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Analysis of serum Haptoglobin using glycoproteomics and lectin immunoassay in liver diseases in Hepatitis B virus infection

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) due to Hepatitis B viral (HBV) infection is a major cause in Asia-Pacific countries. Its early detection is of paramount importance using a marker having both sensitivity and specificity. The present study promises diagnostic and prognostic markers by the identification of site-specific glycoforms on Haptoglobin (Hp) using LC-MS/MS and lectin ELISA in liver diseased conditions in HBV infection.
Methods: Three groups of patients: chronic, liver cirrhosis and HCC with HBV infection along with controls were enrolled. Hp was purified using affinity column chromatography and, peptide sequence, N-glycosylation site, glycan composition and glycoforms were identified using mass spectrometry. Quantitative lectin ELISA was used to measure levels of fucosylation on Hp in liver diseases due to HBV.
Results: Hp levels were significantly lower in HCC when compared with Non-HCC cases ($p < .05$). Fucosylated glycoforms were significantly increased at site Asn184, Asn207 and Asn211 in liver diseased stages versus controls. A significant association was observed between the Fuc-Hp/Hp Elisa index and, advanced liver disease stages and controls using lectin Elisa ($p < .001$).
Conclusion: Quantitation of fucosylation levels on Hp protein using Lectin ELISA may be useful glycobiomarker either alone or in combination (AFP + DCP + FucHp; AUC = 0.94) in HBV HCC diagnosis in clinical practice.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is a leading cause of cancer-related death worldwide [1]. HCC is the sixth most common cancer and it accounts for approximately 5.7% of all new cancer cases [2]. Chronic HBV infection (CHB) has a well-known association with HCC [3]. It is estimated that Hepatitis B Virus (HBV) is responsible for 50% to 80% of HCC cases worldwide. India comes under intermediate endemicity for HBV infection (with an average of 4%), with a disease burden of about 50 million [4,5].

The established diagnostic and prognostic serum biomarker, alpha-fetoprotein (AFP) is neither sensitive nor specific for detection of HCC [1,6,7]. AFP exists in controversies for its clinical usefulness for HCC detection. American Association for the study of Liver disease (AASLD) guidelines for the management of HCC have excluded diagnostic use of serum AFP (cut off 200 ng/ml) due to false positivity [8]. However, European and Asian Pacific guidelines still recommend its use for diagnosis of HCC [9].

Protein glycosylation is ubiquitous post translational modification (PTM), playing an important role in pathophysiological processes. In humans, approximately 70% of proteins are glycosylated [10]. Glycosylation of several human plasma glycoproteins is proved to be biomarkers for various diseases including cancer [11,12]. Recently, changes in the glycosylation of proteins associated with liver diseases have received much attention. Haptoglobin (Hp), is an acute phase and polymorphic protein that is produced in the liver and plays an important role in scavenging heme in haemolytic conditions. Hp, a

Abbreviations: HCC, Hepatocellular carcinoma; HBV, Hepatitis B Virus; Hp, Haptoglobin; CHBV, Chronic Hepatitis B infection; LC, Liver cirrhosis; ELISA, Enzyme Linked Immunosorbent Assay; AFP, Alpha-fetoprotein; LC-MS, Liquid Chromatography mass spectrometry; AFP-L3, Alpha-fetoprotein *Lens culinaris* agglutin 3; DCP, Des-gamma-carboxyprothrombin; PCR, Polymerase chain reaction; MW, Molecular weight; Fuc-Hp, Fucosylated haptoglobin; Ab, Antibody; AAL, Aleuria Aurantia Lectin; BSA, Bovine Serum Albumin; PBS, Phosphate Buffered Saline; TMB, 3,3′,5,5′-Tetramethylbenzidine; HRP, Horseradish peroxidase; ROC, Receiver operating characteristic; AUC, Area under curve; Se, Sensitivity; Sp, Specificity; SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

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tetramer glycoprotein, consists of two beta chains and two alpha chains interconnected by disulphide bonds. Hp is characterized by molecular heterogeneity caused by genetic polymorphism [13]. Hp has three phenotypes: Hp1–1, Hp2–1 and Hp2–2 which are controlled by autosomal codominant alleles identified as HP^{1F}, HP^{1S} and HP^{2}. Haptoglobin polymorphism reflects variations in the α-chain due to differences between the α1-chain and the α2-chain. This polymorphism results in Hp with different molecular masses. Hpt1–1 consists of α1 and β chains, Hpt2–2 consists of α2 and β chains. Hpt2–1 consists of two α1, multiple α2, and β chains. The alpha-2 chains are capable of binding each other at two sites, whereas the alpha-1 chain has only one binding site. Hence, Hp 1–1 produces a single homodimer while Hp2–1 and Hp2–2 forms polymer [13]. The β-chain of Hp is not polymorphic and is identical in all phenotypes. Only Hp β-chain contains four N-glycosylation sites at Asn184, Asn207, Asn211 and Asn241 [14]. Many studies have demonstrated various changes in N-glycosylation status of Hp protein in HBV infection using glycomic approach [15–18], but only a few studies have shown site-specific glycosylation in Hp protein in various liver diseased conditions by HBV infection using glycoproteomic approach [19,20].

