Assessment of Hepatocellular Carcinoma Metastasis Glycobiomarkers Using Advanced Quantitative N-glycoproteome Analysis

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Hepatocelluar carcinoma (HCC) is one of the most common malignant tumors with high incidence of metastasis. Glycosylation is involved in fundamental molecular and cell biology process occurring in cancer including metastasis formation. In this study, lectin microarray, lectin blotting, lectin affinity chromatography and tandem ^{18}O stable isotope labeling coupled with liquid chromatography-mass spectrometer (LC-MS) analysis were applied to quantify the changes in N-glycosite occupancy for HCC metastasis serum. Firstly, lectin microarray was used to screen glycoforms and Phaseolus vulgaris Leucoagglutinin (PHA-L) reactive structure (β1,6-GlcNAc branched N-glycan) was found to be increased significantly in HCC patients with metastasis compared with those with non-metastasis. Then, PHA-L affinity glycoproteins were enriched followed by N-glycosite occupancy measurement with strategy of tandem ^{18}O stable isotope labeling. 11 glycoproteins with significantly changed N-glycosite occupancy were identified, they were associated with cell migration, invasion and adhesion through p38 mitogen-activated protein kinase signaling pathway and nuclear factor kappa B signaling pathway. Quantification of N-glycosite occupancy for PHA-L reactive glycoproteins could help to discover important glycoproteins of potential clinically significance in terms of HCC etiology. Also, understanding of N-glycosite occupancy alterations will aid the characterization of molecular mechanism of HCC metastasis as well as establishment of novel glycobiomarkers.

Keywords: hepatocellular carcinoma, metastasis, N-glycosite occupancy, lectin, tandem ^{18}O stable isotope labeling

INTRODUCTION

Hepatocellular carcinoma (HCC) as the major primary liver cancer is the third leading cause of cancer-related death and account for 70–85% of the liver cancers worldwide (Jemal et al., 2011). Despite the medical techniques have experienced significant advances, the clinical prognosis still remains extremely poor and the 5-year survival rate in HCC patients after surgical resection is only 20–30% (Yamamoto et al., 2001; Pang et al., 2008; Yang et al., 2013). High incidences of recurrence and metastasis rate contribute to the long-term prognosis unsatisfactory (Tung-Ping Poon et al., 2000; Jia et al., 2011).

HCC metastasis was due to the comprehensive effect of manifold causes and always began by HCC cells breaking through the walls of nearby lymph or blood vessels. It is very important to identify the changed biomolecular associated with HCC metastasis so that it could predict the risk of metastasis and the prognostic value, which may contribute to better treatments for the patients.

Glycosylation is one of the most prominent forms of posttranslational protein modification and more than 50% of human protein are presumed to have undergone glycosylation (Apweiler et al., 1999). Glycosylation plays a major role in regulating critical cellular functions and assembly of complex multicellular organs and organisms. It is involved in cell-cell and receptor-ligand interactions, signal transduction, and endocytosis (Varki and Lowe, 2009; Karve and Cheema, 2011; Rakus and Mahal, 2011). Abnormal glycosylation is associated with malignant transformation (Kannagi et al., 2004; Mi et al., 2012). In recent years, a handful of glycoproteins as cancer biomarkers have completed the program from discovery to verification and validation (Kim and Misek, 2011; Kuzmanov et al., 2013; Shah et al., 2015). One typical example is the Lens Culinaris Agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3). The tumor marker AFP is widely used for HCC's surveillance (Blomme et al., 2009; Xia et al., 2012), while on account of AFP-negative HCC is frequently observed, AFP-L3 has been a preferred HCC biomarker in early diagnosis of HCC and in predicting prognosis after treatment (Sato et al., 1993; Okuda et al., 1999; Kumada et al., 2014). It was reported that complement C3, ceruloplasmin, histidine-rich glycoprotein, CD14, hepatocyte growth factor (HGF) (Liu, Y. et al., 2010), hemopexin, fetuin-A (Comunale et al., 2009) and haptoglobin (Zhang et al., 2016) could be potential glycobiomarkers for distinguishing HCC. However, some glycobiomarkers are not unique for HCC progression, which could be observed in most gastrointestinal (GI) cancers and may provide clinical assistant diagnosis for HCC (Dempsey and Rudd, 2012; Ren et al., 2016).

Glycoproteins have been found to play important roles in invasion and metastasis of tumors. Accurate characterization of glycoproteins with multiple glycosylation sites and assessment of the glycan macroheterogeneity (glycosite occupancy) and microheterogeneity (glycan structure) are urgently needed for understanding the functions of glycans in HCC. Especially, N-glycosite occupancy is associated with the enzymatic activity and the physical stability of glycoproteins (Baboval et al., 2000; Alsenaidy et al., 2014), which might contribute to the metastasis of HCC. An endoplasmic reticulum-retained green fluorescent protein (GFP) biomarker was reported, whose fluorescence was lost when it was N-glycosylated. This marker was a highly sensitive indicator of N-glycosite occupancy of multiple cell lines. But it could not be used to measure glycosite occupancy of other target glycoproteins (Losfeld et al., 2012). Xu et al. (2015) developed SWATH-MS-based methods were developed for automated measurement of glycosite occupancy in N-glycoproteins from the yeast cell wall and from human whole saliva. Sumer-Bayraktar et al. (2012) performed a MS-driven glycoproteomics and glycomics combined with exoglycosidase treatment to determine glycosite occupancies of serum-derived Hshbg. A universal workflow for site-specific N- and O-glycopeptide analysis of pronase treated glycoproteins was described and glycosite occupancy of IgG3 was reported (Stavenhagen et al., 2015).

A novel strategy using tandem ^{18}O stable isotope labeling (TOSIL) could quantify N-glycosite occupancy by measuring the intensity ratios of ^{18}O/^{16}O for glycosylated (6 Da) and for non-glycosylated (4 Da) peptides (Liu, Z. et al., 2010). This method could quantify the changes of N-glycosite occupancy in complex protein mixtures and produce a 6 Da difference among differently labeled glycopeptides which was easily observed. In this study, lectin microarray was used to screen metastasis-related glycoforms which were validated by lectin blotting analysis. PHA-L reactive structure (β1,6-GlcNAc branched N-glycan) was found to be increased significantly in HCC patients with metastasis compared with those with non-metastasis. Then, PHA-L affinity glycoproteins were enriched and 11 glycoproteins with changed N-glycosite occupancy were identified using TOSIL strategy coupled with LC-MS analysis. What was more, p38 mitogen-activated protein kinase signaling pathway (p38 MAPK) and nuclear factor kappa B signaling pathway (NF-κB) were found to be significant nodes in IPA network, indicating that these glycoproteins played important roles in biological processes of HCC metastasis.

MATERIALS AND METHODS

Clincial Specimens
Serum samples from 80 HCC patients were collected at First Affiliated Hospital of Dalian Medical University and stored at −80°C. The clinicopathological data of the patients were provided in **Table 1**. Pooling sera of 10 HCC patients with extrahepatic metastasis (metastasis) and 10 HCC patients with non-metastasis were used for analyses. Four biological repeats were measured independently to guarantee the reproducibility of experiment. Written informed consent was obtained from each patient. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of First Affiliated Hospital of Dalian Medical University. All methods in this study were performed in accordance with the human experimentation guideline of the People's Republic of China.

