in HBV patients and healthy individuals. A set of different *N*-glycan patterns of Hp β chain in various liver diseases has been determined. Thus, the sialylated and fucosylated glycoforms of Hp β chain might be related to early hepatocarcinogenesis and also might be useful as novel differential markers for LC and HCC patients.

Keywords haptoglobin; liver diseases; site-specific *N*-glycan; mass spectrometry

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Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths and frequently diagnosed only at an advanced stage when limited therapeutic options are available [1]. α-Fetoprotein (AFP) is an established biomarker for the progression of liver disease to liver cancer [2], but it is not efficient to distinguish various forms of chronic liver diseases. Furthermore, serum AFP levels can be elevated under many physiological circumstances, thus it affords restricted specificity discriminating the pathological states of liver diseases. The establishment of auxiliary diagnosis biomarkers may attenuate these complications and aid early differential diagnosis of liver disease. On the basis of our previous investigation, we have examined serum haptoglobin (Hp) as a candidate biomarker for liver disease [3–5].

Human Hp is a tetrameric protein composed of two α and two β chains [6,7]. The Hp β chain contains four potential sites of *N*-glycosylation and it can be digested into three glycopeptides by trypsin. In our previous report, the protein expression level of Hp β chain has been found to be higher in HCC patients compared with those in liver cirrhosis (LC) patients [3] and the overall glycan structure has been found to be different as well [4,5]. In this study, we analyzed *N*-glycan structures on the Hp β chain in a site-specific fashion and quantified the *N*-glycans present on each glycopeptide of the Hp β chain in liver diseases.

Glycopeptide analysis using liquid chromatography mass spectrometry (LC-MS) allows simultaneous characterization of glycan structure and attached peptide site. However, the ionization efficiency of glycopeptide is different from that of non-modified peptide, as the chemical properties are quite different between a hydrophilic glycan and a hydrophobic peptide [8]. Higher energy collision dissociation (HCD) on the Orbitrap-XL platform performs well for the analysis of modified peptides [9] and it is also of benefit in structure elucidation, because significant sequence information together with diagnostic immonium ions and immonium-related ions can be obtained. For glycopeptides, the glycosidic linkage is

weaker than the peptide backbone. HCD-MS/MS will mainly produce glycosidic fragmentation to characterize the glycan structure attached to different *N*-glycosylation sites. Compared with non-modified peptides, glycopeptides usually have lower content in samples and poorer electrospray ionization efficiency. In this study, nano LC flow conditions and HCD-MS/MS provided glycan oxonium ions such as m/z 366 and m/z 528 that could obtain extracted ion chromatograms (XIC) of glycopeptides.

It is necessary to develop qualitative and quantitative methods to analyze oncologically interesting glycoproteins. Klapoetke *et al*. [10] employed ultra performance liquid chromatography-electrospray ionization (ESI) Time of Flight MS to identify the glycopeptides and deglycopeptide of human IgA1. Grass *et al*. [11] measured the intensity of the base peak of summed spectrum covering the entire analyte peak in XIC. The approaches for quantitative glycoproteomics are attractive, because they use label-free steps for highly precise abundance measurements and are comparable to stable isotope labeling methods. In this study, the same glycopeptides of Hp β chain were compared in liver diseases by calculating the percentage of one glycoform at each glycosylation site without labeling.

Materials and Methods

Preparation of specimens

The serum specimens used in this study were obtained from the First Affiliated Hospital of Guangxi Medical University (Nanning, China). Informed consent was obtained from each patient and the protocols were approved by the First Affiliated Hospital of Guangxi Medical University Research Ethics Committee and the Institutional Review Board of the National Cancer Center. The pathological data of the patients were listed in **Table 1**. All serum samples were collected according to a standard protocol and stored at −80°C until use.