Hence, it prompted us to undertake this glycoproteomic study to uncover and validate candidate marker for prognosis and diagnosis of the liver diseases in HBV infection. The major advantage of studying glycoproteins/peptides as a potential biomarker is their non-template biosynthesis in response to trigger and disease status as against the usual protein production from a gene. We investigated the complete characterization of Hp glycoprotein including identification, glycosylated site location and glycan structure elucidation. In the present study, we used a combinational approach with LC-MS/MS based quantitative glycoproteomics and lectin-based ELISA to investigate serum Hp glycosylation in various liver diseased conditions due to HBV infection.

2. Materials and methods

2.1. Subject recruitment

This Study was approved by the Institutional Ethics Committee (IEC—I), Seth GS Medical College and King Edward Memorial (K.E.M.) Hospital and, informed consent was taken from each individual. Total 75 patients were recruited from the outpatient department (OPD) and inpatient department (IPD) of Gastroenterology in K.E.M. Hospital, Mumbai during the year 2013 to 2017. They were subcategorized into three groups viz. chronic hepatitis B viral infection (CHBV), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) based on Asian Pacific Association for the Study of the Liver guidelines. The diagnosis of liver diseases was done using serological, biochemical, virological, radiological and pathological (whenever biopsy possible) findings. The HCCs were assessed using Barcelona Clinic Liver Cancer staging system. Thirty healthy controls aged between 18 and 65 years were included in this study with no history of HBV, HCV and HIV. Clinical pathological data of patients were obtained from medical records.

2.2. Laboratory testing

Total Hp (ICL, USA), Alpha-fetoprotein (Calbiotech, USA), alpha-fetoprotein-L3 (ImmunoTag, USA) and Des-gamma-carboxyprothrombin (ImmunoTag, USA) levels were quantified using ELISA kit according to manufacturer's instructions. HBV DNA was extracted from plasma using viral nucleic acid isolation- Nucleo spin Dx virus kit (Macherey-Nagel, Germany). HBV quantitative PCR (Mylab Lifesolutions PathoDetect HBV quantitative PCR kit) was carried out to quantify plasma levels of HBV DNA using Taqman assay with a detection range of 10–10^8 IU/ml on Step One real time PCR system (Applied Biosystems). The viral load was measured using a range of four quantification calibrators provided in the kit.

2.3. Purification of Hp β from serum

Five hundred µl of patient's serum was applied to HiTrap NHS activated column (GE Healthcare, USA) coupled with 300 µl of anti-Hp antibody according to the manufacturer's instructions. Bound Hp protein was eluted using elution buffer (0.1 M glycine, 0.5 M NaCl, pH 3.0) and concentrated using 10KDa MW cutoff centrifugal device (Pall Corporation, USA). Purified Hp β was separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. In-gel protein digestion was carried out using modified trypsin (Promega) and, the protein was identified and confirmed using mass spectrometry MALDI-TOF/TOF (Bruker Daltonik GmbH) as described previously [21].

2.4. Hp digestion, ESI-LC-mass spectrometry conditions and data analysis

Purified Hp samples were denatured with 0.6 M GnHCl, pH 8.5, reduced by 5 mM DTT and alkylated using 10 mM IAA. The samples were desalted and buffer exchanged with 50 mM ammonium bicarbonate using Amicon Ultra-0.5 ml centrifugal filters (cut-off 10 KDa). Then, digested using trypsin and Endoproteinase GluC at enzyme to substrate ratio 1:25 (w/w). The peptide samples were separated using a modified Eksigent nanoLC 425 with a flow controller system coupled to Triple TOF 5600 + mass spectrometer (AB Sciex, Framingham, MA, USA). Five hundred ng of the sample was loaded onto column. Mobile phase consisted of 100% water with 0.1% formic acid (Solvent A) and 100% Acetonitrile with 0.1% Formic acid (Solvent B). The peptides and glycol peptides bound to C18 analytical column (90 Å, 2.7 µm, 100 × 0.5 mm, Eksigent) were eluted at 15 µl per min using following gradient: 5% B to 50% B in 28 min, increased to 90% B in 3.5 min, and then to 5% B for 8 min. The MS and MS/MS data of the eluted peptides were performed using Triple TOF 5600+ system (AB Sciex, Framingham, MA, USA) and, analyst (version 1.6) software was used for data acquisition. All the experiments were performed in the positive polarity mode. It was operated in the data dependent mode to switch between MS and MS/MS acquisition. RAW files generated by XCalibur software were used to database search with MASCOT. 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. For each glycopeptide found in the chromatogram, the peak intensity was identified and calculated using Xcalibur software by either comparing the pre-generated mass list using GlycoMod tool, or by adding and subtracting the sugar mass and also, by confirming with MS/MS data.

2.5. Lectin based ELISA for estimation of fucosylated Hp

Lectin based ELISA was performed according to previously described protocol [22,23]. Briefly, 96 well ELISA plates (maxisorp, Thermo Scientific) were coated with anti-Hp antibody resolved in coating buffer and incubated at 4 °C overnight. Washings were given with PBST (0.05% Tween 20 in PBS, pH 7.4), 3% BSA in PBS was used as blocking agent. After washing, 100 µl of 1000 fold diluted serum sample was added into each well and incubated at room temperature for 1.5 h on the orbital shaker at 120 rpm. After washing, 100 µl of biotinylated Aleuria Aurantia Lectin (1 µg/ml diluted in PBST) was added into each well and incubated at room temperature for 2 h on the shaker at 100 rpm. After washing, 100 µl of diluted HRP-conjugated streptavidin in 3% BSA in PBS was added into each well and incubated at room temperature for 1 h. After washing, TMB substrate solution and later, stop solution were added into each well. Absorbance was read at 450 nm using microplate reader Infinite M200 pro (TECAN, Switzerland).