Each pooling sera was mixed by equivalent volume of individual sera (6 μL), and the total volume of pooling sera was 60 μL. Albumin and IgG were depleted by ProteoExtract® Albumin/IgG removal kit (Calbiochem, Billerica, MA, USA) from pooling sera according to the manufacturer's description.

Lectin Microarray Analysis
One microgram proteins were biotinylated by Lightning-Link Biotin Labeling Kit (Innova Biosciences, Cambridge, UK). A lectin microarray was produced using 50 lectins (Vector Laboratories, Burlingame, CA, USA; Sigma-Aldrich, Castle Hill, NSW, Australia). The name and the binding specificity of 50 lectins were provided in **Table S1**. The workflow for lectin microarray was described in **Figure S1**: after blocking the non-specific binding sites with 2% bovine serum albumin (BSA)-phosphate buffer saline (PBS), the lectin microarray was incubated with equal biotinylated proteins (non-metastatic or metastatic) and Cy5 labeled streptavidin (Life technologies,

TABLE 1 | General information and clinical characteristics of HCC patients for screening.

	Non-metastatic $n = 40$	Metastatic $n = 40$	p
Age (years)	56 ± 10	57 ± 12	0.855[a]
Gender (male/female)	37 (92.5%)/3 (7.5%)	38 (95%)/2 (5%)	0.644[b]
AFP (IU/ml)	353.2 ± 332.7	437.3 ± 326.6	0.578[a]
ALT (IU/L)	52.7 ± 42.1	59.3 ± 43.2	0.708[a]
AST (IU/L)	71.3 ± 44.1	81.5 ± 65.7	0.703[a]
HbsAg (yes/no)	36 (90%)/4 (10%)	39 (97.5%)/1 (2.5%)	0.166[b]
PT (s)	13.2 ± 1.8	14.7 ± 2.5	0.121[a]

[a]Student's T-test.

[b]Chi-square test

The values supplied were mean ± standard deviation. AFP, Alpha fetoprotein; ALT, Alanine aminotransferase; AST, Aspartate transaminase; HbsAg, Hepatitis B surface antigen; PT, Prothrombin time.

Waltham, MA, USA) in turn. LuxScan 10K/A scanner system (CapitalBio, Beijing, China) was used to scan and data were analyzed as described previously (Xin et al., 2014).

Lectin Blotting Analysis

Twenty microgram proteins for identification were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with biotinylated Datura Stramonium Agglutinin (DSA), Maackia Amurensis Lectin-I (MAL-I), PHA-L 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-L agarose was washed and resuspended with the lectin-binding solution (10 mM Tris-HCl, pH7.5, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Then, 3 mg proteins from different assemblages (non-metastatic or metastatic) were added into PHA-L agarose and incubated at 4°C overnight with a round shaker. Lectin-binding solution was used to wash the agarose and the bound fraction was eluted by 200 mM N-acetyl-D-(+)-glucosamine. The eluted fraction was separated by SDS-PAGE

and stained by PhastaGel^{TM} Blue R. The gels containing all bands were cut and processed for in-gel digestion.

In-gel Digestion and Strategy of Tandem ^{18}O Stable Isotope Labeling

The destained gel pieces were reduced and alkylated with Tris-(2-carboxy-ethyl)-phosphine hydrochloride (TCEP, Sigma, Castle Hill, NSW, Australia) and iodoacetamide (IAA, Sigma, Castle Hill, NSW, Australia), respectively. Subsequently, the gel pieces were re-dehydrated with 100% ACN, and then digested in trypsin solution (5 ng/μL) at 37°C overnight. TOSIL strategy was performed as described previously (Liu, Z. et al., 2010), and the workflow was shown in **Figure S2**. HCC patients with metastasis were treated in $H_2^{18}O$ and those with non-metastasis treated in $H_2^{16}O$.

LC-MS Analysis

The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA, USA) connected to a quadrupole-Orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. 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 350–1,800) were acquired with a mass resolution of 70 K, followed by 10 sequential high energy collisional dissociation (HCD) MS/MS scans with a resolution of 17.5 K. In all cases, one microscan was recorded using dynamic exclusion of 30 s.

Datebase Searching and Quantification

The deglycosylated glycopeptides and non-glycosylated peptides were searched against SWISS-PROT human database using the MaxQuant 1.5.3.30, a quantitative proteomics software package. The parameters for searching were set: enzyme, partial trypsin; missed cleavages allowed, two; fixed modification, carboxyamidomethyla-tion (Cys); variable modifications, deamidation (Asn), deamidation plus ^{18}O (Asn), ^{18}O C-term and oxidation (Met); peptide tolerance, 10 ppm; MS/MS tolerance, 0.05 Da. The relative quantities of N-glycosylated and its parent protein levels were obtained simultaneous by measuring the intensity ratios of ^{18}O/^{16}O for glycosylated (6 Da) and for non-glycosylated (4 Da) peptides from the same proteins respectively. A comparison of these two ratios can be utilized to evaluate the change of N-glycosite occupancy between HCC patients with metastasis (^{18}O labeling) and those with non-metastasis (^{16}O labeling) by the Equation as follows:

$$
\begin{aligned}
\text{Change of N-glycosite occupancy} &= \frac{\text{Intensity of }^{18}\text{O deglycosylated glycopeptide/Average intensity of }^{18}\text{O non-glycosylated peptides}}{\text{Intensity of }^{16}\text{O deglycosylated glycopeptide/Average intensity of }^{16}\text{O non-glycosylated peptides}} \\
&= \frac{\text{Intensity of }^{18}\text{O deglycosylated glycopeptide}}{\text{Intensity of }^{16}\text{O deglycosylated glycopeptide}} \\
&\quad \times \frac{\text{Average intensity of }^{16}\text{O non-glycosylated peptides}}{\text{Average Intensity of }^{18}\text{O non-glycosylated peptides}} \\
&= \frac{^{18}\text{O}/^{16}\text{O ratio for deglycosylated glycopeptide}}{^{18}\text{O}/^{16}\text{O ratio for protein}}
\end{aligned}
$$

