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Original Article

Glycosylation patterns and PHA-E-associated glycoprotein profiling associated with early hepatic encephalopathy in Chinese hepatocellular carcinoma patients

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Abstract: Hepatic encephalopathy (HE) as a severe neuropsychiatric complication is commonly present in the end stage of Hepatocellular Carcinoma (HCC). However, widely accepted biomarkers for diagnosing early HE are still absent. Here, we screened glycosylation patterns of serum proteins from Chinese HCC patients with or without early HE by lectin microarray. Then, phaseolus vulgaris erythroagglutinin (PHA-E) as a lectin binding with bisecting GlcNAc structure which was significantly decreased in sera from Chinese HCC patients with early HE, was chosen to perform lectin affinity chromatography, following by in-gel digestion, Mass Spectrometry (MS) analysis and bioinformatics analysis. Here we found, 13 lectins showed statistically significant reduction suggesting GalNAc, terminal α-1,3 Man, bisecting GlcNAc, (GlcNAc)n, O-GlcNAc, Neu5Ac, tetra-antennary complex-type N-glycan and GalNAc α/β1-3/6 Gal were decreased in serum glycoproteins from Chinese HCC patients with early HE. Furthermore, a total of 141 PHA-E-associated glycoproteins were identified in MS, of which 12 serum glycoproteins only in Chinese HCC patients without early HE and 26 serum glycoproteins only in Chinese HCC patients with early HE. In addition, bioinformatics analysis revealed the PHA-E-associated serum glycoproteins only in Chinese HCC patients with early HE might be related to early HE occurrence through p38 MAPK signaling pathway and MAPK/ERK signaling pathway. Collectively, this was the first glycomics study of serum proteins in HCC patients with early HE and it could provide a database for discovering and developing serum biomarkers to identify and predict early HE in HCC patients.

Keywords: Hepatocellular carcinoma, hepatic encephalopathy, glycoproteomics, lectin microarray, lectin-affinity chromatography, LC-MS/MS

Introduction

Hepatocellular carcinoma (HCC) is a major primary liver cancer, accounting for 70-85% of the known liver cancers [1]. Hepatic encephalopathy (HE) is often described as a prevalent complication of liver insufficiency including portal hypertension and cirrhosis that results in cognitive, psychiatric and motor impairments in these patients after exclusion of other known brain disease. There are several different stages of HE, of which early HE is the stage with abnormal results of established psychometric or neuropsychological tests, but without clinical manifestations [2]. It was reported that the prevalence of early HE in patients with Child B/C liver dysfunction is 50% [3]. Recently, it's worth noting that HE is commonly present in the end stage of HCC. These HCC patients with HE will follow a course from some cognitive/behavioral decay to null response, and finally slip into coma [4, 5]. By this token, HE severely affects the HCC patient's prognosis. At present, the biological markers in clinical diagnosis for HE still lack, especially for early HE. It is currently not possible to diagnose HE with a high degree of certainty until relatively late in the disease process. Hence, the importance of

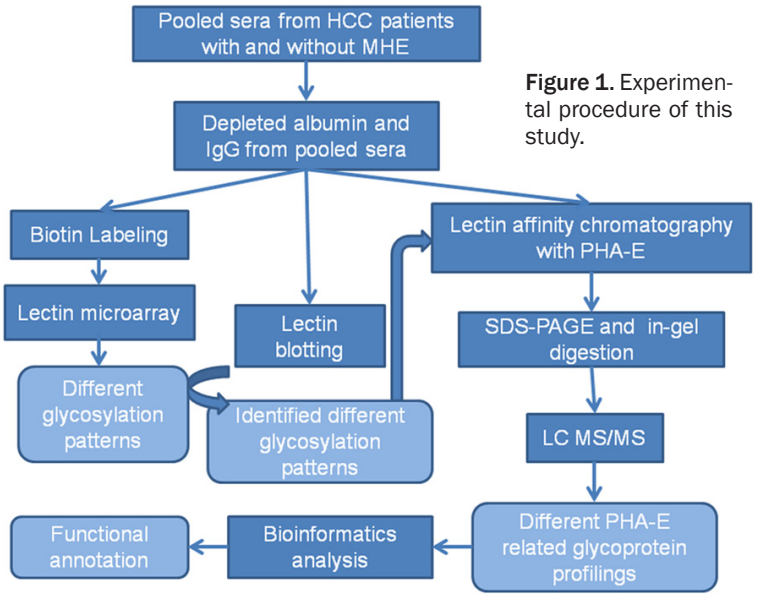

Figure 1. Experimental procedure of this study.

diagnosis and treatment of early HE in the HCC patients is always neglected in such a clinical context, leading to most HCC patients with early HE missing the best opportunity for intervention in time. To date, the diagnosis of HE dependents mainly on psychometric tests and brain activity measurement [6], and only several molecules have been assumed as possible markers of HE in patients with cirrhosis [7-9], but definitive serologic predictors for early HE in HCC patients are still absent [10].

Glycosylation is an important protein post-translational modification (PTMs), there are more than 50% of proteins are presumed to have undergone glycosylation in nature [11], being involved in many different biological events such as protein folding, transport, cell-cell interactions and antigenicity [12, 13]. However, aberrant glycosylation is one of the most common phenomena linked to human diseases either by influencing protein functionalities or as nonfunctional participants [14]. Increasing evidence shows that the alteration in glycosylation could promote invasive behavior of cells and play an important role in cancer metastasis [15-17]. Moreover, most of current serum biomarkers utilized in clinical practice are glycoproteins including alpha fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), carbohydrate antigen 12-5 (CA12-5) and carbohydrate antigen 15-3 (CA15-3). Screening aberrant glycan as diseases biomarkers or drug targets has become an attractive field of research, which might have unique strengths in disease prediction compared with proteins themselves. The representative example is Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), an extensively proven disease glycobiomarker. AFP has widely been used for HCC's surveillance, but AFP-negative HCC is frequently missing diagnosis because of its low specificity for prediction or diagnosis. In contrast, AFP-L3, as a specific marker which could remarkably distinguish HCC and other hepatic disease by the glycoprotein part of AFP, has been a preferred HCC biomarker in early diagnosis of HCC and in predicting prognosis after treatment [18, 19]. For all these above, glycosylation and glycoproteins might play superior roles in diagnosis and prediction of early HE in HCC patients. Seeking a novel glycoprotein biomarker of early HE might reduce the diagnostic window between the time of HE occurrence and diagnosis. Therefore, the serum glycosylation pattern and glycoprotein profiling from HCC patients with early HE are urgently needed.

In the present study, we screened glycosylation patterns of serum glycoproteins from Chinese HCC patients with and without early HE, and then, enriched glycoprotein fractions by lectin affinity chromatography with specific lectin, identified the specific lectin related proteins by modern Mass Spectrometry (MS), and further performed bioinformatics analysis (**Figure 1**). This study aimed to achieve a novel glycoprotein database for digging out potential markers of identifying early HE in Chinese HCC patients.