2.6. Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS), version 24.0 (SPSS, Inc., Chicago, IL, USA). Normally distributed variables were presented as mean and SD; non-normally distributed variables were presented as the median and interquartile range (IQR)—the distance between the 25th and 75th percentiles. The Mann-Whitney *U* test was used to compare the variables between the different groups. Receiver operating characteristic (ROC) analysis with area under curve (AUC) was applied for the diagnosis of HCC using GraphPad Prism version 7.0. The value of the AUC and the corresponding 95% confidence intervals (CI) were calculated. Youden index was calculated to obtain optimal cut off for each biomarker's differentiating ability by giving equal weightage to sensitivity and specificity. Predicted probabilities were calculated by applying a logistic regression model on markers as independent variables, and presence of HCC as dependable variable. ROC analysis helped to evaluate the performance of combination of markers. Glycan structures were generated using GlycoWorkBench (version 1.0) [24].

3. Results

3.1. Patients' characteristics

The demographic and laboratory parameters of enrolled participants are summarized in Supplementary Material 1. Mean age of study participants was 40.63 ± 11.16 years with preponderance of males (74.3%). Twenty one patients (28%) showed active infection of HBV i.e. HBeAg positivity. HBV HCC patients had higher HBV DNA level than non-HCC. Liver enzymes and bilirubin levels were significantly associated with liver diseased stages ($p < .05$).

3.2. Hp β purification and identification

Purified Hp from sera of patients with chronic hepatitis, LC, HCC and healthy controls were subjected to SDS-PAGE followed by staining with Coomassie brilliant blue. Supplementary Material 2 illustrates the separation of Hp β subunit (MW ~40 kDa) from tetramer structure (MW ~130 kDa). The results of MASCOT search confirmed the protein bands corresponding to Hp β protein after in-gel tryptic digestion and MALDI-TOF/TOF, suggesting the feasibility of purification method for further processing as shown in Supplementary Material 2.

3.3. Site-specific N-glycans on Hp β subunit using glycoproteomic approach

Hp β was digested with trypsin and GluC which generated four glycopeptides, MVSHH***N***^{184}LTTGATLINE, NLFL***N***^{207}HSE, ***N***^{211}ATAK, VVLHP***N***^{241}YSQVDIGLIK. Every peak was identified based on either comparing the pre-generated mass list using GlycoMod tool or, by adding and subtracting the masses of sugars. It was found that Asn184 contained eight glycoforms including bi-, tri- and fucosylated. Nine glycoforms including bi-antennary, tri-antennary and fucosylated glycans were present at Asn207. Asn211 and Asn241contained bifucosylated tetra antennary and biantennary glycans respectively as shown in Table 1.

N-Glycan microheterogeneity, the diversity of N-glycans at glycosylation sites were analysed by LC-MS/MS. Precursor ions were selected for fragmentation and the fragments were measured in subsequent product ion scans. For example, Fig. 1 demonstrates MS/MS spectrum of Hp peptide VVLHP***N***^{241}YSQVDIGLIK with 5Hex: 4HexNAc: 2SA glycoform attached at Asn241 glycosylation site. It was observed as the precursor ion m/z 1000.66 with charge state 4+. In our MS/MS spectra of N-glycopeptide, we observed glycan oxonium ions (1+), peptide fragment ions (1+), glycopeptide ions (2+).

The relative abundance of glycoforms at different sites of Hp β subunit in variable liver diseased conditions is shown in Fig. 2. Triantennary Glycoform with sialic acid was relatively increased in controls at site Asn184 when compared to that in liver diseased cases. Whereas, biantennary fucosylated structure (5Hex:4HexNAc:2SA:1Fuc) at Asn184 site was distinctly increased in patients with HCC compared to other liver pathologies of HBV infection and, controls. Notably, triantennary glycoform 6Hex:5Hex:3SA at Asn207 was increased as the liver disease progressed to HCC. Bifucosylated and biantennary glycoform 4Hex:4HexNAc:1SA:2Fuc was increased in all liver diseased stages versus controls. 5Hex:4HexNAc:2SA was not observed in healthy controls at site Asn207 signifying its distinction in chronic hepatitis ($p = .005$), LC ($p = .010$), HCC ($p = .016$) against subjects. The unique bifucosylated tetra-antennary glycan at Asn211 was predominantly present in HCC ($p = .028$, HCC vs controls), suggesting its usefulness as HCC marker. Results showed increased levels of fucosylation at different sites in serum Hp β in HCC. In addition, glycoform 5Hex:4HexNAc:2SA at the site Asn241 was able to distinguish between chronic hepatitis and HCC ($p = .06$).

3.4. Confirmation of expression change in fucosylated Hp using lectin based ELISA assay

From mass spectrometry analysis, we identified increased levels of fucosylation at various sites in serum Hp protein which could be used for HCC detection. An AAL-based ELISA was used to confirm the fucosylation changes in serum Hp protein without depletion of abundant proteins. ELISA Index was calculated as OD value of fucosylated Hp, divided by OD value of protein Hp. It was used to reveal Hp fucosylation status on its protein level in sera of patients and controls. Serum Hp levels were measured using ELISA. Levels of Hp were 121.5 (50.2–197.8) mg/dl in Chronic hepatitis, 41.1 (17.9–140) mg/dl in LC, 57.37 (3.1–110) mg/dl in HCC and 114.2 (90.5–157) mg/ dl in controls as shown in Fig. 3a.