Fold changes >1.5 or <0.667 were considered to be significant. Fold changes between 1.2 and 1.5 (1.2–1.5) or between 0.667 and 0.833 (0.667–0.833) were considered as minor.
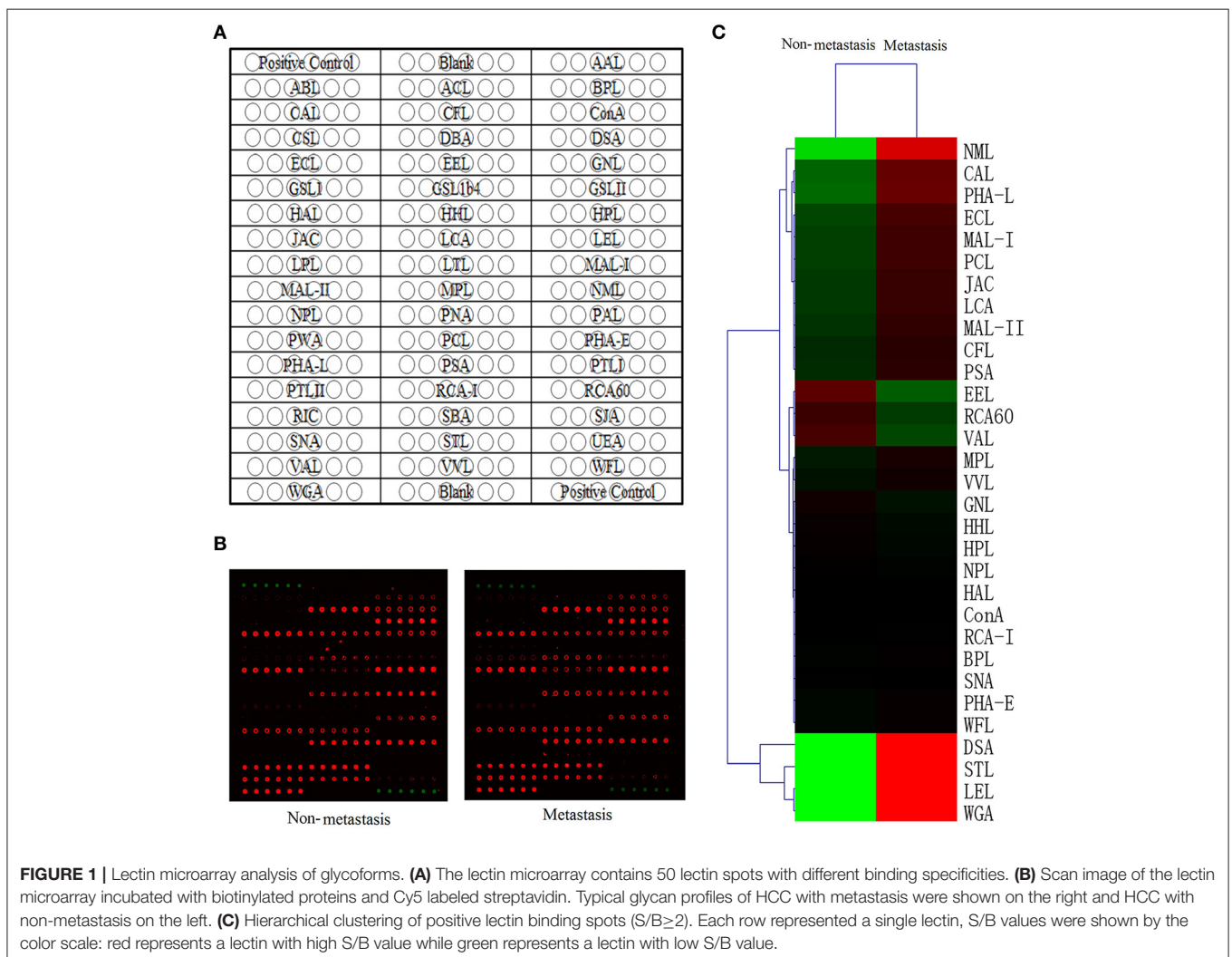
Functional Annotation and Patterns Analysis

Functional categories of 11 N-glycoproteins with changed N-glycosite occupancy were analysis using OmicsBean (http://www.omicsbean.com). Ingenuity Pathway Analysis (IPA) analysis (QIAGEN, Redwood City, CA, USA) was used to investigate biological interactions. Motif extractor (Motif-X, http://motif-x.med.harvard.edu) was used to identify overrepresented glycosylation motifs. Proteins annotated in International Protein Index (IPI) human proteome database were used as background and the significance value was set as 0.000001 (Schwartz and Gygi, 2005; Chou and Schwartz, 2011; Wang et al., 2014; Zhang et al., 2015).

RESULTS

Altered Glycoforms of HCC Metastasis Sera

A high-throughput lectin microarray (**Figure 1A**) which included 50 lectins, 2 positive controls and 2 blank controls in each block was applied to detect different glycoforms between HCC patients with metastasis and HCC patients with non-metastasis. The positive controls were albumin coupling with Cy3 which showed by Cy3 scanning, and the blanks in each block were the negative controls (**Figure 1B**). The Spot Intensity Median (S) and the Background Intensity Median (B) were extracted and S/B was calculated. Using S/B ≥ 2 as cutoff, 31 lectins were defined as positive lectin binding spots. Hierarchical clustering of them was mapped by The MeV 4.8.1. (**Figure 1C**).
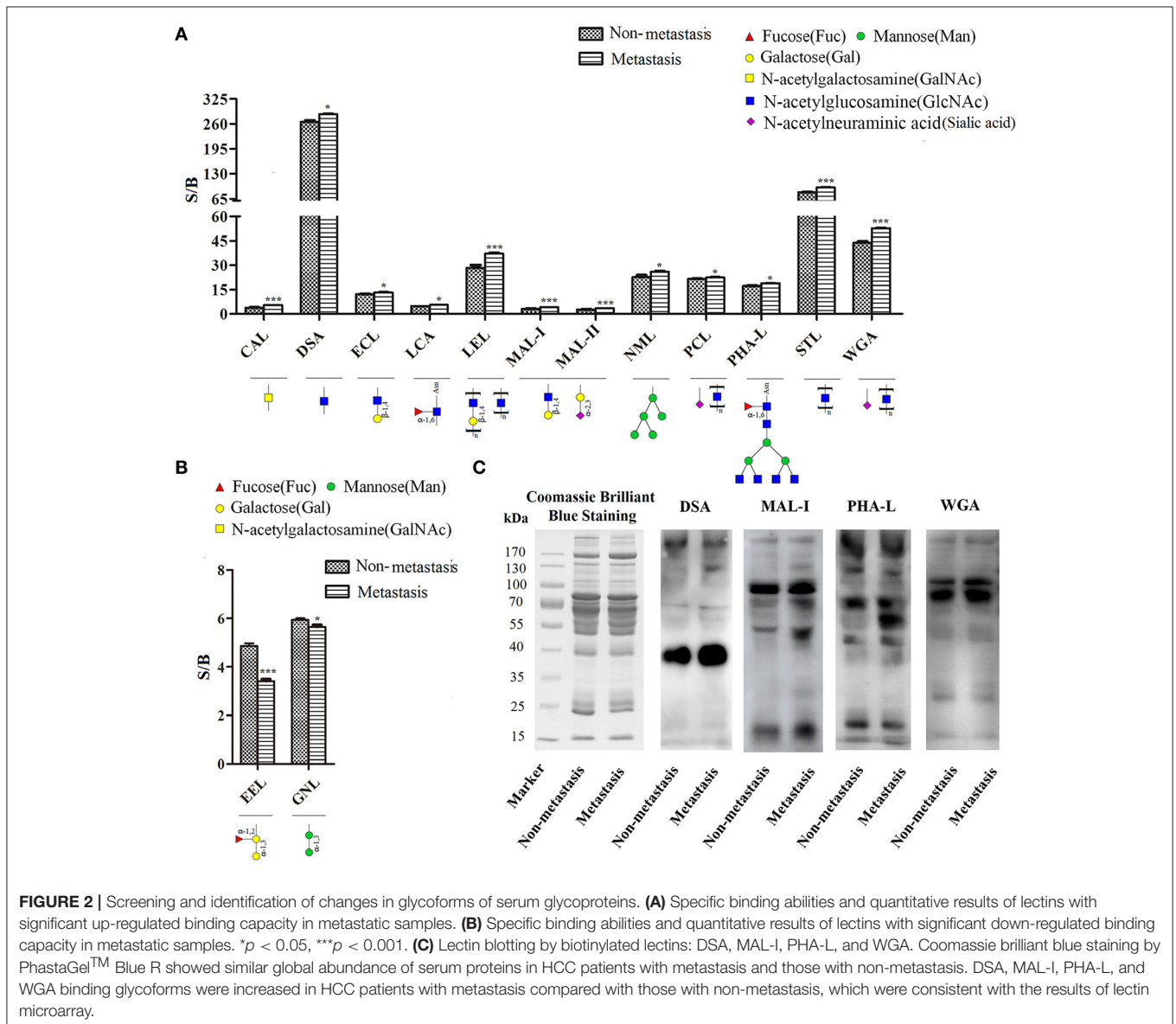
Among these 31 lectins, 14 lectins had statistical significance ($p < 0.05$) and we divided protein-lectin binding intensities of them into 3 grades: weak binding ($5 > S/B \geq 2$), medium binding ($15 > S/B \geq 5$) and strong binding ($S/B \geq 15$). In non-metastatic HCC samples, Caragana Arborescens Lectin (CAL),