Materials and methods

Clinical specimens

Serum samples from 72 Chinese HCC patients (36 HCC patients with early HE, the other 36 HCC patients without early HE were controls) were collected at First Affiliated Hospital of Dalian Medical University (Liaoning, China) in this study. The diagnosis of HCC was deter-

Table 1. Clinical characteristic of Chinese HCC patients

	HCC patients without early HE	HCC patients with early HE
Number of individuals	36	36
Gender (male/female)	27 (75%)/9 (25%)	26 (72.2%)/10 (27.8%)
Age (years)	59 ± 10	61 ± 11
ALT (IU/L)	49.8 ± 29.7	57.1 ± 42.3
AST (IU/L)	75.4 ± 56.3	88.7 ± 61.5
AFP (IU/ml)	352.6 ± 378.1	410.2 ± 439.4
PT (s)	14.9 ± 3.6	15.2 ± 2.9

Mean ± standard deviation; ALT, Alanine aminotransferase; AST, Aspartate transaminase; AFP, alpha fetoprotein; HbsAg, hepatitis B surface antigen; PT, Prothrombin time.

mined by liver biopsy with imaging tests, and the diagnosis criteria for the early HE was according to the 2014 Practice Guideline by the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases that include the minimal HE and grade I HE [2, 20]. The exclusion criteria was as the same as previously reported [21] that included history of autoimmune diseases, infectious diseases, diabetes, immune deficiency disorder, psychiatric illness, severe cognitive impairment, or a systemic or central nervous system infection two weeks before sample collection. All of the samples were stored at -80°C for further analyses. Clinical characteristic of these HCC patients were provided in **Table 1**. Pooled serum samples from 10 HCC patients without early HE and 10 HCC patients with early HE were used for screening glycosylation patterns and identifying PHA-E-associated glycoprotein profiling by MS. Three biological repeats were measured independently to guarantee the reproducibility of experiment. Other 12 samples were used to re-identifying the results from MS, respectively. The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and all of projects in the study were approved by Ethics Committee in the First Affiliated Hospital of Dalian Medical University. Informed consent was obtained from each patient.

Lectin microarray

Pooled serum samples were processed by ProteoExtract® Albumin/IgG removal kit (Calbiochem, Billerica, MA, USA) to deplete albumin and IgG. Then, Lightning-Link Biotin Labeling Kit (Innova Biosciences, Cambridge, UK) was used to biotinylate serum proteins according to the manufacturer's description. Glycosylation patterns were screened by a lectin microarray constructed in our previous study [22] including 50 lectins form Vector Laboratories (Burlingame, CA, USA) and Sigma-Aldrich (Castle Hill, NSW, Australia), the protocol was summarized as follows: Firstly, the lectin microarray was blocked by 2% bovine serum albumin (BSA)-TBS solution. Secondly, incubated it with equal biotinylated proteins from pooled serum samples from 10 HCC patients without early HE and 10 HCC patients with early HE, respectively. And then, Cy5 labeled streptavidin (Life technologies, Waltham, MA, USA) was used for combining with the microarray. LuxScan 10K/A scanner system (CapitalBio, Beijing, China) was used to scan the lectin microarray and extract the data, which was analyzed as described previously [23] and three biological repeats were measured independently.

Lectin blotting

Albumin and IgG-depleted serum proteins from 10 HCC patients without early HE and 10 HCC patients with early HE, respectively were separated by SDS-PAGE and analyzed by lectin blotting. In brief, proteins in the gels were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with biotinylated jacalin (JAC), phaseolus vulgaris erythroagglutinin (PHA-E), ricinus communis agglutinin I (RCA-I), solanum tuberosum lectin (STL), wisteria floribunda lectin (WFL) and wheat germ agglutinin (WGA, Vector Laboratories, Burlingame, CA, USA), respectively. The membranes were washed with 0.1% TBS-Tween20 (TBST, 50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.6), and then incubated with Streptavidin Horseradish Peroxidase (HRP) Conjugate (Invitrogen, Waltham, MA, USA). Amersham ECL prime western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) were used to detect the bands on the membranes.

Lectin affinity chromatography

PHA-E-agarose was washed and supplemented with lectin-binding solution (10 mM Tris-HCl,

0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.5). Serum samples depleted albumin and IgG from 10 HCC patients without early HE and 10 HCC patients with early HE, respectively were added into PHA-E-agarose and incubated at 4°C overnight. Then, the agarose was washed by lectin-binding solution and followed by elution of bound fraction with 200 mM N-acetyl-D-(+)-glucosamine. The eluted fraction was separated by SDS-PAGE and stained by PhastGel™ Blue R (GE Healthcare, Piscataway, NJ, USA). Gels containing all bands were cut into slices and processed for in-gel digestion; three biological repeats were performed independently.

In-gel digestion and nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Gel slices were destained, then Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP, Sigma, Castle Hill, NSW, Australia) and iodoacetamide (IAA, Sigma, Castle Hill, NSW, Australia) were used to reduce and alkylate, respectively. Subsequently, after redehydrating with 100% acetonitrile (ACN), those gel slices were digested in trypsin solution at 37°C overnight and extracted by extraction buffer (50% ACN, 0.1% trifluoroacetic acid). The dried solutions were resuspended with 12 μl solvent A (A: water with 0.1% formic acid; B: ACN with 0.1% formic acid) and then separated by nanoLC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA) connected to a quadrupole-Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. 8 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm×2 cm), with a flow of 10 μl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm×25 cm) with a linear gradient, from 2% B to 45% B in 75 min. The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 300 nL/min and column temperature was maintained at 40°C. The electrospray voltage of 2.0 kV versus the inlet of the mass spectrometer was used.

The Q-Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350-1600) were acquired with a mass resolution of 70K, followed by fifteen sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 17.5K. For MS, the automatic gain control (AGC) was set to 1,000,000 ions, with maximum accumulation times of 20 ms. For MS/MS, precursor ions were activated using 30% normalized collision energy, an isolation window of 2 m/z and the AGC was set to 200,000 ions, with maximum accumulation time of 120 ms. Single charge state was rejected and dynamic exclusion was used with one microscan and 60 s exclusion duration.

Enriching and identification of PHA-E-associated matrix metalloproteinase-9 (MMP9)

PHA-E-associated MMP9 was enriched in 6 serum samples from Chinese HCC patients without early HE and 6 serum samples from Chinese HCC patients with early HE by lectin affinity chromatography with PHA-E-agarose as described before. Then, the enriched PHA-E-associated MMP9 was separated by a 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% Bull Serum Albumin (BSA), membranes were incubated with a polyclonal goat anti-human MMP9 antibody (R & D, Minneapolis, MN, USA) at 4°C overnight. Washed the membranes with 0.1% TBST and incubated them with HRP-conjugated secondary antibodies (KangChen Biotech, Shanghai, China), and then the membranes were washed by 0.1% TBST again. Amersham ECL detection (GE Healthcare, Piscataway, NJ, USA) was used to visualize the bands.