Serum Fuc-Hp levels measured using lectin-based ELISA were 19.7 (15.7–25.3) RU/ml in chronic hepatitis B, 25 (18.7–49.44) RU/ml in LC, 39.91 (19.12–51.32) RU/ml in HCC and 18.41 (15.63–24.29) RU/ml in healthy controls. Levels of serum Fuc-Hp were significantly higher in LC ($p = .025$) and HCC ($p = .009$) when compared with chronic hepatitis B patients. There was no significant difference in Fuc-Hp levels in LC and HCC ($p = .44$) as given in Fig. 3b. However, Fuc-Hp/Hp ratio, the Elisa Index was significantly increased in LC and HCC patients compared to chronic hepatitis patients ($p < .001$). A distinct significant association was observed between the Fuc-Hp/Hp and, advanced liver disease stages and controls ($p < .001$) as depicted in Fig. 3c.

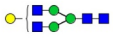
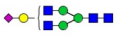
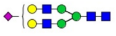
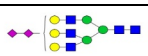
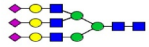
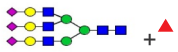
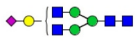
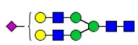
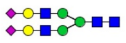
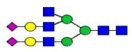
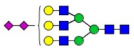
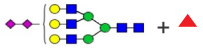
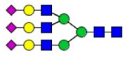
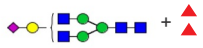
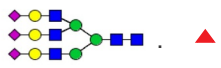
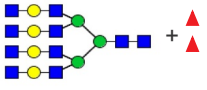
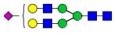
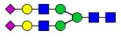
As shown in Fig. 4, Median values of AFP, DCP, Fuc-Hp and ratio of OD of Fuc Hp/Hp were significantly higher in HCC cases when compared with Non-HCC cases. In detail, median AFP, AFP-L3, DCP, Fuc-Hp, ELISA index were 2.2 ng/ml (range, 1.2–4.0) vs 204.5 ng/ml (range, 30.55–500), 0.64 ng/ml (range, 0.63–1.8) vs 0.64 ng/ml (range, 0.63–2.24), 1.18 ng/ml (range, 0.65–2.30) vs 2.62 (range, 0.98–6.18), 20.0 RU/ml (range, 16.32–28.11) vs 39.91 RU/ml (range, 19.1–51.3), 0.25 (range, 0.14–0.65) vs 1.01 (range, 0.6–4.3) in patients without HCC and with HCC, respectively. Whereas, levels of Hp were lower in HCC (57.37 mg/dl; range 3.13–90.5) when compared with Non-HCC (97.54 mg/dl; range 46–181).

4. Discussion

We successfully used ESI-MS/MS quantitative glycoproteomics in conjunction with lectin-based ELISA to investigate glycoforms in N-linked glycopeptide of serum Hp in liver diseased conditions i.e. chronic hepatitis, LC, HCC by HBV infection. In the Indian context, it is first reported study about already existing markers and site-specific Hp glycosylation in liver diseased conditions due to HBV infection.

It has been already identified that Hp comprised of two different types of alpha chains and one type of two beta chains. Hp is a

Table 1
Glycopeptide mass and oligosaccharide composition of each glycopeptide of Hp β protein.

No	Glycopeptide mass	Oligosaccharide composition	Glycan structure
Site-specific N-glycans on MVSHH*N*[184]LTTGATLINE (peptide MW: 1736.8515 Da)			
S1-1	3197.2411	4Hex:4HexNAc	
S1-2	3285.3979	4Hex:3HexNAc:1SA	
S1-3	3650.5406	5Hex:4HexNAc:1SA	
S1-4	3941.644	3Hex:4HexNAc:3Fuc	
S1-5	4087.693	5Hex:4HexNAc:2SA:1Fuc	
S1-6	4306.7708	6Hex: 5HexNAc:2SA	
S1-7	4597.8741	6Hex:5HexNAc:3SA	
S1-8	4743.9314	6Hex:5HexNAc: 3SA:1Fuc	
Site-specific N-glycans on NLFL*N*[207]HSE (peptide MW: 972.4665 Da)			
S2-1	2521.0149	4Hex:3HexNAc:1SA	
S2-2	2886.1767	5Hex:4HexNAc:1SA	
S2-3	3177.25	5Hex:4HexNAc:2SA	
S2-4	3251.2859	6Hex:5HexNAc:1SA	
S2-5	3542.3847	6Hex:5HexNAc:2SA	
S2-6	3688.4855	6Hex:5HexNAc: 2SA:1Fuc	
S2-7	3833.489	6Hex:5HexNAc:3SA	
S2-8	3957.6251	4Hex:4HexNAc:1SA:2Fuc	
S2-9	3979.5811	6Hex:5HexNAc:3SA:1Fuc	
Site-specific N-glycans on *N*[211]ATAK (MW: 503.2703 Da)			
S3-1	3979.7811	7Hex:10HexNAc:2Fuc	
Site-specific N-glycans on VVLHP*N*[241]YSQVD (1269.6353 Da)			
S4-1	3707.6459	5Hex:4HexNAc:1SA	
S4-2	3998.624	5Hex:4HexNAc:2SA	

Hex: Hexose (Gal, Man); HexNAc: N-acetyl glucosamine (GlcNAc); Fuc: Fucose; SA: Sialic acid.