Purification of Hp β chain from sera

A total of 500 μl of sera were centrifuged at 12,000 *g* for 10 min and albumin and IgG were depleted by using an Albumin/IgG Removal Kit (Merck KGaA, Darmstadt, Germany). The depleted sera were applied to a HiTrap (GE Healthcare, Uppsala, Sweden) [12] column packed with n-hydroxysuccinimide-activated resin coated with 300 μl of anti-human haptoglobin antibody (4.5 mg). The haptoglobin bound to the column was eluted with elution buffer (pH3.0, 100 mM Glycine, 0.5 M NaCl) and concentrated by acetone precipitation. Purified haptoglobin was displayed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate β chain and then stained with coomassie brilliant blue.

Tryptic digestion and LC-ESI-HCD-MS/MS analysis

The SDS-PAGE gels were destained, reduced, alkylated and then digested with trypsin at an enzyme-to-substrate ratio of 1:30 (w/w). The non-modified peptides and glycopeptides were bound to a Magic C18 AQ reverse phase column (100 μm × 50 mm; Michrom Bioresources, Auburn, USA) and eluted with a three-step linear gradient, starting from 5% B to 45% B in 40 min (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid), increased to 80% B in 3 min, and then to 5% B in 2 min. The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 500 nl/min and the column temperature was maintained at 35 °C. The electrospray voltage of 1.2 kV versus the inlet of the mass spectrometer was used.

The LTQ Orbitrap XL mass spectrometer (Thermo Electron Corp., Bremen, Germany) was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 400−2000) were acquired in the Obitrap with a mass resolution of 60,000 at m/z 400, followed by five sequential HCD MS/MS scans. The automatic gain control was set to

Table 1 Characteristics of healthy individuals and patients with HBV, cirrhosis, and HCC

Group	*N*	HBV	LC[c]	HCC[c]
Number	20	20	20	20
Sex (F/M)	5/15	5/15	2/18	2/18
Age (years)	29.6 ± 3.8	21.6 ± 4.1	50.7 ± 5.2	50.4 ± 6.1
HBV DNA (copy)[a]	NA	1.8×10^6 (1.2×10^3–2.6×10^6)	1.7×10^5 (1.8×10^3–6.7×10^5)	2.3×10^4 (1.2×10^3–1.5×10^5)
AFP (ng/ml)[b]	NA	1.2 (0.9–2.6)	16.2 (3.1–38.7)	4120.4 (56.0–35,350.0)
$HbsAg^+$(%)	0	100	100	100
AST (U/L)	20.5 (12–30)	43.6 (15–110)	120.5 (39–692)	110.5 (35–237)
ALT (U/L)	12.5 (11–27)	65.7 (20–211)	120.6 (36–569)	100.2 (43–382)

[a]HBV DNA was detected with fluorescent quantitative polymerase chain reaction and has a detection limit of sensitivity of approximately 1×10^3 genome equivalents per ml.

[b]AFP was determined using standard kits (Abbott Labs, Chicago, USA) and 20 ng/ml was considered the upper limit of normal.

[c]LC and HCC diagnoses were confirmed by ultrasound imaging and biopsy.

1,000,000 ions to prevent over-filling of the ion trap. The minimum MS signal for triggering MS/MS was set to 20,000. In all cases, one microscan was recorded. MS/MS scans were acquired in the Obitrap with a mass resolution of 7500. Dynamic exclusion was used with one repeat count, 10-s repeat duration, and the m/z values triggering MS/MS were put on an exclusion list for 30 s. For MS/MS, precursor ions were activated by using 40% normalized collision energy and an activation time of 30 ms.

Database searching and glycopeptide quantification

RAW files generated by XCalibur software were used to database search with MASCOT. The parameters for this search were as follows: one missed cleavage allowed from tryptic digestion, ±10 ppm tolerance for precursors, ±0.3 Da tolerance for MS/MS, oxidation of methionine (variable modification), carbamidomethyl of cysteine (fixed modification). Glycan oxonium ions were used to identify the XIC of glycopeptides and the peaks of the diverse charged ions of glycopeptides were used to calculate intensities of these glycopeptides.