Data base searching and bioinformatics analysis

Thermo Scientific Proteome Discoverer software version 1.4 with the MASCOT v2.3.2 search engine was used for all searches of the database. The database was the Human UniProtKB/Swiss-Prot database (Release 2015-03-11, with 20199 sequences). Raw files generated by the Q-Exactive instrument were searched directly using a 10 ppm precursor mass tolerance and a 50 mmu fragment mass tolerance. The enzyme specificity with trypsin was used. Up to two missed cleavages were

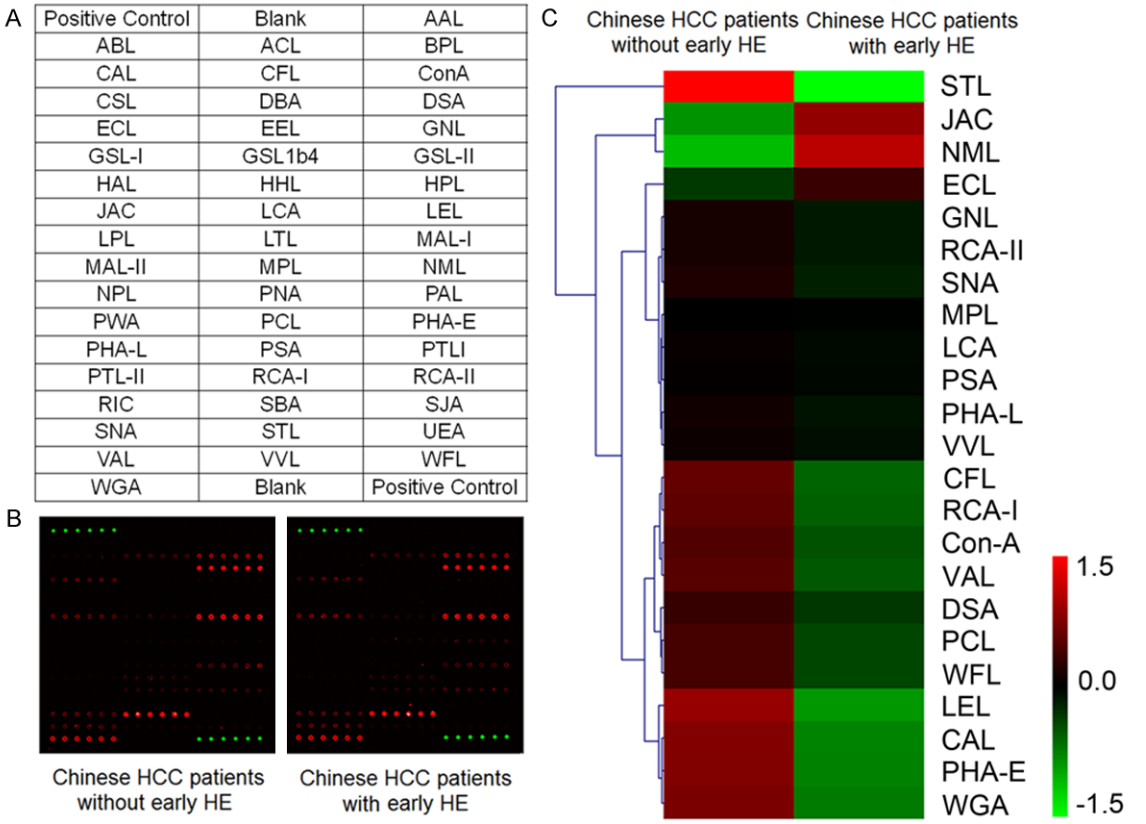

Figure 2. Lectin microarray analysis. A. The arrangement of lectins in the lectin microarray. B. Scan images of the lectin microarray incubated with sera from Chinese HCC patient with and without early HE, respectively. C. Hierarchical clustering of positive lectin binding spots (S/B≥2). Each row represented a lectin, and red represents a lectin with high S/B value, while green represents a lectin with low S/B value.

allowed and peptides with at least 7 amino acids were retained. Carbamidomethyl on cysteine was set as a fixed modification. Oxidation on methionine and Deamidation on asparagine were set as variable modifications. Use the percolator algorithm to control peptide level false discovery rates (FDR) lower than 1%.

Uniport database (http://www.uniport.org) was used to evaluate the identified PHA-E-associated proteins and the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) was used to further analyze functional categories of the different groups of PHA-E-associated glycoproteins. Moreover, we investigated biological interactions by Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA, USA).

Statistical analysis

In the lectin microarray analysis, the Spot Intensity Median (S) and the Background Intensity Median (B) were extracted by LuxScan 10K/A scanner system and S/B was calculated. S/B≥2 was used as cutoff to define positive lectin binding spots. T-test was used to statistically analyze the S/B of each kind of positive lectin spots binding different samples and the difference was statistically significant for $P<0.05$.

Statistical analysis was performed by SPSS 16.0 statistical packages (SPSS Inc., Chicago, IL, USA). Quantitative variable was evaluated using Student's T-test. $P<0.05$ was considered statistically significance.

Results

Glycosylation patterns of sera from HCC patients with and without early HE

The lectin microarray constructed in our previous study [22], which included 50 different kinds of lectins, 2 blank controls and 2 positive