Hex: Hexose (Gal, Man); HexNAc: *N*-acetyl glucosamine (GlcNAc); Fuc: Fucose; SA: Sialic acid. Glycan structures were generated using GlycoWorkBench [24].

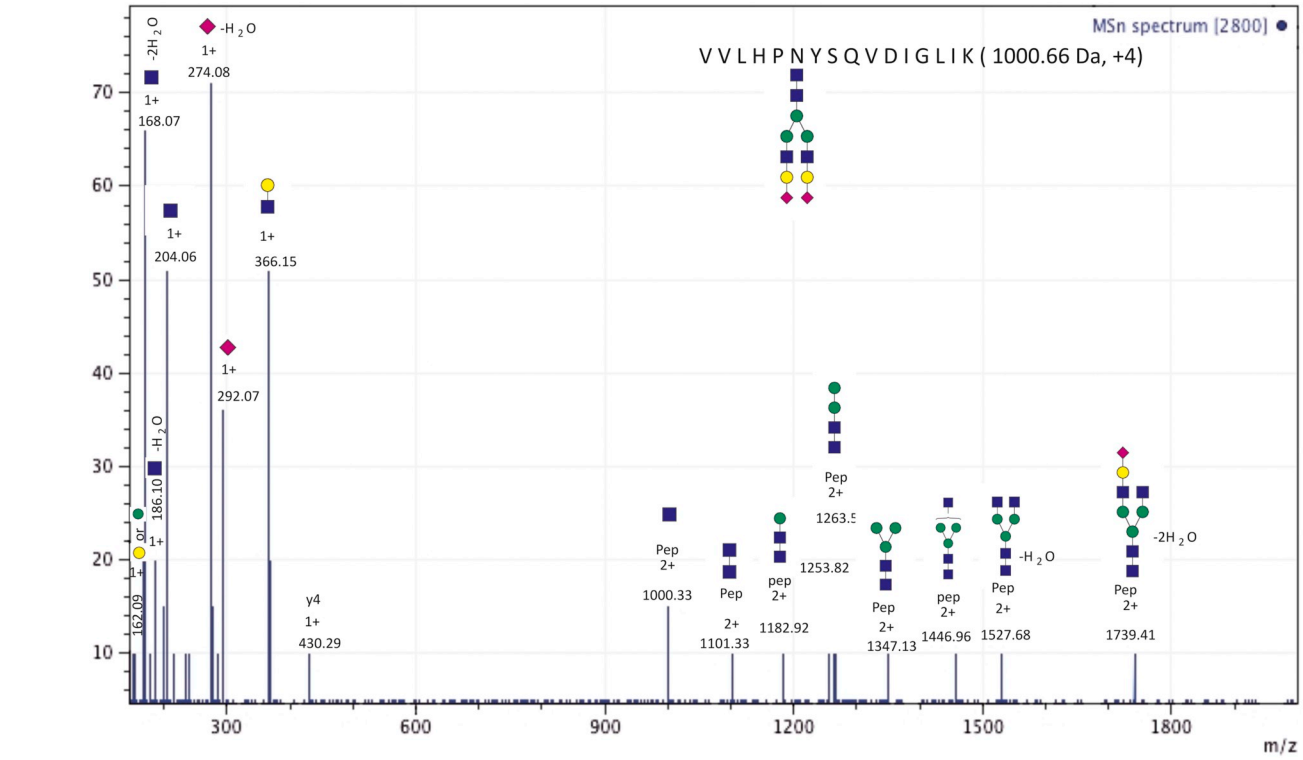


Fig. 1. Glycopeptide MS/MS spectrum of glycopeptide VVLHP**N**YSQVDIGLIK with 5Hex: 4HexNAc: 2SA glycoform attached at Asn241 glycosylation site. Potential structures of fragment ions are labelled.

polymorphic protein; there are three major phenotypes which are produced by differences in alpha chains [13]. Hp phenotypes show major structural and functional differences and it is already known along with Hp values for each phenotype. [25] In this study, we did not demonstrate about correlation of genotype and phenotype of Hp with HBV related liver diseased conditions. Rather, we focused on glycosylation of haptoglobin-beta chain which is identical in all phenotypes.

In the present study, we identified 20 glycoforms at various N-glycosylation sites of Hp beta chain. Initially, we isolated serum Hp protein using anti-Hp affinity column which was evaluated on SDS PAGE followed by MALDI-TOF analysis as shown in Supplementary Material 2. As majority of the serum glycoproteins are hepatic in origin, it is inferred that liver diseases associated with abnormal glycosylation can exhibit significant changes in glycoproteins [26]. Infection with viral hepatitis and neoplastic transformation initiate acute phase response which in turn releases cytokines to modify glycosylation machinery of hepatocyte and alters glycosyltransferase expression at transcriptional level [27].