FIGURE 1 | Lectin microarray analysis of glycoforms. **(A)** The lectin microarray contains 50 lectin spots with different binding specificities. **(B)** Scan image of the lectin microarray incubated with biotinylated proteins and Cy5 labeled streptavidin. Typical glycan profiles of HCC with metastasis were shown on the right and HCC with non-metastasis on the left. **(C)** Hierarchical clustering of positive lectin binding spots (S/B≥2). Each row represented a single lectin, S/B values were shown by the color scale: red represents a lectin with high S/B value while green represents a lectin with low S/B value.

Euonymus Europaeus Lectin (EEL), MAL-I, Maackia Amurensis Lectin-II (MAL-II) were weak binding; Erythrina Cristagalli Lectin (ECL), Galanthus Nivalis Lectin (GNL) and Lens Culinaris Agglutinin (LCA) were medium binding; DSA, Lycopersicon Esculentum Lectin (LEL), Naja Mossambica Lectin (NML), Phaseolus Coccineus Lectin (PCL), PHA-L, Solanum Tuberosum Lectin (STL), and WGA were strong binding. While, in metastatic samples, EEL, MAL-I, MAL-II were weak binding; CAL, ECL, GNL, and LCA were medium binding; DSA, LEL, NML, PCL, PHA-L, STL, and WGA were strong binding.

Quantitative results of S/B and specific binding abilities of the 14 lectins were shown in **Figures 2A,B**, 12 lectins: GalNAc binder CAL, GlcNAc binder DSA and STL, β-1,4Gal binder ECL, Fucα-1,6GlcNAc binder LCA, Poly-LacNAc or (GlcNAc)n binder LEL, α-2,3Sia or β-1,4Gal binder MAL-I and MAL-II, exopolysaccharide binder NML, Sia binder PCL, β1,6-GlcNAc branched N-glycan binder PHA-L and (GlcNAc)n or multivalent Sia binder WGA showed increasing trend in metastatic HCC samples compared to non-metastatic HCC samples; However, α-1,3Gal binder EEL and α-1,3mannose binder GNL were lectins showed decreasing trend. Among them, the p-values of lectins CAL, LEL, MAL-I, MAL-II, STL, WGA, and EEL were less than 0.001, while, the p-values of lectins DSA, ECL, LCA, NML, PCL, PHA-L, and GNL were less than 0.05. It suggested that structures such as GalNAc, GlcNAc, β-1,4Gal, Fucα-1,6(GlcNAc)n, Sia and β1,6-GlcNAc branched N-glycan were increased significantly in HCC patients with metastasis; while, α-1,3Gal and α-1,3mannose were decreased significantly.

FIGURE 2 | Screening and identification of changes in glycoforms of serum glycoproteins. **(A)** Specific binding abilities and quantitative results of lectins with significant up-regulated binding capacity in metastatic samples. **(B)** Specific binding abilities and quantitative results of lectins with significant down-regulated binding capacity in metastatic samples. *$p < 0.05$, ***$p < 0.001$. **(C)** Lectin blotting by biotinylated lectins: DSA, MAL-I, PHA-L, and WGA. Coomassie brilliant blue staining by PhastaGel^{TM} Blue R showed similar global abundance of serum proteins in HCC patients with metastasis and those with non-metastasis. DSA, MAL-I, PHA-L, and WGA binding glycoforms were increased in HCC patients with metastasis compared with those with non-metastasis, which were consistent with the results of lectin microarray.

Confirmation of the Changed Glycoforms by Lectin Blotting

Lectin blotting was performed to validate changed glycoforms using biotinylated lectin DSA, MAL-I, PHA-L, and WGA. Coomassie brilliant blue staining showed similar global abundance of serum proteins in HCC patients with metastasis and those with non-metastasis. GlcNAc (which binds to DSA), α-2,3Sia or β-1,4Gal (which binds to MAL-I), β1,6-GlcNAc branched N-glycan (which binds to PHA-L) and (GlcNAc)n or multivalent Sia (which binds to WGA) were increased in HCC patients with metastasis compared with those with non-metastasis, which were consistent with the results of lectin microarray (**Figure 2C**).

Among them, β1,6-GlcNAc branched N-glycan was significantly changed. This structure was catalyzed by UDP-N-acetylglucosamine: α-6-D-manno-side β1–6-N-acetylglucosaminyltransferase (EC2.4.1.155) which was known as GnT-V. Expression levels of β1,6-GlcNAc branched N-glycan and GnT-V were associated with metastasis in human digestive cancers such as colorectal carcinoma and gastric cancer (Seelentag et al., 1998; Kim et al., 2008; Huang et al.,

2013; Huang, B. et al., 2014). In our previous studies, we have found this glycoform was increased in epithelial mesenchymal transition (EMT) process of Huh7 HCC cell and it might be a metastasis-promoting glycoform in HCC (Li, S. et al., 2013).

Quantification of N-glycosite Occupancy for PHA-L Reactive Glycoproteins

Then, PHA-L affinity chromatography was chosen to enrich serum N-glycoproteins and a total of deglycosylated glycopeptides from 14 glycoproteins were quantified in HCC patients with metastasis compared with those with non-metastasis (**Table 2**). The cutoff of fold change was determined by experiments: the same sera sample was divided into two equal parts for $^{16}O/^{18}O$ labeling, which indicated expected ratio of 1:1 (fold change = 1). The average (five replicates) measured ratios of N-glycosite occupancy was 1:1.19 (fold change = 1.19), which indicated the cutoff of fold change was 1.19. Considering complexity of sera, the cutoff was set as 1.5 (data not shown). Among these deglycosylated glycopeptides, there were 6 deglycosylated glycopeptides displayed significant changes in N-glycosite occupancy (fold changes>1.5 or <0.667, highlighted in bold) and 7 deglycosylated glycopeptides with minor changes

TABLE 2 | Changes of N-glycosite occupancy in HCC patients with metastasis compared with those with non-metastasis.