Results

Hp β chain purification and identification

Hp has been purified from sera of patients with hepatitis B virus (HBV), patients with LC, HCC patients, and healthy individuals. It is a glycoprotein having an $(\alpha\beta)_2$ tetramer structure (MW ≈ 130,000 Da). The β chain (MW ≈ 40,000 Da) contains four N-linked glycosylation sites. The affinity-purified Hp was subjected to SDS-PAGE. **Figure 1** showed the results of SDS-PAGE analysis of Hp β chain from eight healthy individuals, eight HBV patients, eight LC patients, and eight HCC patients. Proteins were then excised from the gels, subjected to trypsin digestion and analyzed by LC-ESI-HCD-MS/MS. The results of MASCOT searching confirmed that these protein bands corresponded to Hp, suggesting that the purification method was feasible.

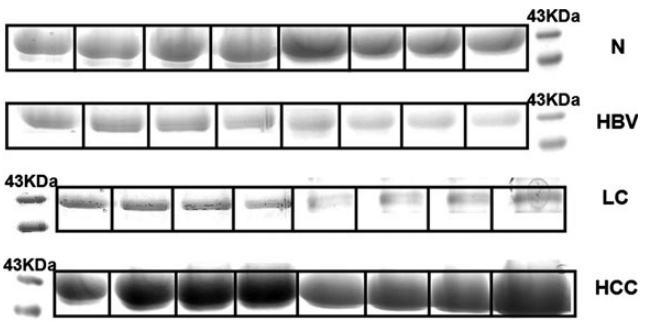


Figure 1 Purification of Hp β chain from equal volume of 8 healthy, 8 HBV, 8 LC and 8 HCC patients' sera The purified Hps were subject to SDS-PAGE and stained with coomassie brilliant blue.

Hp is known to be readily digested with trypsin [13] and this enzyme digestion generates three glycopeptides, MVSHHN^{184}LTTGATLINEQWLLTTAK (T1), NLFLN^{207}HSEN^{211}ATAK (T2) and VVLHPN^{241}YSQVDIGLIK (T3). Glycosylation site occupancy at Asn241 has been found to change significantly in HCC patients [14]. Here, automatic database searching was employed to match peptides against the known Hp sequence and the matched non-glycosylated peptide was shown in bold red (**Supplementary Fig. S1**). The non-glycosylated form of VVLHPN^{241}YSQVDIGLIK could be identified in LC-ESI-HCD-MS/MS, while the non-glycosylated forms of T1 and T2 were not observed. We also found the non-glycosylated form of this glycopeptide in MS together with its glycosylated form as shown in **Supplementary Fig. S1**. These data suggested that Asn241 was partially occupied and there might be difference in glycosylation site occupancy.

Site-specific *N*-glycans on sera Hp β chain

Glycopeptides often produce low molecular weight glycan oxonium ions [15,16] which help locate glycopeptides from the base peak chromatogram (**Fig. 2**). The retention time for T1, T2, and T3 was identified to be 35–36, 20–21 and 31–32 min, respectively. The shorter time for T2 was expected, suggested its increased hydrophilicity.

For each cluster of glycopeptides found in the complex LC-MS chromatogram, the peak was identified based either on the pregenerated mass list or by adding and subtracting the masses of *N*-acetylhexosamine (HexNAc), hexose (Hex), fucose (Fuc), and sialic acid (SA) residues [17]. In this study, tandem MS of most peaks in the MS spectra was acquired to assign the structure of the *N*-glycan. MS/MS spectra of the precursor peak at m/z 1222 ($z = 4$) and 1237 ($z = 3$) were shown in **Fig. 3**. Since the glycan moiety is more labile than the peptide backbone, the diagnostic ions are commonly observed from *N*-glycan fragmentation (m/z 204, 366 and 657) [18] and the inner most GlcNAc attached at the glycosylation site of the peptide (GlcNAc + peptide) can be used to analyze the complex glycopeptide map of Hp β chain. **Tables 2, 3 and 4** listed glycoform mass and oligosaccharide composition of each glycopeptide which were found in pooling sera of 20 healthy people, 20 HBV patients, 20 LC patients, and 20 HCC patients. Here, it was found that NLFLN^{207}HSEN^{211}ATAK contained seven glycoforms including bi-, tri-, and fucosylated glycans. Five glycoforms including bi-, tri-, and fucosylated glycans were present at Asn184. Asn241 contained seven glycoforms including bi-, tri-, tetra-, and fucosylated glycans.