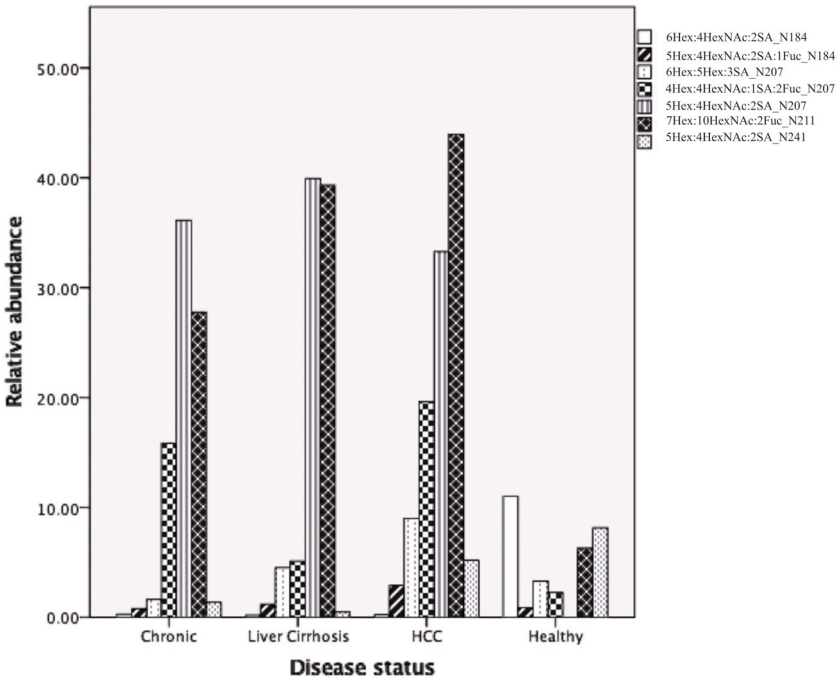


Fig. 2. Relative abundance of N-glycans in Hp β in sera of controls and, patients with chronic hepatitis, LC and HCC. Fucosylated glycoforms were significantly increased at Asn184, Asn207 and Asn211 in liver diseased stages as against controls. No sialylated glycoform was found in serum Hp of controls. Distinct bifucosylated tetra-antennary glycan was predominantly identified in HCC versus other groups.

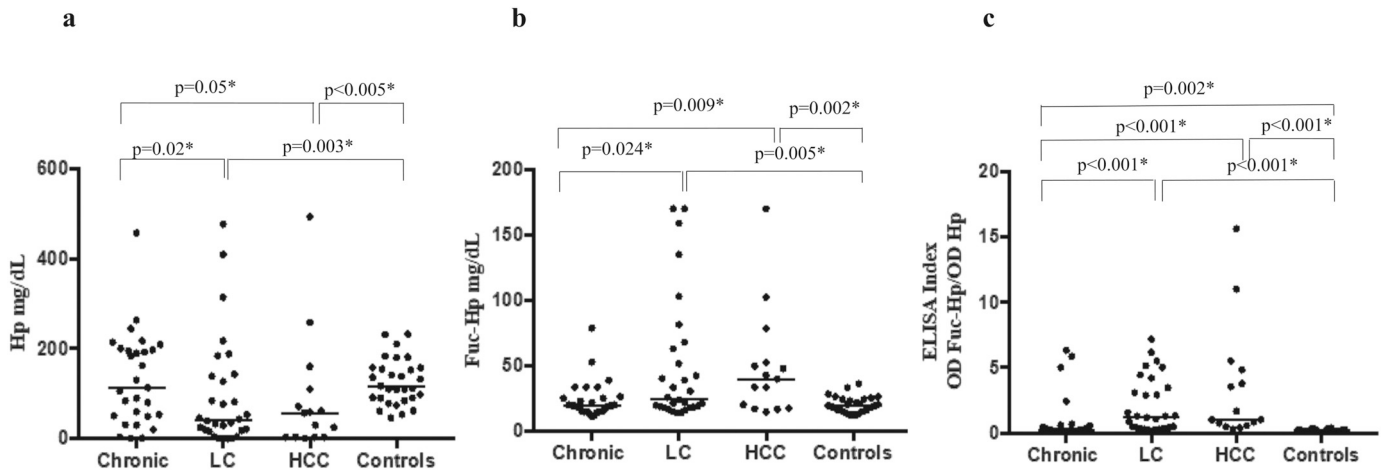


Fig. 3. (a)Association of serum Hp levels in patients with liver disease conditions and controls. Mann-Whitney Wilcoxon test for serum Hp levels among the groups: Chronic Hepatitis vs LC: $p = .02$, chronic hepatitis vs HCC: $p = .05$, LC vs controls: $p = .003$, HCC vs controls: $p = .005$. (b) Mann-Whitney Wilcoxon test for serum Fuc-Hp levels among groups: chronic hepatitis vs LC: $p = .024$, chronic vs HCC: $p = .009$, LC vs controls: p = .005, HCC vs controls: $p = .002$ (c) Mann-Whitney Wilcoxon test for ELISA index (ratio of OD of Fuc-Hp/ OD of Hp) among groups: chronic vs LC: $p < .001$, chronic hepatitis vs HCC: p < .001, chronic hepatitis vs controls: p = .002, LC vs controls: p < .001, HCC vs controls: $p < .001$.