UniProt number	Protein name	Peptide sequence[a]	$^{18}O/^{16}O$ ratio for deglycosylated glycopeptide	Average $^{18}O/^{16}O$ ratio for protein	Change in N-glycosite occupancy (Metastasis/Non-metastasis)[b]
P00450	Ceruloplasmin	EHEGAIYPDN#TTDFQR	0.483	0.573	0.843
		EN#LTAPGSDSAVFFEQGTTR	*0.767*	*0.573*	*1.340*
P00738	Haptoglobin	**VVLHPN#YSQVDIGLIK**	**1.300**	**0.852**	**1.525**
P01008	Antithrombin-III	**SLTFN#ETYQDISELVYGAK**	**0.446**	**0.915**	**0.487**
P01009	Alpha-1-antitrypsin	**YLGN#ATAIFFLPDEGK**	**0.438**	**0.736**	**0.595**
P01011	Alpha-1-antichymotrypsin	*FN#LTETSEAEIHQSFQHLLR*	*0.658*	*0.913*	*0.721*
P02790	Hemopexin	SWPAVGN#CSSALR	0.577	0.498	1.159
P07996	Thrombospondin-1	VVN#STTGPGEHLR	1.587	1.421	1.117
P08603	Complement factor H	**ISEEN#ETTCYMGK**	**0.394**	**0.800**	**0.492**
P0C0L5	Complement C4-B	**GLN#VTLSSTGR**	**0.254**	**0.525**	**0.484**
P10909	Clusterin	**KKEDALN#ETR**	**0.456**	**0.957**	**0.476**
		HN#STGCLR	*0.722*	*0.957*	*0.754*
P23142	Fibulin-1	*CATPHGDN#ASLEATFVK*	*1.260*	*1.658*	*0.760*
Q08380	Galectin-3-binding protein	*AAIPSALDTN#SSK*	*0.499*	*0.667*	*0.747*
		ALGFEN#ATQALGR	*0.809*	*0.667*	*1.213*
		DAGVVCTN#ETR	0.659	0.667	0.987
		GLN#LTEDTYKPR	0.630	0.667	0.944
Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	LPTQN#ITFQTESSVAEQ EAEFQSPK	0.865	0.737	1.173
Q99784	Noelin	*LDPVSLQTLQTWN#TSYPK*	*0.644*	*0.817*	*0.787*
		VQN#MSQSIEVLDR	0.759	0.817	0.928

[a]*The # denotes the residue site of N-glycosylation.*
[b]*Fold changes >1.5 or <0.667 were considered to be significant (highlighted in bold). Fold changes between 1.2 and 1.5 (1.2–1.5) or between 0.667 and 0.833 (0.667–0.833) were considered as minor (highlighted in italics).*
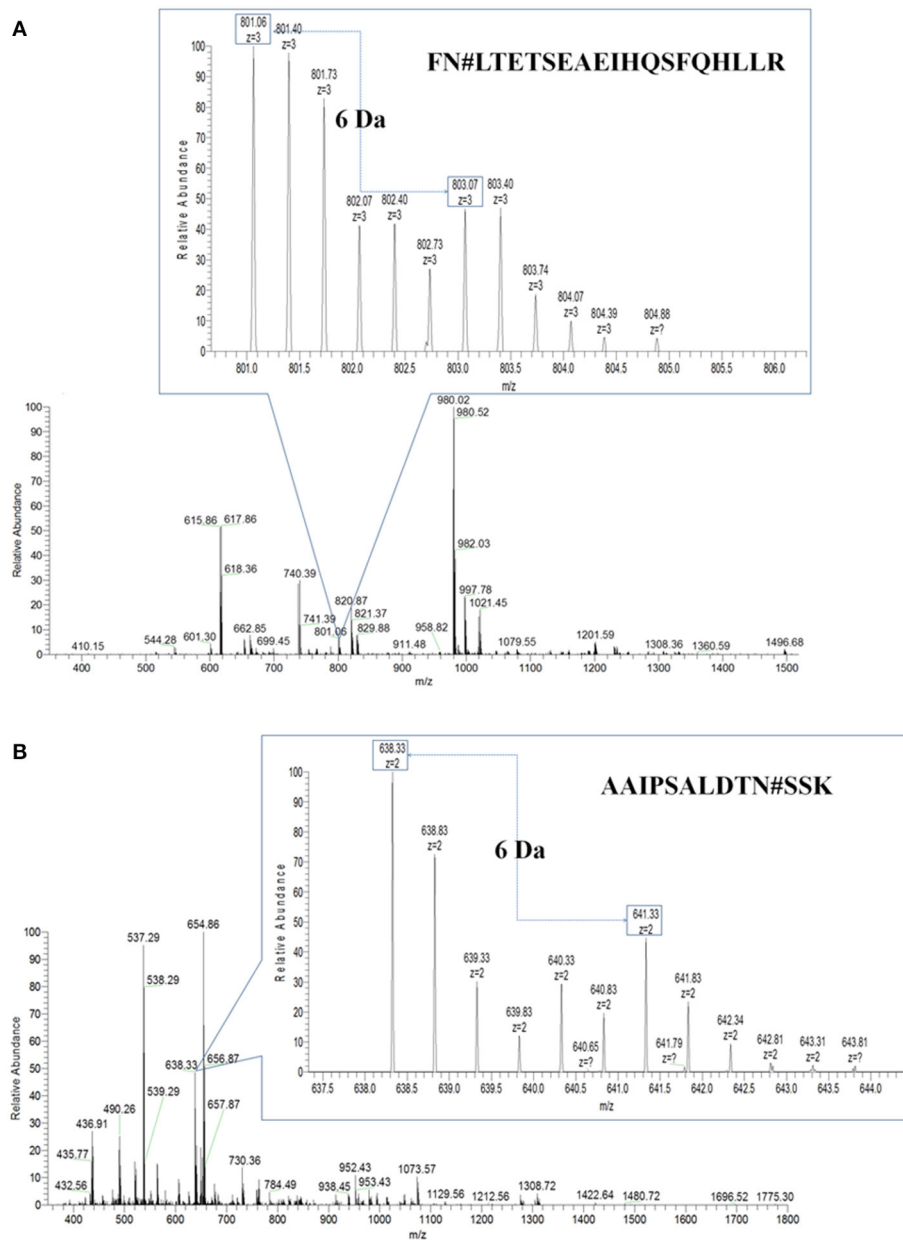
FIGURE 3 | Deglycosylated glycopeptides were examined by the TOSIL strategy with LC-MS. (A) Quantitative analysis of deglycosylated glycopeptides FN#LTETSEAEIHQSFQHLLR from alpha-1-antichymotrypsin by LC-MS. (B) Quantitative analysis of deglycosylated glycopeptides AAIPSALDTN#SSK from fibulin-1 by LC-MS. A unique mass shift of 6 Da was shown for N-glycosylated peptide with single glycosylation site.

(fold changes 1.2–1.5 or 0.667–0.833, highlighted in italics). **Figure 3** showed representative MS spectra of deglycosylated glycopeptides FN#LTETSEAEIHQSFQHLLR from alpha-1-antichymotrypsin and AAIPSALDTN#SSK from fibulin-1. MS spectra of non-glycosylated peptides ADLSGITGAR from alpha-1-antichymotrypsin and LADGGATNQGR from fibulin-1 were shown in **Figures 4A,B**, respectively. Characteristic 6 or 4 Da shift in mass could be observed via TOSIL strategy.

