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Glycoproteomic Discovery of Serological Biomarker Candidates for HCV/HBV Infection-Associated Liver Fibrosis and Hepatocellular Carcinoma

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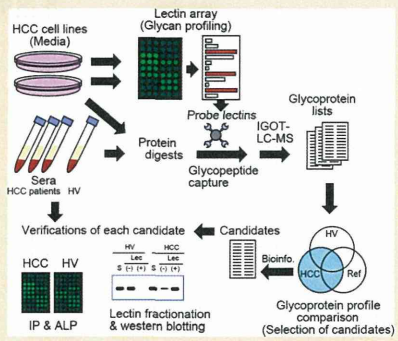
Supporting Information

ABSTRACT: We previously proposed a high-throughput strategy to discover serological biomarker candidates of cancer. This strategy focuses on a series of candidate glycoproteins that are specifically expressed in the original tissues (cells) of the target cancer and that carry glycan structures associated with carcinogenesis [Narimatsu, H., et al. *FEBS J.* **2010**, *277*(1), 95−105]. Here, we examined the effectiveness of our strategy in identifying biomarkers to assess progression of liver fibrosis and for the early detection of hepatocellular carcinoma (HCC). On the basis of the results of lectin array analyses in culture media of hepatoma cell lines, we captured glycopeptides carrying AAL-ligands (fucosylated glycans) or DSA-ligands (branched glycans) from digests of culture media proteins and sera from HCC patients with a background of liver cirrhosis (LC). Glycoproteins were identified by the IGOT-LC−MS method. In all, 21 candidates were selected from 744 AAL-bound glycoproteins for further verification according to (i) their abundance in serum, (ii) their specific expression in liver, and (iii) the availability of antibodies to the glycoproteins. All selected candidates showed enhancement of AAL-reactivity in sera of HCC patients compared with that of healthy volunteers (HV). These results indicate that our glycoproteomic strategy is effective for identifying multiple glyco-biomarker candidates in a high-throughput manner.



KEYWORDS: *glycoprotein, glycan alteration, hepatocellular carcinoma, liver fibrosis, biomarker, lectin array, glycoproteomics*

INTRODUCTION

In Japan, hepatocellular carcinoma (HCC) often develops in association with chronic liver diseases such as liver cirrhosis (LC) caused by persistent infection with hepatitis B or C virus followed by liver fibrogenesis.[1] The annual incidence of HCC increases with the degree of liver fibrosis and reaches 7−8% for the LC patients (fibrosis stage = F4).[2] Therefore, it is important to understand the degree of progression of liver fibrosis for patients with chronic hepatitis. This will enable identification of high-risk patients with progressed liver fibrosis, who should then undergo further detailed examinations such as computed tomography (CT) to detect evidence of early stage HCC. Presently, the progression of liver fibrosis is diagnosed by percutaneous biopsy; however, the method is both invasive and painful.[3,4] In addition, this method may result in sampling errors because the portion of the organ subjected to examination is very small.[5,6] These concerns prompted us to develop a serum biomarker to quantitatively evaluate the progression of liver fibrosis. Hence, we aimed to identify a marker that is applicable to a rapid, low cost, accurate, sensitive, and minimally invasive test. Furthermore, development of a serum HCC biomarker with increased sensitivity is required in order to diagnose early stage HCC at a size that is applicable for treatment such as radiofrequency ablation, thereby enhancing survival rates.[7,8]

Thus far, serological biomarkers have been screened by proteomic analyses using sera of HCC patients.[9,10] However, early stage cancer cells often express almost the same proteome as their originating cells because they have a common lineage. Hence, it is difficult to discover, verify, and validate practical serological marker candidates by comparing the levels of individual protein concentrations in serum between patients and healthy or disease controls. The problem can be seen by reference to a simple example. Suppose that the cancerous liver cells comprise 1% of the entire liver and secrete a 10-fold excess of a certain protein into the blood compared with the noncancerous cells. The blood concentration of such a protein in the cancer patient will be 1.1-fold that of the healthy individual.