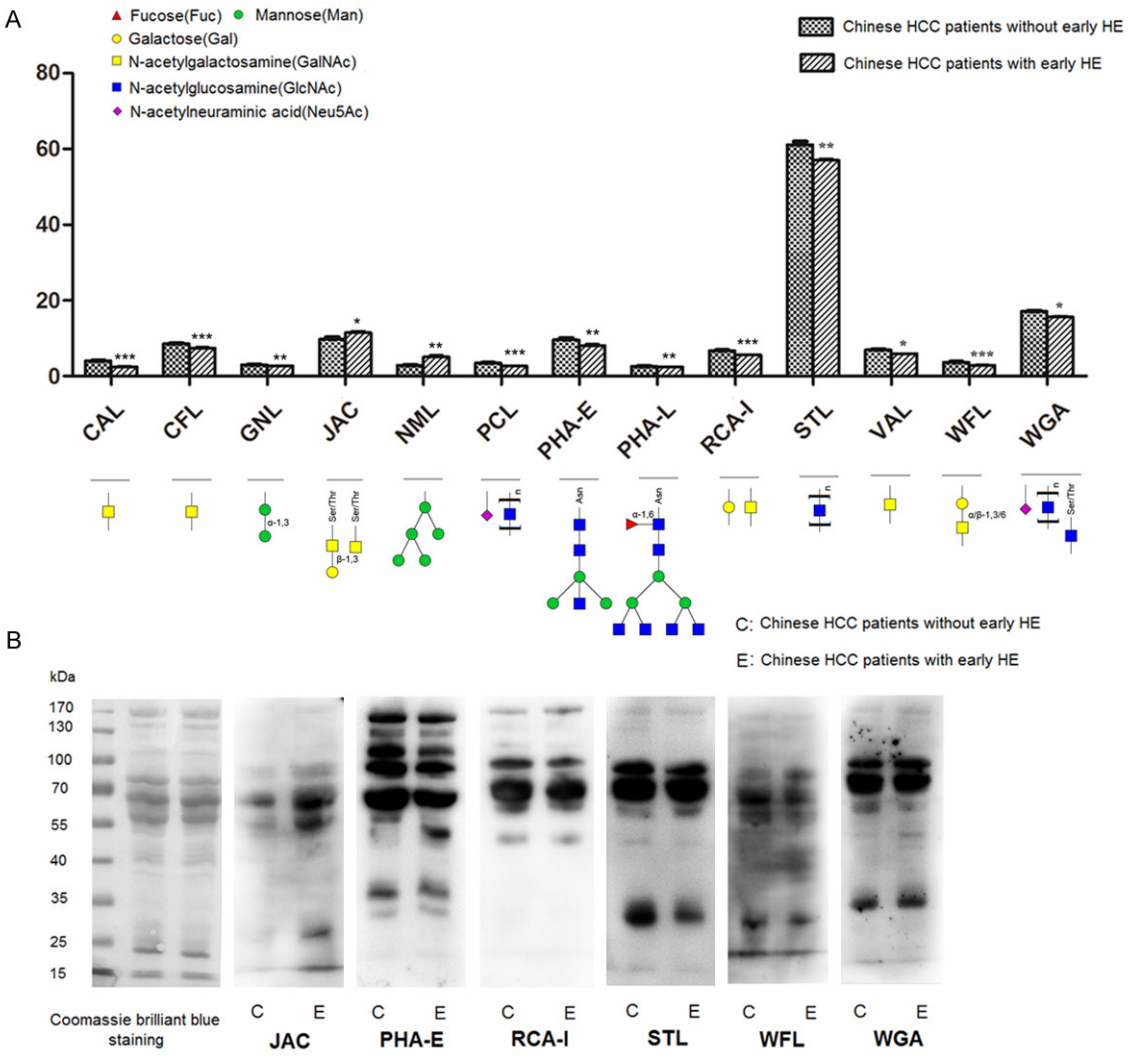

Figure 3. Different glycosylation patterns in sera from Chinese HCC patients with and without early HE. A. Specific carbohydrate epitopes and quantitative results of lectins had statistical significance between sera form Chinese HCC patients with and without early HE. Data was the average ± SD, *P<0.05, **P<0.01, ***P<0.001. B. Validation of different glycosylation patterns by lectin blotting. Coomassie brilliant blue staining was used to show the protein loading. Signal strength of JAC was increased, while signal strengths of PHA-E, RCA-I, STL, WFL and WGA were all decreased in sera from Chinese HCC patients with early HE compared with in sera from Chinese HCC patients without early HE.

controls in each block (**Figure 2A**), was applied to screen glycosylation patterns of sera from Chinese HCC patients with and without early HE. Fluorescence intensities were scanned and showed in **Figure 2B**. Hierarchical clustering of 23 positive lectins in sera from both HCC patients with and without early HE was mapped by the MeV 4.8.1. (**Figure 2C**).

To facilitate the analysis, statistically significant 13 lectins (lectin signal pattern of sera from Chinese HCC patients with HE vs. pattern of sera from Chinese HCC patients without early HE, P<0.05) were divided into 3 grades according to protein-lectin binding intensities: weak binding (5>S/B≥2), medium binding (8>S/B≥5) and strong binding (S/B≥8). Stronger combining capacity meant more carbohydrate epitopes. In sera from Chinese HCC patients with early HE, caragana arborescens lectin (CAL), galanthus nivalis lectin (GNL), naja mossambica lectin (NML), phaseolus coccineus lectin (PCL), phaseolus vulgaris leucoagglutinin (PHA-L), WFL were weak binding; RCA-I, viscum album lectin (VAL) were medium binding; codium fragile lectin (CFL), JAC, PHA-E, STL and

Table 2. PHA-E-associated glycoproteins only identified in sera from Chinese HCC patients without early HE

Uniprot Number	Protein Name	Gene Name
P01596	Ig kappa chain V-I region CAR	KV104
P0C0L4	Complement C4A	CO4A
A6NMB1	Sialic acid-binding Ig-like lectin 16	SIG16
Q15485	Ficolin-2	FCN2
P04070	Vitamin K-dependent protein C	PROC
P02786	Transferrin receptor protein 1	TFR1
Q8NBJ4	Golgi membrane protein 1	GOLM1
O75144	ICOS ligand	ICOSL
Q9Y6R7	IgGFc-binding protein	FCGBP
Q10588	ADP-ribosyl cyclase 2	BST1
Q9NPR2	Semaphorin-4B	SEM4B
P04003	C4b-binding protein alpha chain	C4BPA

WGA were strong binding. While, in sera from Chinese HCC patients without early HE, CAL, GNL, PCL, PHA-L and WFL were weak binding; CFL, NML, RCA-I and VAL were medium binding; JAC, PHA-E, STL and WGA were strong binding.

Quantitative results of S/B and binding structures of these 13 lectins were shown in **Figure 3A**. Only 2 lectins: galactosyl (β-1,3) N-acetylgalactosamine binder JAC and branched mannose (branched Man) binder NML, had more increased S/B in sera from Chinese HCC patients with early HE compared to patients without early HE, while S/B of other lectins were all decreased in Chinese HCC patients with early HE, which suggested that specific carbohydrate epitopes like N-acetylgalactosamine (GalNAc, recognized by CAL, CFL, RCA-I and VAL), terminal α-1,3 Man (recognized by GNL), bisecting N-acetylglucosamine (bisecting GlcNAc, recoginzed by PHA-E), $(GlcNAc)_n$ (recognized by PCL, STL and WGA), O-GlcNAc (recognized by WGA), N-Acetyl Neuraminic Acid (Neu5Ac, recoginzed by PCL and WGA), tetra-antennary complex-type N-glycan (recoginzed by PHA-L) and GalNAc α/β1-3/6 Gal (recoginzed by WFL) were all decreased in sera from Chinese HCC patients with early HE.

Validation of different glycosylation patterns

Lectin blotting was performed by using biotinylated lectin JAC, PHA-E, RCA-I, STL, WFL and WGA to further validate the different glycosylation patterns observed in lectin microarray; meanwhile, coomassie brilliant blue staining of total proteins was also performed to show similar loading quantity of different samples. It was showed that results of lectin blotting analysis by ECL detection reagents were consistent with results of lectin microarray: signal strength of JAC was increased, while signal strengths of PHA-E, RCA-I, STL, WFL and WGA were all decreased, in sera from Chinese HCC patients with early HE (**Figure 3B**). Among these lectins, PHA-E was chosen to perform subsequent lectin affinity chromatography, based on its strong combining capacity and specific recognition for the bisecting GlcNAc.