EN#LTAPGSDSAVFFEQGTTR from ceruloplasmin and ALGFEN#ATQALGR from galectin-3-binding protein were reduced in N-glycosylated and parent protein levels, but they displayed increasing trends in N-glycosite occupancy. CATPHGDN#ASLEATFVK from fibulin-1 were up-regulated in N-glycosylated and parent protein levels, but N-glycosite occupancy of it was down-regulated. It was noteworthy that different N-glycosite from the same parent protein had the different occupancy, for example, AAIPSALDTN#SSK and
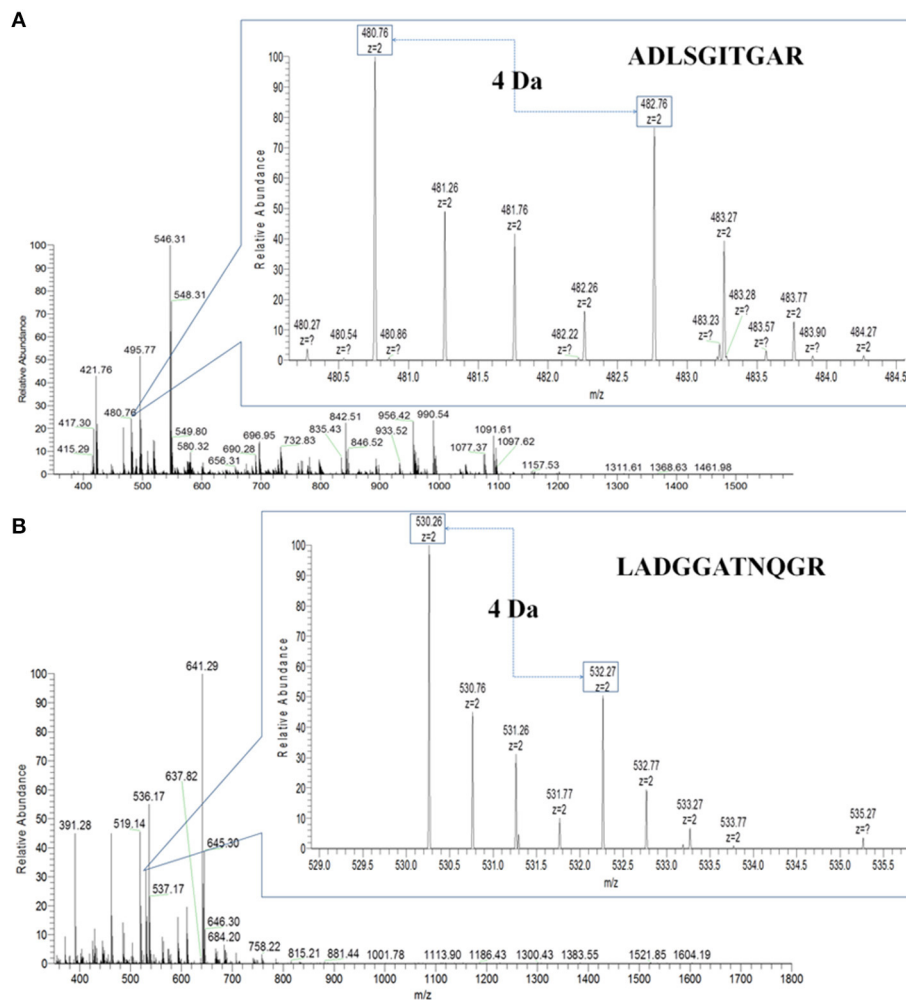
FIGURE 4 | Non-glycosylated peptides were examined by the TOSIL strategy with LC-MS. **(A)** Quantitative analysis of non-glycosylated peptide ADLSGITGAR from alpha-1-antichymotrypsin by LC-MS. **(B)** Quantitative analysis of non-glycosylated peptide LADGGATNQGR from fibulin-1 by LC-MS. A mass difference of 4 Da was shown for nonglycosite peptide.

ALGFEN#ATQALGR from galectin-3-binding protein, had fold changes of 0.747 and 1.213 in N-glycosite occupancy, respectively. In our previous study, N-glycosite occupancy of VVLHPN#YSQVDIGLIK was changed significantly in HCC patients compared with patients with hepatitis B virus infection (HBV) and liver cirrhosis (LC) (Zhang et al., 2012).
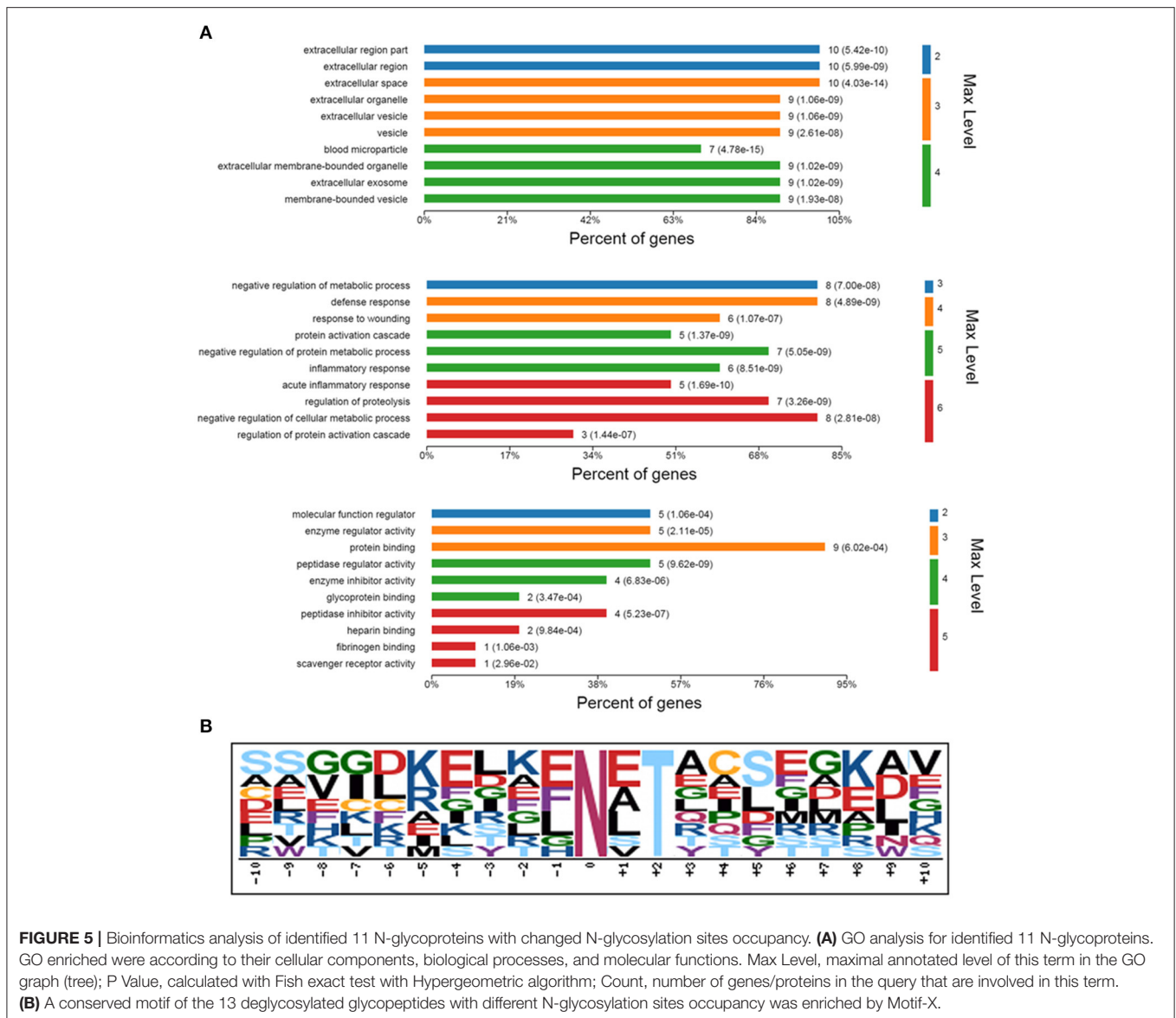
Functional Categories and Patterns Discovery