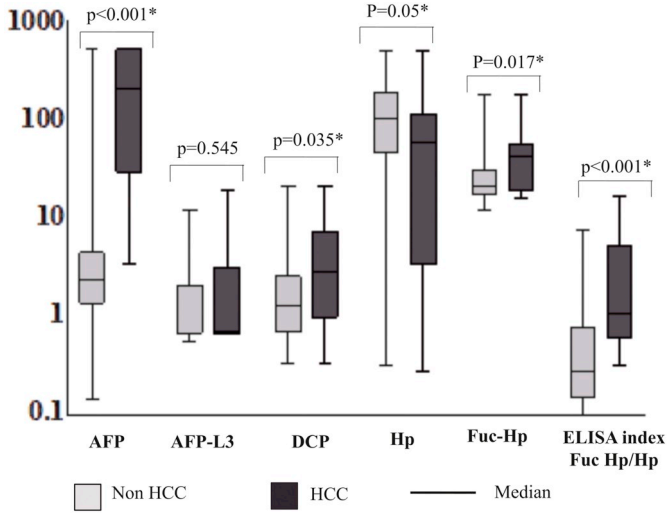


Fig. 4. Median AFP, AFP-L3, DCP, Hp, Fuc-Hp, ratio of Fuc Hp/Hp levels in patients with and without HCC.

A complete characterization of glycoprotein includes identification of glycosylation site and elucidation of glycan structure which can be achieved by using advanced mass spcectrometry with MS/MS analysis [28]. Hp protein was digested with dual enzymes; trypsin and GluC to access the peptide backbone between two closely spaced glycosylation sites at Asn207 and Asn211. Glycan composition on various glycopeptide was identified on the basic of observed *m/z* and analyzing MS/MS fragment ion spectra resulting from collision-induced dissociation (CID) of N-linked glycopeptides. The interpretation of CID MS/MS spectra of N-glycopeptide requires an understanding of specific spectral characteristics including glycan oxonium ions, intact peptide ions (Y ions), glycan B ions and additional glycopeptide Y ions consisting of intact peptide with partially fragmented glycans. [29] Glycan oxonium ions, singly protonated mono- and oligosaccharide ions resulting from fragmentation of glycan and glycopeptides are highly sensitive markers of glycopeptide fragmentation [30]. As shown in Fig. 1 Hex+ (*m/z* 162.9), HexNAc+ (m/z 204.06), NeuAc+ (m/z 292.07), HexNAc-Hex + (m/z 366.82) oxonium ions were prevalent in MS/MS spectra. The peak at 430.29 represents intact peptide fragment ions for the peptide VVLHPN^{241}YSQVDIGLIK. Also, doubly charged glycopeptide Y ions (m/z 1000.33, 1101.33, 1182.92, 1253.81, 1263.81, 1348.13, 1456.96, 1527.68, 1739.41) were visible in the spectrum. Similarly, glycoforms were evaluated for each glycopeptide.

It was observed that bifucosylation (3957.6251 Da) and tetra anteannary Hp, especially bifucosylated tetraantennary (3979.7811 Da) may serve as a potential marker for predicting progression of HCC from cirrhosis which is, in concordance with the earlier report on western population [15]. In Japanese population, Asazawa et al. have shown increase in serum Fuc-Hp levels using ELISA in advanced liver diseases which is in accordance with our glycopeptide analysis using mass spectrometry and lectin based ELISA [31]. Quantitative results by Zhang et al. have revealed that sialylation of glycopeptide (NLFLN^{207}HSEN^{211}ATAK) and fucosylated structure at each glycopeptide were significantly increased in LC and HCC patients. However, sialylated glycoforms 6Hex:5HexNAc:3SA were increased in our LC and HCC cases under study whereas, 4Hex:4HexNAc:2Fuc:2SA and 5Hex:4HexNAc:3SA glycoforms were decreased in LC and increased in HCC at site Asn 207. Fucosylated structure was increased at Asn 211 in advanced liver disease stages as shown in Fig. 2 [19]. Changes in the glycosylation status of Hp using different approaches in other cancer types are mentioned in Table 2. Raised Fuc-Hp levels, therefore, are not disease specific. Fucosylation and sialylation on Hp may influence its legend-receptor interactions with CD163, CD22, CCR2, CD18 which in turn cause a shift in the strength of receptor signalling in pathways and hence affecting tumour microenvironment. [14] Takahashi S et al. have found no difference in Hp N-glycan but observed remarkable differences in the linkage of fucosylation with different gastroenterological cancers. [32] Several other serum glycomarkers have been studied extensively such as hemopexin (Hpx) which has no polymorphism and contains five N glycosylation sites. Debruyne E et al. have demonstrated that Fuc-Hpx expression in HCC patients was high, indicating that Fuc-Hpx is an effective biomarker for HCC [33]. However, Hpx glycan markers showed no significant difference between HCC and cirrhotic cases whereas, in present study, glycans 5Hex:4HexNAc:2SA (Asn207) and 7Hex:10HexNAc:2Fuc (Asn211) on Hp protein showed significant association in distinguishing HCC from Non-HCC. Additionally, in our study glycoform 5Hex:4HexNAc:2SA at the site Asn241 was able to distinguish between chronic hepatitis and HCC ($p = .06$). Also, Hpx glycan marker for HCC showed lowered sensitivity when compared with Hp in our study.

We measured Hp levels along with already existing biomarkers for diagnosis of HCC. As shown in Fig. 4, level of Hp was decreased in HCC

Table 2
Glycosylation alteration in Haptoglobin in various cancer types using various approaches.