Mass spectrometric identification of PHA-E associated glycoproteins

Proteins were enriched by PHA-E affinity chromatography in sera from Chinese HCC patients with and without early HE, and identified by LC-MS/MS. All of identified proteins were searched by the Uniport database and totally 141 glycoproteins were identified in three biological repeats. Among them, 103 glycoproteins like alpha-2-macroglobulin were identified in sera from HCC patients both with and without early HE (Table S1). **Figure 4A** and **4B** showed representative MS spectrum of peptide HNVYINGITYTPVSSTNEK from alpha-2-macroglobulin in sera from Chinese HCC patients without and with early HE, respectively. There were 12 glycoproteins in **Table 2** only identified in sera from Chinese HCC patients without early HE. Representative MS spectra of peptide LDSTDFTGTIK form transferrin receptor protein 1 in sera from Chinese HCC patients without early HE was showed in **Figure 4C**. While, it was worth noting there were still 26 glycoproteins only identified in sera from Chinese HCC patients with early HE, though total bisecting GlcNAc-modified was decreased in sera from Chinese HCC patients with early HE (**Table 3**). Representative MS spectra of peptide NIETIINTFHQYSVK from MMP9 in sera from Chinese HCC patients with early HE was showed in **Figure 4D**.

Western blotting analysis of the enriched PHA-E-associated MMP9 in sera from Chinese HCC patients with and without early HE by lectin affinity chromatography was showed in **Figure 4E**. There was no band in sera from Chinese HCC patients without early HE (C1-C6); but

Table 3. PHA-E-associated glycoproteins only identified in sera from Chinese HCC patients with early HE

Uniprot Number	Protein Name	Gene Name
A0M8Q6	Ig lambda-7 chain C region	LAC7
O00391	Sulfhydryl oxidase 1	QSOX1
O75015	Low affinity immunoglobulin gamma Fc region receptor III-B	FCG3B
O95445	Coagulation factor XII	FA12
P01854	Ig epsilon chain C region	IGHE
P02766	Transthyretin	TTHY
P02788	Lactotransferrin	TRFL
P02790	Hemopexin	HEMO
P05160	Coagulation factor XIII B chain	F13B
P05164	Myeloperoxidase	PERM
P06276	Cholinesterase	CHLE
P08294	Extracellular superoxide dismutase [Cu-Zn]	SODE
P0C0L5	Complement C4B	CO4B
P10721	Mast/stem cell growth factor receptor Kit	KIT
P12259	Coagulation factor V	FA5
P13671	Complement component C6	CO6
P14780	Matrix metalloproteinase-9	MMP9
P17813	Endoglin	EGLN
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1
P27169	Serum paraoxonase/arylesterase 1	PON1
P35916	Vascular endothelial growth factor receptor 3	VGFR3
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	CA2D1
P55058	Phospholipid transfer protein	PLTP
Q02413	Desmoglein-1	DSG1
Q12913	Receptor-type tyrosine-protein phosphatase eta	PTPRJ
P00748	Coagulation factor XII	FA12

three positive bands in 6 serum samples from Chinese HCC patients with early HE (E1-E6).

Functional annotation analysis

To clearly understand these identified PHA-E-associated glycoproteins, we carried out functional annotation analysis by DAVID. The group of glycoproteins identified in sera from Chinese HCC patients both with and without early HE were classified according to biological process (BP), cellular component (CC) and molecular function (MF). **Figure 5A** showed that most of these glycoproteins identified in sera from Chinese HCC patients both with and without early HE were involved in response to wounding, inflammatory response, defense response, and so on. Cellular components of them were focus on extracellular region, extracellular space, vesicle lumen and platelet alpha granule (**Figure 5B**). Furthermore, these glycoproteins were involved in many different kinds of molecular function: inhibitor activity, like peptidase inhibitor activity and endopeptidase inhibitor activity; binding activity, like collagen binding, calcium ion binding, pattern binding, heparin binding and lipid binding (**Figure 5C**). On the other hand, the above-mentioned BP, CC and MF were not only implicated in Chinese HCC patients with early HE but also in Chinese HCC patients with early HE, suggesting that they might not play key roles in the occurrence of early HE in HCC patients.

We further analyzed the other group of glycoproteins. Firstly, the group of 12 glycoproteins was only identified in sera from Chinese HCC patients without early HE, which were involved in humoral immune response, response to wounding, defense response and so on (**Figure 5D**). Besides, these glycoproteins were mainly localized on extracellular region, rather than the abundant cellular components of glycoproteins identified in sera from Chinese HCC patients both with and without early HE (not shown here). Then, the 26 glycoproteins only identified in sera from Chinese HCC patients with early HE were analyzed. In addition to the biological processes mentioned above (just like response to wounding, wound healing and so on), these 26 glycoproteins showed special processes and might be closely linked with blood coagulation, hemostasis, enzyme linked receptor protein signaling pathway, regulation of cellular protein metabolic process and oxidation reduction (**Figure 5E**) and mainly localized on extracellular space and extracellular region (**Figure 5F**). Among these 26 glycoproteins, voltage-