Quantitative analysis of altered glycopeptides

Samples from eight healthy people, eight HBV patients, eight LC patients, and eight HCC patients were analyzed individually by LC-ESI-HCD-MS/MS. Analytical parameters of instrument remained the same when analyzing different

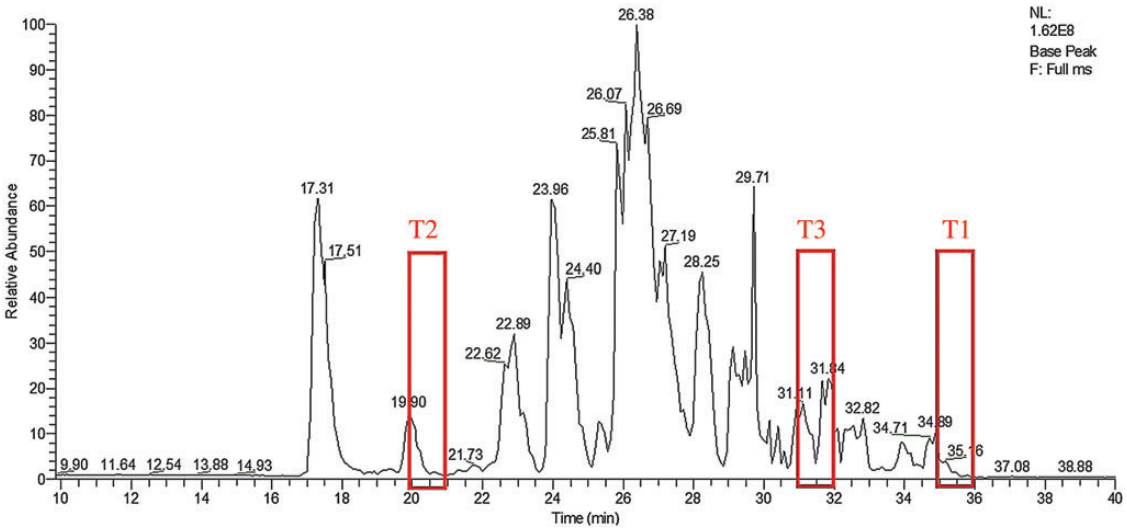


Figure 2 Base peak chromatogram of LC-ESI-MS analysis for Hp digest with trypsin The retention time for T1, T2, and T3 was identified to be approximately 35–36, 20–21, and 31–32 min, respectively.