Cancer types	Glycosylation alteration in Hp	Year/study population	Cases (n)	Controls (n)	Approach used	References
Pancreatic	Increased fucosylation	2018/ Japanese	118	135	c	[34]
	Increased Fucosylation at site Asn241	2017/Polish	76	26	a, b, d	[35]
	Increased branching with α-1 → 3 outer-arm fucosylation and minor core fucosylation	2009/Spanish	9	8	b, d	[36]
Prostate	Increased monosialyl tri-antennary structures	2009/American	21	9	b, d	[37]
	Increased branching, Increased triantennary mono sialylation and fucosylation, Increased tri-antennary glycoforms, Increased fucosylation on antenna, O-glycosylation	2008/Japanese	8	8	b	[38]
	Increased Core fucosylation	2016/Japanese	39: other cancer; 26: prostate cancer	5	a, d	[32]
Colon	Increased fucosylation	2009/Korean	17	23	d, e	[39]
	α1-3/4 fucosylation at Asn 241	2011/Korean	46	52	a, d, f	[40]
	Increased fucosylation	2011/Japanese	77	22	c	[41]
Gastric	Increased branching, antenna fucosylation and sialylation mainly sLe^x	2016/Korean	30	30	b, d	[42]
	Increased fucosylated and sialylated complex-type glycan at site Asn 211, Asn241 and increased fucosylated complex-type glycan at Asn 241	2018/Korean	10	15	a	[43]
Breast	Increased levels of β(1,6)-branched N-linked glycan structures, Increased tetra antennary structures	2008/Georgian	5	0	b	[44]
Ovarian	Increased fucosylation	2014/American	62	15	c	[45]
Hepatocellular carcinoma[#]	Increased fucosylation, sialylation and branching	2018/Indian	75	30	a, c	

Approach used- a: glycoproteomic approach; b:glycomic approach; c:lectin based Elisa; d: lectin blot, e: NP-HPLC, f: lectin micro array.
[#] Present study.

when compared with non-HCC cases ($p = .027$) which is in concordance with the earlier reports. [15,46] Baxi A et al. have revealed that Hp levels were constantly low in most of the cirrhotic cases in Indian population which may be due to Hp2, lower hemoglobin-binding capacity and increase in free iron-mediated oxidative stress causing predisposing factor for hepatocarcinogenesis [47]. Fig. 3 depicts significantly increased levels of fuc-Hp in LC and HCC cases. ELISA index was calculated as a fraction by dividing OD value of fucosylated Hp by OD value Hp which reflects on its fucosylation magnitude. It was significantly increased in HCC patients compared to controls ($P < .001$) as given in Fig. 4. There was no correlation between Hp and Fuc-Hp ($p = .99$), suggesting that mechanisms of expression and glycosylation of Hp may be different. Thompson et al. have concluded in their study about the increased magnitude of Fuc-Hp than total Hp and total fucose in active rheumatoid arthritis. Overlap in the range of values for total Hp and total fucose made them unsuitable for use in discriminatory role. However, Fuc-Hp in combination with C-Reactive protein (CRP) showed the highest correlation with the clinical assessment of inflammatory joint disease [48]. Our study demonstrates that ELISA index (AUC = 0.81) achieved better performance with Se (93.33%) and Sp (64.44%) when compared with Hp (AUC = 0.65) and Fuc-Hp (AUC = 0.69) alone as given in Supplementary Material 3. As shown in the Supplementary Material 3, combination of markers: AFP + FucHp, AFP + Elisa index resulted in an AUC of 0.93 and 0.92 respectively. Also, the AUC of three combinational markers: AFP + AFP-L3 + FucHp was 0.93 for distinguishing HCC cases from controls whereas, AFP + DCP + FucHp had AUC of 0.94 as illustrated in Supplementary Material 3. Asazawa et have demonstrated the advantage of Fuc-Hp along with the conventional HCC markers such as AFP and DCP to differentiating liver pathologies [31]. Thus, a combination of markers may be very useful for diagnosis of HCC instead of a single marker.

In conclusion,

By using affinity columns coupled to antibody-based purification, mass spectrometry and lectin-based ELISA, we identified site-specific glycoforms on the haptoglobin beta chain in different liver diseases due to HBV infection. We showed discrete N-glycosylation abnormalities on Hp beta chain using mass spectrometry.

A lectin-based ELISA was developed to confirm the fucosylation changes in serum Hp protein alleviating the need for depletion of abundant proteins. It may provide a simple and practical solution for clinical diagnosis and prognosis of HBV HCC especially, without the need for costly mass spectrometry instruments and expertise in software analysis. This may be useful diagnostic glycobiomarker either alone or in combination with AFP + DCP + FucHp (AUC: 0.94) in HBV HCC.

Further work may require to evaluate the practicability of glycosylated Hp along with its correlation with phenotypes and fucose linkage analysis in different liver pathologies.

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Author contribution

Study Concept, design and supervision: SA.
Acquisition, analysis and interpretation of data: DK, DB, SA, BS, SA^1.
Provision of clinical samples and information: SA^1, BS.
Interpretation of data: DK, DB, SA, BS, SA^1.
Drafting of the manuscript: DK.
Review of the draft manuscript: DB, SA, SA^1, BS.

(DK-Dalal Kruti, DB- Dalal Bhavik, SA^1- Shukla Akash, BS-Bhatia Shobna, SA- Shankarkumar Aruna)

Declaration of interest

None.

Compliance with ethical standards

This Study was approved by the Institutional Ethics Committee for research on human subjects and, informed consent was obtained from all individual participants included in the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.04.072.

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