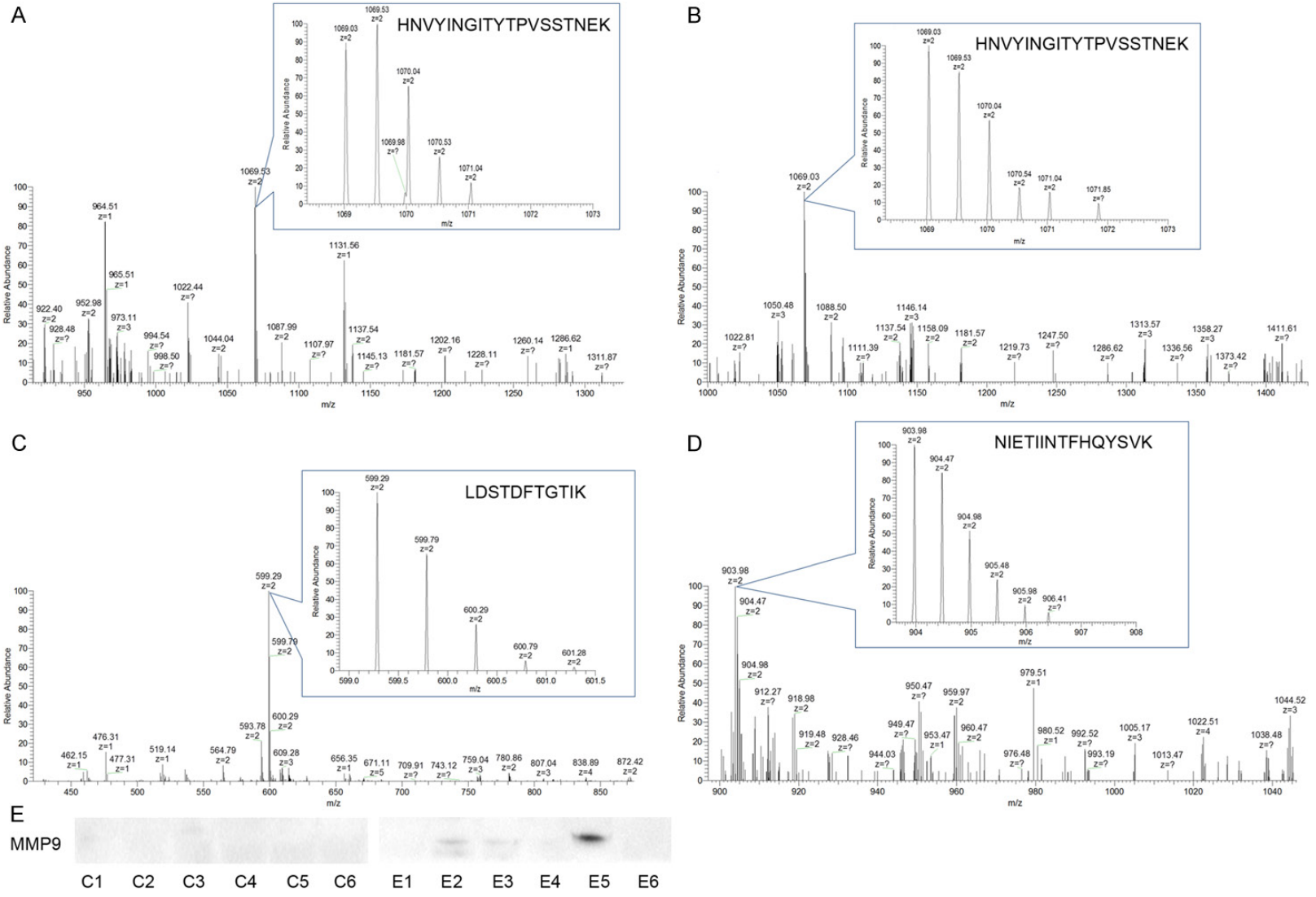

Figure 4. PHA-E-associated proteins were examined by LC MS/MS. A and B. MS spectra of HNVYINGITYTPVSSTNEK from alpha-1-antichymotrypsin in sera from Chinese HCC patients without and with early HE, respectively. C. MS spectra of LDSTDFTGTIK from transferrin receptor protein 1 which only identified in serum from Chinese HCC patients without early HE. D. MS spectra of NIETIINTFHQYSVK from matrix metalloproteinase-9 which only identified in serum from Chinese HCC patients with early HE. E. PHA-E-associated MMP9 glycoproteins enriched in serum from 6 HCC patients without early HE (C1-C6) and 6 HCC patients with early HE (E1-E6) were subjected to western blotting.

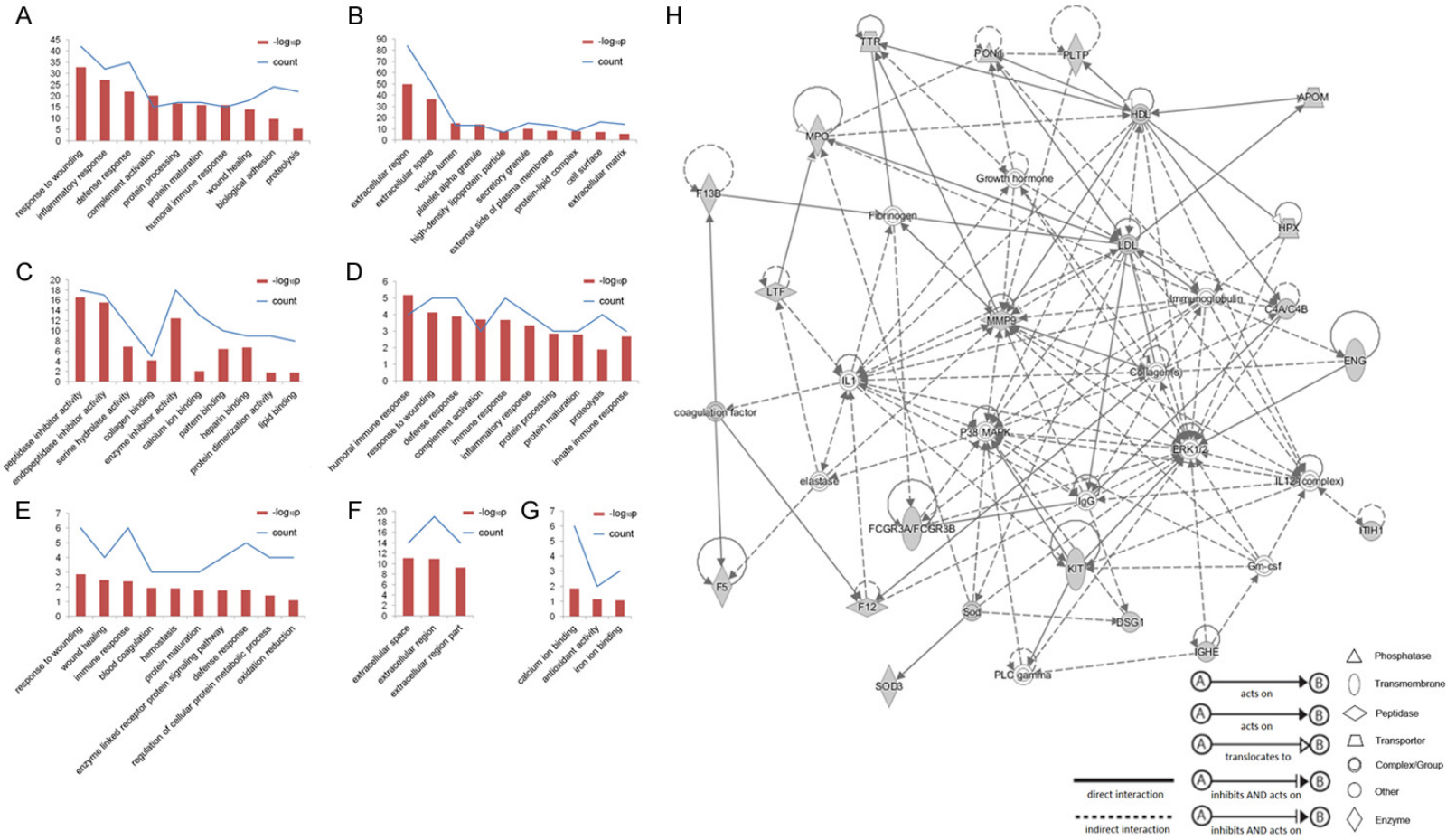

Figure 5. Functional annotation by bioinformatics analysis. A-C. the biological processes, cellular components and molecular function of the PHA-E-associated glycoproteins identified in sera from Chinese HCC patients both with and without early HE. D. The biological processes functional annotation of the PHA-E-associated glycoproteins only identified in serum from Chinese HCC patients without early HE. E-G. the biological processes, cellular components and molecular function of the PHA-E-associated glycoproteins only identified in serum from Chinese HCC patients with early HE. H. Corresponding network of the PHA-E-associated glycoproteins only identified in sera from Chinese HCC patients with early HE by IPA analysis.