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If the secretion increases 100-fold, the blood concentration of the protein becomes approximately 2-fold; however, the difference is still less than that of individual variability. Alpha-fetoprotein (AFP), a clinical marker for HCC, is elevated from 3 ng/mL (i.e., average level in healthy individuals) to ≥20 ng/mL in a cancer patient,[11] which indicates HCC cells secrete >500-fold AFP by comparison to noncancerous cells. In fact, AFP was found to be a major component of the culture medium of a human hepatoma cell line (HepG2; data not shown). Thus, it is relatively straightforward to identify proteins that are significantly overexpressed by cancer cells (e.g., AFP). However, such proteins are not always found. These considerations reveal the difficulty of finding a "cancer-specific biomarker" based on the quantification of proteins.

We have established an integrated strategy for high-throughput discovery of serum biomarker candidates by utilizing a number of advanced technologies. Specifically, we developed techniques to analyze glycan biosynthesis-related genes (glycogenes), glycan structures, and glycoproteins as described previously.[12,13] We designed the strategy based on a well-known fact that the glycan structure on the cell surface and secretory proteins derived from cancerous cells is different from that derived from the same kind of noncancerous cells. Thus, we believe a qualitative alteration in the glycan structure, rather than a quantitative change in the amount of protein, will be a good index for developing serum biomarkers. The strategy comprises a 4-step analyses: (1) glycogene expression profiling by quantitative real-time PCR array for cancer cell lines, to predict possible and significant glycan structural motifs such as α1,6-fucose, bisecting GlcNAc (GlcNAcβ1-4Man), β1,6-branch-(GlcNAcβ1-6Man), poly-N-acetyllactosamine extension, Lewis antigen, and LacdiNAc (GalNAcβ1-4GlcNAc); (2) comparative glycan profiling by lectin array analysis of proteins secreted from cancer cells into the culture media, to determine cancer-associated glycan alteration and to select lectins to capture glycoproteins or glycopeptides displaying the altered glycans; (3) identification of core proteins carrying the cancer-associated glycans by LC–MS analysis; and (4) selection of promising glycoprotein candidates by comparative profiling of the identified glycoproteins in cancerous samples and healthy controls, followed by bioinformatics to establish tissue specificity and the expression level in the liver.

In accordance with this strategy, we digested glycoprotein mixtures prepared from the culture media of hepatoma cell lines, HepG2 and HuH-7, and also from the sera of HCC patients having a background of LC and those of healthy volunteers (HV). Glycopeptides were separately captured using AAL, DSA, and RCA120 lectins. Many glycoprotein biomarker candidates were identified (744 for AAL and 234 for DSA). In all, 21 AAL-bound glycoproteins were selected to determine the differences in their glycan structure between HCC patients and HV. All selected glycoproteins showed increases of AAL-reactivity in HCC sera, suggesting these would be valuable biomarkers of HCC, LC, or fibrosis progression. Our results confirm the effectiveness of this strategy in identifying cancer glycobiomarker candidates in a high-throughput manner.

METHODS

Materials

We collected sera from 8 HCC patients at Nagoya City University Hospital (NCUH) and control sera from 14 randomly selected healthy volunteers (HV) of 28–48 years of age at AIST, after obtaining the approval from the respective institutional ethics committees and the informed consent from all individuals for the use of clinical specimens. Clinical information of the HCC patients and information on sex and age of HV are presented in Supplementary Table 1. HV did not have hepatitis, other hepatic diseases, renal failure, clinical cardiovascular disease, or malignancies. Because we aimed at discovering not only biomarker candidates of HCC development but also specific markers for various stages between early phase fibrosis and severe liver cirrhosis, we used sera of HV, who were relatively younger than HCC patients, as control samples. To inactivate hepatitis viruses, each serum sample (both HV and HCC patients) was diluted 10-fold with Dulbecco's phosphate buffer containing 0.15 M NaCl (D-PBS) and 0.2% SDS and heated at 98 °C for 15 min before use. Human hepatoma cell lines (HepG2 and HuH-7) were obtained from RIKEN Cell Bank (Ibaraki, Japan). Details of all antibodies used in this study are presented in Supplementary Table 2.