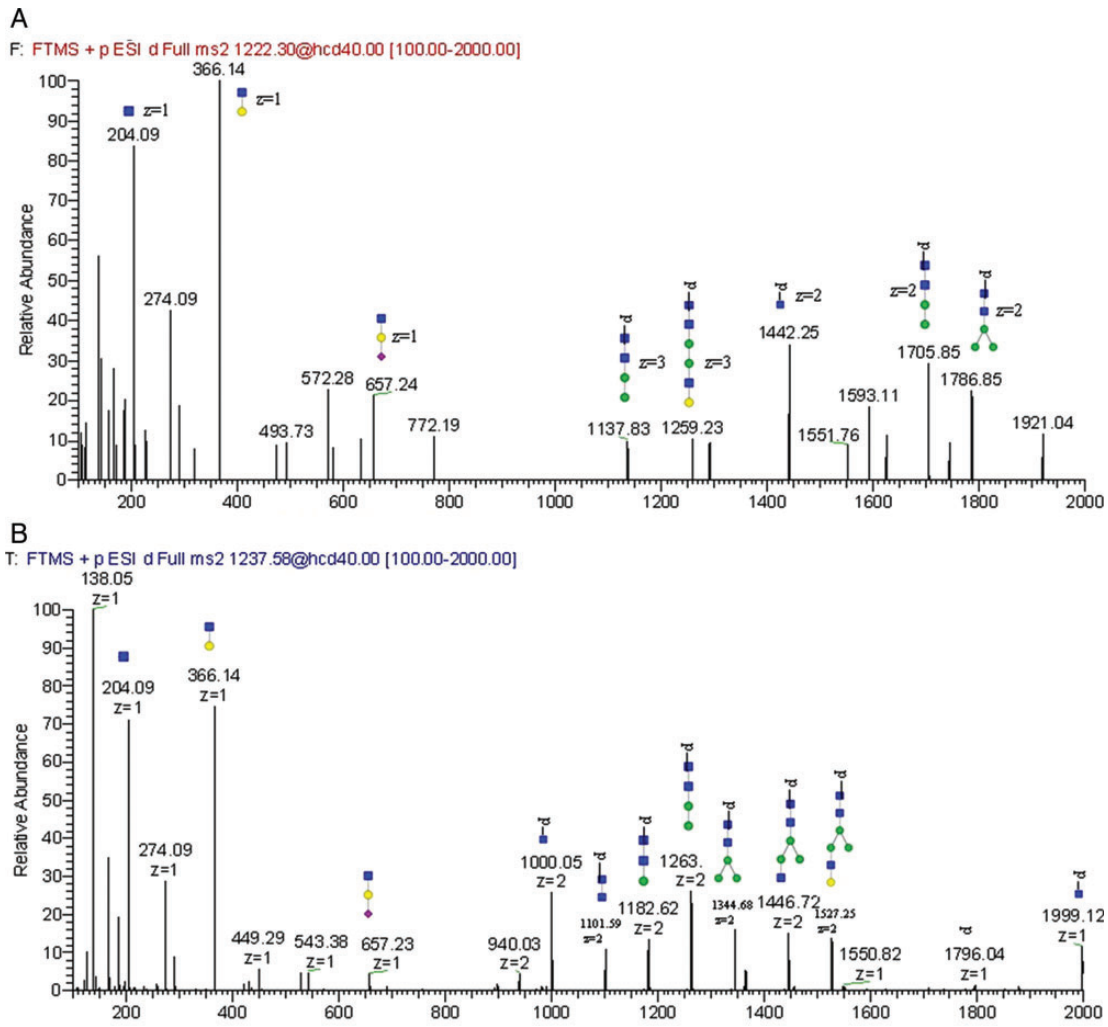
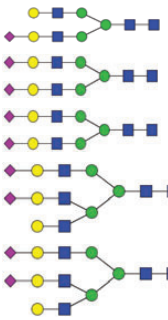


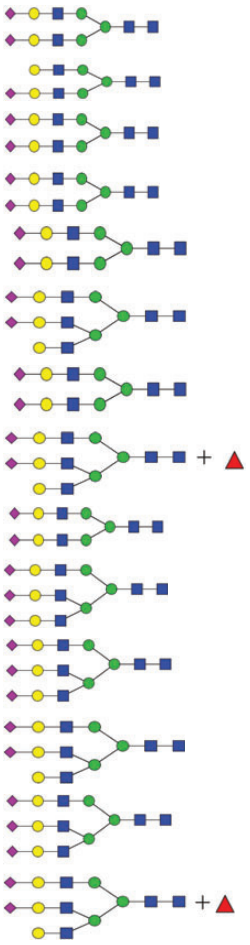
Figure 3 MS/MS spectra of the precursor peak at m/z 1222 (A) and 1237 (B) Blue square, GlcNAc; green circle, mannose; yellow circle, galactose; pink diamond, sialic acid; p, peptide.