dependent calcium channel subunit alpha-2/delta-1, coagulation factor V, desmoglein-1, inter-alpha-trypsin inhibitor heavy chain H1, MMP9 and myeloperoxidase were involved in calcium ion binding; myeloperoxidase and extracellular superoxide dismutase in antioxidant activity; hemopexin, lactotransferrin and myeloperoxidase in iron ion binding (**Figure 5G**). **Figure 5H** showed the top corresponding network of 19 glycoproteins among the 26 glycoproteins only identified in sera from Chinese HCC patients with early HE by IPA analysis, which is based on the Ingenuity Knowledge database to collect and generate information about molecule-to-molecule interactions, biological networks and canonical pathways. Seven molecules were enzyme, transcription, complex/group, peptidase, phosphatase, transporter, and other, respectively. Nobatly, MMP9, Low affinity immunoglobulin gamma Fc region receptor III-B among these 19 glycoproteins, and other molecules like p38 MAPK, ERK1/2, IL1 and IL12 (complex) were enriched in the network.

Discussion

Glycosylation is one of the most common and complex forms of post-translational modification of proteins and regulates a lot of critical cellular processes. Nowadays, impressive progress in exploring the structure and roles of glycans in various diseases, especially in cancer, has contributed to the discovery of glycans as promising biomarkers and highlighted their application in the clinical setting as appealing targets [24]. We had previously reported the alterations of cell surface glycan in HCC EMT [22], and a series of glycoprotein biomarkers of HCC occurrence and metastasis like paraoxonase 1 (PON1) and expression of alpha-1-antitrypsin (SERPINA1) [25, 26]. Glycoproteomics is a focus in the glycobiology, however, research in this field is difficult because of limited techniques exploring the vast number of glycan motifs and potential glycosylation sites of glycoproteins. There are various glycoproteins secreted or shed from cell surfaces or released from tissue into serum, which enables serum as the most available sample to be the primary clinical specimen in disease diagnosis and biomarker discovery [27]. Here, we paid our attention to glycoproteins in sera from Chinese HCC patients with and without early HE based on an integrated strategy of lectin-related techniques like lectin microarray, lectin bolting, lectin affinity chromatography combines with LC-MS/MS to provide early HE related glycosylation pattern and glycoprotein profiling, and tried to provide the database for early HE biomarker discovery in HCC patients.

Our results from lectin microarray analysis demonstrated specific carbohydrate epitopes: GalNAc, terminal α-1,3 Man, bisecting GlcNAc, (GlcNAc) n, O-GlcNAc, Neu5Ac, tetra-antennary complex-type N-glycan and GalNAc α/β1-3/6 Gal were all decreased in sera from Chinese HCC patients with early HE. Although there are not plenty of researches focused on glycoproteomics in HE, results from other researches of neurodegenerative diseases could be used as references. O-GlcNAc-modification, which plays key roles in maintenance and function of the synapse, could be recognized by WGA [28]. Mounting evidence shows O-GlcNAc-modified proteins were decreased in Alzheimer's disease (AD) brain, and the disruption of glucose metabolism was not only a consequence of the disease but also a causative agent for AD [29, 30]. In addition, O-GlcNAc can reduce protein phosphorylation at specific sites by modify competitively, which was associated with amyotrophic lateral sclerosis (ALS) [31]. PHA-L reactive structure, β1,6-GlcNAc branched N-glycan, was catalyzed by glycosyltransferase GnT-V. GnT-V deficiency in T cells could negatively regulate experimental autoimmune encephalomyelitis (EAE) and spontaneous inflammatory demyelination [32]. In this study, most glycosylation modification were decreased in HCC patients with early HE, suggesting that the occurrence of early HE in HCC patients might be accompanied by deglycosylation. This is in line with previously published data, showing that inhibiting deglycosylation of glycoprotein might be an innovative way to suppress abnormal phosphorylation in neurodegenerative diseases [33].

As a plant lectin, which has strong combining capacity and could specifically recognize the bisecting GlcNAc structure, PHA-E was chosen for subsequent lectin affinity chromatography. In the lists of PHA-E-associated glycoprotein profiling of sera from Chinese HCC patients, there were some interesting findings. PHA-E-associated complement C4A and C4B, although they were both as isoforms of C4

[34], they belonged to different PHA-E-associated glycoprotein groups. Complement C4A was only identified in sera from Chinese HCC patients without early HE, while complement C4B was only identified in sera from Chinese HCC patients with early HE. According to the covalent linkage with the target cells or immune complexes through the highly reactive thioester carbonyl group, C4A and C4B are distinguished: activated C4A tends to form an amide bond with amino groups on antigens, while activated C4B is strongly reactive towards hydroxyl groups on glycerols or glycosylated antigens [35]. Increased frequency of complement C4A deficiency could be identified in various diseases, like subacute sclerosing panencephalitis, a chronic neurodegenerative infection of the central nervous system [36]. Furthermore, PHA-E-associated MMP9 was only identified in sera from Chinese HCC patients with early HE. Previous researches had shown that upregulated MMP9 could induce the blood-brain barrier (BBB) permeability during the later stages of HE [37, 38]. On the other hand, in this study we could not detect PHA-E-associated MMP9 in sera from Chinese HCC patients without early HE, suggesting that serum PHA-E-associated MMP9 showed a promising diagnostic accuracy in Chinese HCC patients with early HE and might be a potential biomarker to identify HCC patients with early HE. It was noting that the roles of MMP9 in tumor invasion and progression were prominent; down-regulation of MMP9 could inhibit the metastasis of HCC cells [39-41]. According to previous studies, higher expression of MMP9 always was associated with tumor invasion and metastasis. While bisecting GlcNAc modification, which is recognized by PHA-E, could be a suppressor in the tumor metastasis [42]. That might be the reason why PHA-E-associated MMP9 was so few in HCC without early HE, in contrast, detecting PHA-E-associated MMP9 might identify specifically early HE patients in Chinese HCC patients.

In addition to the glycoprotein MMP9 discussed above, there were other two significant nodes, p38 MAPK and ERK1/2, only identified in Chinese HCC patients with early HE in the IPA network of PHA-E-associated glycoproteins. It was reported that rats with early HE due to portacaval shunt (PCS) presented increased phosphorylation (activity) of p38 [43]. p38 is a therapeutic target for HE, inhibitors of p38 have been reported to improve or reverse neuropsychiatric manifestations and prevent the onset and progression of HE in patients and animal models [44, 45]. Further analysis showed that ammonia could induce ERK1/2 phosphorylation through the activity of EGF receptor and Src [46]. These results suggest that those PHA-E-associated glycoproteins only identified in sera from Chinese HCC patients with early HE might closely be bound up with the occurrence of early HE in HCC patients and involved in the occurrence of early HE in HCC patients through p38 MAPK signaling pathway and MAPK/ERK signaling pathway.