Other reagents used in this study were as follows. Guanidine-HCl and neuraminidase (*Arthrobacter ureafaciens*, EC 3.2.1.18) were purchased from Nacalai Tesque (Kyoto, Japan). Dithiothreitol (DTT), iodoacetamide (IAA), trichloroacetic acid (TCA), methyl α-D-mannopyranoside, lactose, ethanol, 1-butanol, and trifluoroacetic acid (TFA) were from Wako Pure Chemicals (Osaka, Japan). Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Thermo Fisher Scientific (Waltham, MA). Stable isotope-labeled water ($H_2{}^{18}O$, 99% atom% ^{18}O) was a product of Taiyo Nippon Sanso Corp. (Tokyo, Japan). L-Fucose and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (St Louis, MO). Glycopeptidase F (peptide-*N*-glycanase; PNGase) was from TakaraBio (Kyoto, Japan). The HPLC lectin column (LA-AAL, *Aleuria aurantia* lectin) and chitooligosaccharide were from Seikagaku Biobusiness (Tokyo, Japan). DSA (*Datura stramonium* agglutinin)-agarose and RCA (*Ricinus communis* agglutinin) 120-agarose were from Vector Laboratories, Inc. (Burlingame, CA). All solutions were prepared using ultrapure water. Analytical grade reagents were used unless stated otherwise.

HepG2 and HuH-7 were cultured in a Dulbecco's modified Eagle's medium (D-MEM; Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 units/mL penicillin (Life Technologies), and 100 μg/mL streptomycin (Life Technologies) and maintained at 37 °C in an incubator with 5% CO_2. The cells at 60–80% confluence were washed 3 times with D-PBS and further cultured in FBS- and antibiotics-free D-MEM for 48 h. The final media were harvested, and the supernatants were filtered using a 0.45-μm disc filter (Millipore, Billerica, MA).

Preparation of Tryptic Digests of Culture Medium and Serum Proteins

The pooled HV serum was prepared by mixing equal volumes of heated sera of 14 HV. Proteins in the serum and culture media were collected by TCA precipitation (final concentration of 10%, w/w), S-reduced, and then alkylated as described previously.[14] For discovery experiments, we used 5 HCC sera (S-1 to S-5) listed in Supplementary Table 2. Proteins were digested with TPCK-treated trypsin, using an E/S ratio of 1/100 (w/w), at 37 °C for 16 h. Progression of the digestion was monitored by SDS-polyacrylamide gel electrophoresis followed by CBB staining. Protein concentrations were assayed by the BCA method using bovine serum albumin as a standard.

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Lectin Affinity Capture of Glycopeptides from Tryptic Digests

Lectin affinity chromatography was performed as described previously.[14] Briefly, an aliquot of the tryptic digest was applied to an AAL column (4.6 mm i.d. × 150 mm) equilibrated with 10 mM HEPES-NaOH, pH 7.5. Glycopeptides were eluted with the same buffer containing 5 mM fucose. The pass-through fraction was applied again onto the same column, and residual glycopeptides were recovered using an identical procedure. Similarly, the trypsin digest was applied onto a DSA column (10 mm i.d. × 20 mm) after desialylation treatment with neuraminidase from *Arthrobacter ureafaciens*. Glycopeptides were then eluted with 1% chito-oligosaccharide (GlcNAc oligomers), and the eluate was directly applied onto an online RCA120 column (10 mm i.d. × 20 mm) to remove GlcNAc oligomers. Glycopeptides trapped on the RCA120 column were eluted with 0.1 M lactose. In addition, glycopeptides in the pass-through fractions of the AAL column chromatography were applied onto a RCA120 column (10 mm i.d. × 20 mm). Hereafter a fraction (protein/peptide) bound to a lectin column is denoted as "lectin(+)", e.g., AAL(+). Similarly, a passed (unbound) fraction is referred to as, e.g., DSA(−). Subsequently, all glycopeptide samples prepared (AAL(+), DSA(+), and AAL(−)/RCA120(+)) were further purified by hydrophilic interaction chromatography on a Sepharose CL-4B column as described previously.[14]

Enzymatic Stable Isotope Labeling of Glycopeptides

Purified glycopeptides were treated with PNGase (2.5 mU, 37 °C overnight) in stable isotope ^{18}O-labeled water to remove the glycan moiety and concomitantly label the glycosylated Asn of glycopeptides with the isotope, as described previously.[15] This step is referred to as isotope-coded glycosylation site-specific tagging (IGOT).