Table 2 Site-specific *N*-glycans on MVSHHN184LTTGATLINEQWLLTTAK

Glycoform	Glycoform mass	Oligosaccharide composition	Putative glycan structure
T1-01	4595.8	1SA:5Hex:4HexNAc	
T1-02	4886.2	2SA:5Hex:4HexNAc	
T1-03	5033.0	1Fuc:2SA:5Hex:4HexNAc	
T1-04	5251.4	2SA:6Hex:5HexNAc	
T1-05	5398.2	1Fuc:2SA:6Hex:5HexNAc	

Blue square, GlcNAc; green circle, mannose; yellow circle, galactose; pink diamond, sialic acid; deep red triangle, fucose and p peptide.

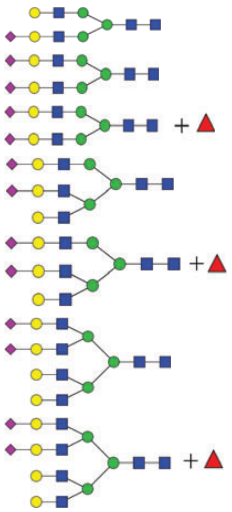
Table 3 Site-specific N-glycans on NLFLN^{207}HSEN^{211}ATAK

Glycoform	Glycoform mass	Oligosaccharide composition	Putative glycan structure
T2-01	5580.2	(1SA:5Hex:4HexNAc) + (2SA:5Hex:4HexNAc)	
T2-02	5871.0	(2SA:5Hex:4HexNAc) + (2SA:5Hex:4HexNAc)	
T2-03	6236.2	(2SA:6Hex:5HexNAc) + (2SA:5Hex:4HexNAc)	
T2-04	6382.2	(1Fuc:2SA:6Hex:5HexNAc) + (2SA:5Hex:4HexNAc)	
T2-05	6528.6	(3SA:6Hex:5HexNAc) + (2SA:5Hex:4HexNAc)	
T2-06	6893.4	(3SA:6Hex:5HexNAc) + (2SA:6Hex:5HexNAc)	
T2-07	7039.8	(3SA:6Hex:5HexNAc) + (1Fuc:2SA:6Hex:5HexNAc)	

Blue square, GlcNAc; green circle, mannose; yellow circle, galactose; pink, sialic acid; deep red, fucose and p peptide.

samples. Identical LC-ESI-HCD-MS/MS conditions were used to compensate the effect of ionization efficiency. Glycopeptides were detected in several charge states depending on their size and composition. Thus, a worthwhile quantification of glycoforms must include all charge states.

Table 4 Site-specific N-glycans on VVLHPN^{241}YSQVDIGLIK

Glycoform	Glycoform mass	Oligosaccharide composition	Putative glycan structure
T3-01	3710.7	1SA:5Hex:4HexNAc	
T3-02	4001.8	2SA:5Hex:4HexNAc	
T3-03	4147.9	1Fuc:2SA:5Hex:4HexNAc	
T3-04	4366.9	2SA:6Hex:5HexNAc	
T3-05	4513.0	1Fuc:2SA:6Hex:5HexNAc	
T3-06	4732.0	2SA:7Hex:6HexNAc	
T3-07	4878.1	1Fuc:2SA:7Hex:6HexNAc	

Blue square, GlcNAc; green circle, mannose; yellow circle, galactose; pink diamond, sialic acid; deep red triangle, fucose and p peptide.

Table 5 The average percentage of one glycoform at each glycosylation site in Hp β chain of eight healthy, eight HBV, eight LC, and eight HCC patients' samples