However, one limitation of this study is that we performed a non-quantitative MS analysis. As mentioned above, proteins identification in our study could be associated with the precision of mass spectrometry system. Thus, future studies performing the quantitative determination by MS could be needed in order to confirm and optimize our results. Currently, our study just presented the difference in glycosylation patterns and PHA-E-associated glycoprotein profiling between sera from Chinese HCC patients with and without early HE, further studies are needed to further explore the relationship between these differences and pathogenic mechanism of early HE in HCC patients.

In conclusion, we performed an integrated strategy of lectin-related techniques and obtained the different glycosylation patterns and PHA-E-associated glycoprotein profiling of sera from Chinese HCC patients with and without early HE. Further bioinformatics analysis showed specific PHA-E-associated glycoproteins might be involved in the occurrence of early HE through p38 MAPK signaling pathway and MAPK/ERK signaling pathway. As we know, this is the first comprehensive glycomics analysis which presented the alteration of glycoproteomics in sera from Chinese HCC patients with and without early HE. Hence, we believe this research will provide potential biomarkers and potential targets in the prophylaxis and treatment of early HE in HCC patients.

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Disclosure of conflict of interest

None.

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Table S1. PHA-E-associated glycoproteins identified in sera from HCC patients both with and without early HE

Uniprot Number	Protein Name	Gene Name
P68871	Hemoglobin subunit beta	HBB
P01023	Alpha-2-macroglobulin	A2MG
P00738	Haptoglobin	HPT
P69905	Hemoglobin subunit alpha	HBA
P10909	Clusterin	CLUS
P01876	Ig alpha-1 chain C region	IGHA1
P05155	Plasma protease C1 inhibitor	IC1
P02647	Apolipoprotein A-I	APOA1
P01871	Ig mu chain C region	IGHM
P02649	Apolipoprotein E	APOE
P01009	Alpha-1-antitrypsin	A1AT
P01024	Complement C3	CO3
P00751	Complement factor B	CFAB
P01591	Immunoglobulin J chain	IGJ
P00450	Ceruloplasmin	CERU
P09871	Complement C1s subcomponent	C1S
P02763	Alpha-1-acid glycoprotein 1	A1AG1
P02671	Fibrinogen alpha chain	FIBA
Q08380	Galectin-3-binding protein	LG3BP
P02679	Fibrinogen gamma chain	FIBG
P80108	Phosphatidylinositol-glycan-specific phospholipase D	PHLD
P19320	Vascular cell adhesion protein 1	VCAM1
P00734	Prothrombin	THRB
P49747	Cartilage oligomeric matrix protein	COMP
P07333	Macrophage colony-stimulating factor 1 receptor	CSF1R
P02787	Serotransferrin	TRFE
P02765	Alpha-2-HS-glycoprotein	FETUA
P10643	Complement component C7	CO7
P51884	Lumican	LUM
P04217	Alpha-1B-glycoprotein	A1BG
P02675	Fibrinogen beta chain	FIBB
P01011	Alpha-1-antichymotrypsin	AACT
O14791	Apolipoprotein L1	APOL1
P01008	Antithrombin-III	ANT3
P01042	Kininogen-1	KNG1
P04004	Vitronectin	VTNC
P22792	Carboxypeptidase N subunit 2	CPN2
P01031	Complement C5	CO5
P23142	Fibulin-1	FBLN1
P02743	Serum amyloid P-component	SAMP
P19652	Alpha-1-acid glycoprotein 2	A1AG2
O75882	Attractin	ATRN
P02750	Leucine-rich alpha-2-glycoprotein	A2GL
P43121	Cell surface glycoprotein MUC18	MUC18
P02751	Fibronectin	FINC
P05090	Apolipoprotein D	APOD

P55290	Cadherin-13	CAD13
P04275	von Willebrand factor	VWF
P01833	Polymeric immunoglobulin receptor	PIGR
P04114	Apolipoprotein B-100	APOB
P00736	Complement C1r subcomponent	C1R
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4
P01877	Ig alpha-2 chain C region	IGHA2
Q6EMK4	Vasorin	VASN
P07225	Vitamin K-dependent protein S	PROS
P49908	Selenoprotein P	SEPP1
P33151	Cadherin-5	CADH5
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2
Q6UX71	Plexin domain-containing protein 2	PXDC2
P03952	Plasma kallikrein	KLKB1
P13688	Carcinoembryonic antigen-related cell adhesion molecule 1	CEAM1
P00747	Plasminogen	PLMN
Q04756	Hepatocyte growth factor activator	HGFA
O14786	Neuropilin-1	NRP1
Q6UXB8	Peptidase inhibitor 16	PI16
P02760	Protein AMBP	AMBP
P40189	Interleukin-6 receptor subunit beta	IL6RB
P48740	Mannan-binding lectin serine protease 1	MASP1
P08697	Alpha-2-antiplasmin	A2AP
Q12860	Contactin-1	CNTN1
P43251	Biotinidase	BTD
P08603	Complement factor H	CFAH
P13591	Neural cell adhesion molecule 1	NCAM1
Q9NZP8	Complement C1r subcomponent-like protein	C1RL
Q7Z7G0	Target of Nesh-SH3	TARSH
P01019	Angiotensinogen	ANGT
P07359	Platelet glycoprotein Ib alpha chain	GP1BA
P15144	Aminopeptidase N	AMPN
P30530	Tyrosine-protein kinase receptor UFO	UFO
P55285	Cadherin-6	CADH6
P02749	Beta-2-glycoprotein 1	APOH
P04196	Histidine-rich glycoprotein	HRG
P15151	Poliovirus receptor	PVR
P16070	CD44 antigen	CD44
P32942	Intercellular adhesion molecule 3	ICAM3
Q99784	Noelin	NOE1
P17936	Insulin-like growth factor-binding protein 3	IBP3
P06681	Complement C2	CO2
O75636	Ficolin-3	FCN3
P43652	Afamin	AFAM
P25311	Zinc-alpha-2-glycoprotein	ZA2G
P20851	C4b-binding protein beta chain	C4BPB
O00533	Neural cell adhesion molecule L1-like protein	CHL1
P02748	Complement component C9	CO9
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3

Q12866	Tyrosine-protein kinase Mer	MERTK
Q14697	Neutral alpha-glucosidase AB	GANAB
P05362	Intercellular adhesion molecule 1	ICAM1
P08195	4F2 cell-surface antigen heavy chain	4F2
Q16610	Extracellular matrix protein 1	ECM1
P35542	Serum amyloid A-4 protein	SAA4
P04278	Sex hormone-binding globulin	SHBG
Q13740	CD166 antigen	CD166