In GO annotation, 11 serum N-glycoproteins with changed N-glycosite occupancy were categorized using OmicsBean according to their cellular components, biological processes, and molecular functions (**Figure 5A**). The annotation defined statistically significant with the P value which was calculated with Fish exact test with Hypergeometric algorithm. Most of the 11 N-glycoproteins were located in the blood microparticle or extracellular space, respectively. The related biological processes including acute inflammatory response ($p = 1.69e-10$), protein

activation cascade ($p = 1.37e-09$), defense response ($p = 4.89e-09$), negative regulation of protein metabolic process ($p = 5.05e-09$), and so on. In molecular function annotation, the 11 N-glycoproteins were main associated with peptidase regulator activity ($p = 9.62e-09$), protein binding ($p = 6.02e-04$), glycoprotein binding ($p = 3.47e-04$) and scavenger receptor activity ($p = 2.96e-02$).

Motif-X was used to extract overrepresented motif of amino acids for the 13 deglycosylated glycopeptide with different N-glycosite occupancy from the 11 N-glycoproteins. Setting up "N" as central character, a conserved glycosylation motif was enriched by the created logo-like representations (**Figure 5B**).

Corresponding network of these 11 N-glycoproteins were obtained using IPA analysis (**Figure 6**). Based on the Ingenuity Knowledge database, information about molecule-to-molecule interactions, biological networks and canonical pathways were collected and algorithmically generated. There were 5 different kinds of molecule shapes: enzyme, peptidase, transporter,
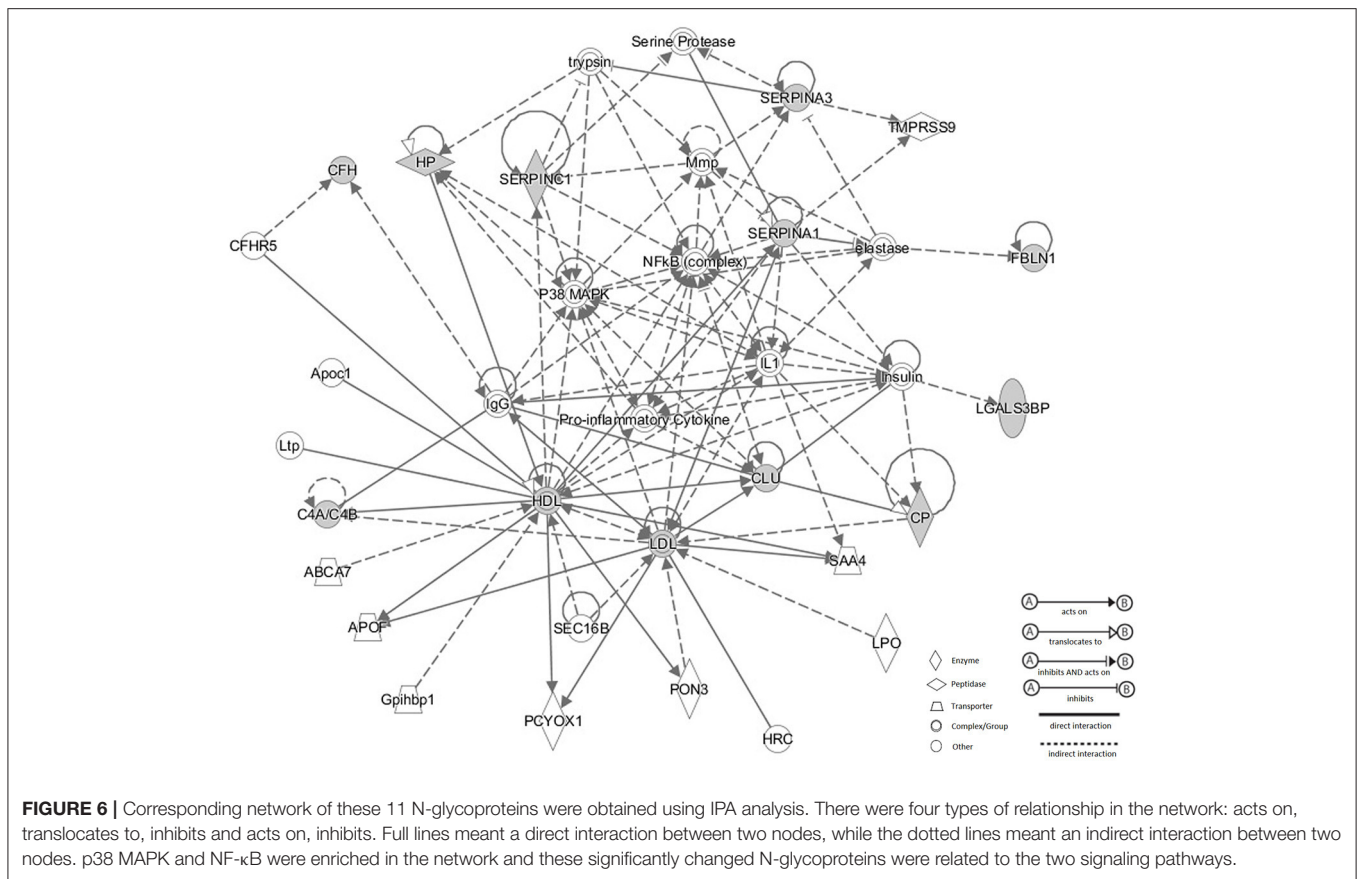
FIGURE 5 | Bioinformatics analysis of identified 11 N-glycoproteins with changed N-glycosylation sites occupancy. **(A)** GO analysis for identified 11 N-glycoproteins. GO enriched were according to their cellular components, biological processes, and molecular functions. Max Level, maximal annotated level of this term in the GO graph (tree); P Value, calculated with Fish exact test with Hypergeometric algorithm; Count, number of genes/proteins in the query that are involved in this term. **(B)** A conserved motif of the 13 deglycosylated glycopeptides with different N-glycosylation sites occupancy was enriched by Motif-X.

complex/group and other. Relationships between two nodes were divided into 4 types, A: acts on, B: translocates to, C: inhibits and acts on and D: inhibits. Full lines in the network meant a direct interaction between two nodes, while the dotted lines meant an indirect interaction. According to the result, p38 MAPK and NF-κB were enriched in the network and these significantly changed N-glycoproteins were related to the two signaling pathways.