T1		**T1-01**	**T1-02**	**T1-03**	**T1-04**	**T1-05**		
N	Average percentage	0.066133	0.879626	0.008231	0.043108	0.002902		
N	SD	0.041	0.054	0.002	0.017	0.03		
HBV	Average percentage	0.058527	0.879843	0.008284	0.049779	0.003568		
HBV	SD	0.047	0.066	0.002	0.030	0.003		
LC	Average percentage	0.035054	0.873313	0.023052	0.043864	0.024717		
LC	SD	0.018	0.044	0.005	0.016	0.020		
HCC	Average percentage	0.046796	0.890965	0.017159	0.0351	0.00998		
HCC	SD	0.031	0.055	0.004	0.018	0.007		
T2		**T2-01**	**T2-02**	**T2-03**	**T2-04**	**T2-05**	**T2-06**	**T2-07**
N	Average percentage	0.062245	0.305407	0.45126	0.124652	0.04549	0.008726	0.002094
N	SD	0.067	0.124	0.078	0.065	0.083	0.012	0.004
HBV	Average percentage	0.084805	0.219335	0.468638	0.175981	0.020895	0.022607	0.007739
HBV	SD	0.128	0.124	0.107	0.054	0.021	0.031	0.011
LC	Average percentage	0.04269	0.212236	0.313105	0.261753	0.099082	0.048151	0.022983
LC	SD	0.040	0.086	0.057	0.050	0.029	0.051	0.022
HCC	Average percentage	0.030072	0.2618	0.296571	0.260829	0.097722	0.043089	0.009917
HCC	SD	0.042	0.183	0.072	0.094	0.087	0.031	0.010
T3		**T3-01**	**T3-02**	**T3-03**	**T3-04**	**T3-05**	**T3-06**	**T3-07**
N	Average percentage	0.043797	0.874887	0.004205	0.067477	0.002315	0.00719	0.000264
N	SD	0.029	0.055	0.001	0.028	0.002	0.004	0.000
HBV	Average percentage	0.037082	0.860817	0.004225	0.085663	0.004192	0.007799	0.000223
HBV	SD	0.034	0.069	0.002	0.039	0.004	0.006	0.000
LC	Average percentage	0.027567	0.824195	0.018443	0.082737	0.035495	0.008226	0.003337
LC	SD	0.023	0.065	0.005	0.030	0.022	0.007	0.004
HCC	Average percentage	0.024831	0.868594	0.019519	0.063935	0.016121	0.005559	0.00144
HCC	SD	0.030	0.049	0.007	0.019	0.013	0.002	0.002

Table 6 ***T*-test for evaluation of glycoform varicance in Hp β chain of eight healthy, eight HBV, eight LC, and eight HCC patient samples**

P	T1-01	T1-02	T1-03	T1-04	T1-05		
N: HBV	0.737	0.994	0.953	0.593	0.659		
N: LC	0.081	0.801	1.24E-05	0.929	0.018		
N: HCC	0.311	0.682	7.53E-05	0.386	0.026		
HBV: LC	0.222	0.820	1.46E-05	0.630	0.021		
HBV: HCC	0.567	0.720	2.20E-05	0.255	0.041		
LC: HCC	0.377	0.490	0.012	0.324	0.081		
P	T2-01	T2-02	T2-03	T2-04	T2-05	T2-06	T2-07
N: HBV	0.666	0.187	0.717	0.109	0.440	0.268	0.202
N: LC	0.493	0.103	0.001	0.000	0.118	0.032	0.033
N: HCC	0.273	0.586	0.001	0.005	0.240	0.018	0.063
HBV: LC	0.400	0.896	0.003	0.005	2.21E-05	0.244	0.110
HBV: HCC	0.280	0.595	0.002	0.044	0.041	0.211	0.677
LC: HCC	0.552	0.503	0.618	0.981	0.967	0.814	0.155
P	T3-01	T3-02	T3-03	T3-04	T3-05	T3-06	T3-07
N: HBV	0.675	0.661	0.983	0.300	0.209	0.814	0.849
N: LC	0.234	0.116	5.66E-05	0.312	0.004	0.700	0.065
N: HCC	0.221	0.814	0.000	0.772	0.019	0.269	0.065
HBV: LC	0.518	0.296	2.17E-06	0.868	0.005	0.896	0.062
HBV: HCC	0.455	0.800	0.000	0.182	0.035	0.357	0.060
LC: HCC	0.841	0.147	0.720	0.153	0.050	0.297	0.236