DISCUSSION

Glycoproteomics was critical to discover altered glycoproteins and glycans in occurrence and development of disease. Lectin microarray technology has been widely used for glycosylation studies in recent years (Kang et al., 2012; Li, Y. et al., 2013; Qin et al., 2013; Xin et al., 2014; Yang et al., 2015). It is a high-throughput technique which could reveal glycoforms using

minimal sample preparation without release or derivatization of glycans and it could observe multiple and distinct binding interactions simultaneously (Pilobello et al., 2005; Fry et al., 2011). In this study, alterations of glycosylation between HCC patients with metastasis and those with non-metastasis were probed by this method. Poly-LacNAc or (GlcNAc)n binder LEL, exopolysaccharide binder NML, Sia binder PCL, β1,6-GlcNAc branched N-glycan binder PHA-L, GlcNAc binder STL, and (GlcNAc)n or multivalent Sia binder WGA showed significant increasing trend with strong binding in HCC patients with metastasis. Especially, PHA-L reactive structures were significantly changed. PHA-L could bind with β1,6-GlcNAc branched N-glycan which might play an important role in digestive cancers metastasis. Salomé et al. reported that the increasing β1,6 GlcNAc branched structures could decrease bisecting GlcNAc structures on E-cadherin molecule and lead to disruption of cell-cell contacts (Pinho et al., 2009). Qi et al. found

FIGURE 6 | Corresponding network of these 11 N-glycoproteins were obtained using IPA analysis. There were four types of relationship in the network: acts on, translocates to, inhibits and acts on, inhibits. Full lines meant a direct interaction between two nodes, while the dotted lines meant an indirect interaction between two nodes. p38 MAPK and NF-κB were enriched in the network and these significantly changed N-glycoproteins were related to the two signaling pathways.

that accumulation of this glycoform could result in enhanced cell migratory capacity by promoting PTPRT's dimerization and decreasing its catalytic activity (Qi et al., 2014).

Then, PHA-L affinity chromatography was applied to enrich the N-glycoproteins containing tetra-antennary complex-type N-glycan, followed by N-glycosite occupancy measurement with strategy of tandem ^{18}O stable isotope labeling. This TOSIL strategy was reported to be advantageous for lowering the cost of experiment (Losfeld et al., 2012) and increasing mass shift (Nettleship et al., 2007). In our previous study, HGF was used to establish EMT model in Huh7 HCC cells. Lectin microarray analysis indicated that cell surface glycans of Huh7 were altered, for example, the binding abilities of PHA-L to glycan were elevated in EMT process (Li, S. et al., 2013). PHA-L could bind with β1,6-GlcNAc branched N-glycan, which is important in digestive cancers metastasis. β1,6-GlcNAc branched N-glycan was reported to be directly associated with metastasis (Dennis et al., 1987) and its specific increase could increase metastatic potential (Seberger and Chaney, 1999).

A total of 13 deglycosylated glycopeptides with changed N-glycosite occupancy were identified and 3 of them exhibited the different tendencies in N-glycosite occupancies compared to glycosylated and parent protein levels, including EN#LTAPGSDSAVFFEQGTTR of ceruloplasmin, CATPHGDN#ASLEATFVK of fibulin-1 and ALGFEN#ATQALGR of galectin-3-binding protein (Gal-3BP).

CP was a copper-binding glycoprotein synthesized by the liver, had ferroxidase activity and would be an iron-regulatory protein. Occurrence, development and metastasis of HCC were consistently characterized by the lack of iron accumulation (Tan et al., 2009). CP could be considered as one of the potentially reliable biomarkers for the detection of HCC as its expression profiles was significantly differential and it also could be used in detecting liver metastasis from digestive cancer (Ferrin et al., 2015). Fibulin-1 was an extracellular matrix glycoprotein often associated with fibronectin and played an important role in cell adhesion and migration along protein fibers (Twal et al., 2001). It was validated that fibulin-1 was highly expressed on the surface of human gliomas and it might be involved in the aggressive nature of tumors (Towner et al., 2013). Gal-3BP is known as tumor-associated antigen 90 K or Mac-2 binding protein. It could combine with several galectins by glycan-dependent interactions and promote intergrin-mediated cell adhesion (Lin, T. W. et al., 2015). In this study, we found that N-glycosite occupancy of Gal-3BP was up-regulated and it might increase galectin-mediated tumor cell aggregation and then lead to increase the survival of cancer cells during the metastatic process. It is noteworthy that only albumin and IgG are deleted and detection of relatively low-abundant sera is affected and suppressed. In the future, we will try to delete most high-abundant proteins of sera and focus on low-abundant proteins.

Bioinformatics analysis was also performed for these 11 serum N-glycoproteins with changed N-glycosite occupancy. We speculated the related biological processes such as acute inflammatory response, protein activation cascade, defense response, negative regulation of protein metabolic process, which might be related with the metastasis of HCC. There were 2 significant nodes in the IPA network: p38 MAPK and NF-κB. Activation of p38 could promote metastasis and suppression of the p38 signaling pathway could inhibit cell migration and reduce the invasion of different tumor cells like gastric cancer cells, chondrosarcoma cells and colorectal cancer cells (Huang, Q. et al., 2014; Ren et al., 2014; Tsai et al., 2015; Yan et al., 2015). NF-κB, as a transcription factors, was frequently activated in tumors. According to previous reports, it could regulate cell migration, invasion and adhesion. Moreover, it was reported to be involved in tumor growth and progression (Tafani et al., 2013). A conserved motif of the 13 deglycosylated glycopeptides with altered N-glycosylation sites occupancy was enriched by Motif-X. This motif was showed as Asn-Xxx-Thr (NXT), and Xxx could be Glu (E), Ala (A), Leu (L), Ser (S), and Val (V). It indicated that occupancy changes of this conserved motif were more likely to occur in HCC with metastasis. It was reported that the occupancy changes of N-glycosylation site, such as NET and NVT of complement factor H, were associated with pancreatic ductal adenocarcinoma and chronic pancreatitis (Pan et al., 2014). Further study will need to uncover the frequency of these alterations and its mechanisms in diseases.

CONCLUSION

In this study, PHA-L reactive structure (β1,6-GlcNAc branched N-glycan) was found to be increased significantly in HCC patients with metastasis compared with those with non-metastasis. Then, 11 PHA-L reactive glycoproteins with significantly changed N-glycosite occupancy were identified, which were associated with cell migration, invasion and adhesion through p38 MAPK and NF-κB signaling pathway. Alterations in N-glycosite occupancy were also related with HCC metastasis. β1,6 GlcNAc branching of N-glycans might be a metastasis-promoting glycoform and we believe quantification of changes in N-glycosite occupancy for PHA-L reactive glycoproteins in HCC metastasis serum could help to discover important glycoprotein of potential clinically significance as well as characterization of molecular mechanism of HCC metastasis.

AUTHOR CONTRIBUTIONS

Study design: SZ, YL. Data acquisition and analysis: TL, SS, WL, XQ, LS. Interpretation of data: TL, SS, WL, LS, SZ, YL. Drafting and revising the work: TL, SS, SZ, YL. Final approval: TL, SS, WL, XQ, LS, SZ, YL. Agreement to be accountable of all aspects of the work: TL, SS, WL, XQ, LS, SZ, YL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00472/full#supplementary-material

Figure S1 | Workflow for detecting glycoforms in serum glycoproteins by lectin microarray.

Figure S2 | Workflow for quantifying N-glycosite occupancy in serum glycoproteins using TOSIL strategy.

Table S1 | Name and binding specificity of 50 lectins used in lectin microarray.

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