The intensity of the most intense isotopic peak of the glycoform was measured in the present study. After the calculation of the percentage of one glycoform at each glycosylation site (**Table 5**), the changes in glycoform intensities were evaluated by using *F*-test and *t*-test. Generally, a change associated with a *P* value less than 0.05 is considered to be significant, suggesting a 5% probability that the difference is not valid [19]. A list of *t*-test *P* values for glycoforms of each glycopeptide was shown in **Table 6**. Peaks T1-03, T1-05, T2-03, T2-04, T2-05, T2-06, T3-03, and T3-05 had *P* values lower than 0.05 and changed remarkably. These quantitative results revealed that the fucosylated structure increased significantly at each glycopeptide of Hp β chain in LC and HCC patients compared with HBV patients and normal subjects (T1-03, T1-05, T2-04, T3-03, and T3-05), while the increased sialylation of LC and HCC patients only occurred on NLFLN^{207}HSEN^{211}ATAK (T2-03, T2-05 and T2-06).

Discussion

Good quantification and reproducibility of glycopeptide profiling by using MALDI and ESI MS have been reported [20]. Compared with glycan profiles, the analysis of glycopeptides circumvents many problems [21]. However, glycopeptide analysis allows the identification of site-specific glycan structures which are essential to understand the microheterogeneity on multiglycosylated proteins and the biological significance of local glycans [22,23]. LC-ESI-MS was selected to analyze glycopeptides as sialic acids are highly labile in MALDI MS [24]. For qualitative analysis, the *N*-glycan structures of Hp β chain were elucidated by using HCD-MS/MS of glycopeptides ions. Tentative confirmation of glycopeptides was obtained by spacing between the peaks of glycoform, which corresponded to monosaccharide residue masses. For quantitative analysis, all charge states of one glycoform were performed to calculate the level of this glycoform on its glycosylation site, since there was distribution of multiple-charge ions of the glycoform.

Glycopeptides T2 with two glycosylation sites eluted earlier and trypsin digestion was unable to cleave the peptide backbone between the closely spaced glycosylation sites at Asn207 and Asn211. Seven glycan compositions could be identified on this glycopeptide on the basis of observed m/z and MS/MS fragments. However, **Table 3** only showed the potential glycan structures of two glycosylation sites. It was not possible to identify the exact glycan at each site when the two glycans differed from each other. For example, we could not determine which site the fucose of T2-04 was attached to. Detailed analysis of these two glycosyaltion sites is still needed in the future.

Fucose and sialic acid play important roles in glycosylation and are associated with many diseases. For example, expression of A and B blood group antigens is lost in many tumors with concomitant increases in H and Lewis^{y}

expression, changes that correlate with poor clinical prognosis [25]. Up-regulation of sialyl Lewis^{x} and sialyl Lewis^{a} has been demonstrated in numerous cancers [26]. This study showed the fucosylated structure increased significantly at each glycopeptide of the Hp β chain in LC and HCC patients compared with HBV patients and normal individuals. In addition, sialylation of NLFLN^{207}HSEN^{211}ATAK was found to be higher in LC and HCC patients.

Despite surgery, radiotherapy, and chemotherapy for HCC, there is still an urgent need for an early diagnostic strategy for detection. Recently, glycoprotein alterations such as AFP-L3 have provided useful clues for biomarker development [27,28]. However, it should be pointed out that the lectin method cannot determine the 'true' amount of glycan on each glycopeptide due to lectin reactivity and separation difficulties [29]. The current LC-ESI-MS technology could be used to demonstrate the relative amount of *N*-glycans expressed at each site. Our finding is considered preliminary and the effect of glycosyl transferases should be considered in addition to a larger group validation. Nevertheless, it is suggested that glycopeptide alterations of Hp β chain, especially the fucosylation and sialylation changes on the NLFLN^{207}HSEN^{211}ATAK glycopeptide, might be useful as novel glyco-biomarkers for LC and HCC patients.

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Supplementary Data

Supplementary data are available at *ABBS* online.

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