Identification of the Labeled Peptides by Nanoflow LC–MS Analysis

Stable isotope-labeled peptides were analyzed by the LC–MS method, as described previously.[16] Briefly, the peptide mixture was injected onto a C18 trap column (0.5 mm i.d. × 1 mm). After washing, the column was connected to a nanoflow LC system (flow rate: 100 nL/min), and the peptides were separated on a reverse phase (C18) tip column (150 μm i.d. × 70 mm) using a linear gradient of MeCN (0–35% in 0.1% formic acid) for 70 min. The eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-TOF Ultima; Waters-Micromass, Beverly, MA). The spectrometer was operated in data-dependent MS/MS mode.

MS/MS spectra were processed using MassLynx software (version 4.0, Micromass) to create peak list files with smoothing by the Savitzky-Golay method (window channels, ±3). The files were processed by the MASCOT algorithm (Version 2.1, Matrix Science) to assign peptides using the refseq protein sequence database (34,276 entries, downloaded on July 2007). The database search was performed by the MS/MS ion search method with the following parameters. Enzyme: Trypsin + Lys-C; Fixed modification: Carbamidomethyl (Cys); Variable modifications: Gln > pyro-Glu (N-term Gln), Oxidation (Met), Pyro-carbamidomethyl (N-term Cys), IGOT (Asn > Asp+^{18}O = +2.988261 Da, custom-made) (Asn); Peptide mass tolerance: 200 ppm; Fragment mass tolerance: 0.5 Da; Max missed cleavage: 2. All results from the peptide search were exported as CSV files and processed by Microsoft Excel. First, we selected the peptides with rank 1 and an expectation value <0.05. Then, we selected the peptides that contained one or more aspartic acids labeled with ^{18}O atoms (IGOT) at the position of Asn in the consensus sequence for *N*-glycosylation, Asn-Xaa-(Ser/Thr), where Xaa is any residue except Pro.

Immunoprecipitation and Lectin Array Analysis of the Glycoprotein Marker Candidates

Selected glycoproteins were enriched with their specific antibodies. Aliquots (10 μL each) of the pooled sera of HCC patients and HV were incubated overnight with 1 μg of biotinylated antibody at room temperature. The resultant complexes were trapped by 20 μL of streptavidin-conjugated magnetic beads (Dynabeads MyOne Streptavidin T1; Invitrogen) and then released by heating with 20 μL of 0.2% SDS in TBS. Glycan profiles of the released proteins were analyzed by the antibody-assisted lectin profiling (ALP) method.[17] Briefly, we analyzed array signals by using LecChip and GlycoStation Reader (GP Biosciences Ltd., Yokohama, Japan) according to a prescribed method.[17] The data were processed with the program software, Array-Pro Analyzer (Ver. 4.5, Media Cybernetics, Inc., Silver Spring, MD).

AAL Fractionation of Serum Proteins

The denatured serum (10 μL each) was incubated with 100 μL of AAL-agarose at 4 °C for 16 h with gentle shaking. After recovery of the supernatant (AAL(−) fraction), the gel was washed three times with PBSTx. Then, the gel was suspended with an AAL elution buffer (PBSTx containing 500 mM L-fucose) and shaken at 4 °C for 16 h. The supernatant (AAL(+) fraction) was then collected. Both AAL(+) and AAL(−) fractions were concentrated by ultrafiltration using an Amicon Ultra unit (Ultracel 3 kDa, Millipore Amicon) to adjust the volume of each fraction.

LCA-AAL Serial Lectin Fractionation of Serum Glycoproteins

The glycoproteins in the pooled sera of HCC patients and HV were separated by serial lectin fractionation using LCA- and AAL-conjugated gels (Kuno, A., et al. manuscript in preparation). Briefly, the 100-fold diluted serum (100 μL) was incubated with 100 μL of LCA-agarose at 4 °C for 5 h with gentle shaking, and the supernatant (LCA(−) fraction) was recovered. After washing, LCA-bound proteins were eluted with 100 μL of an LCA elution buffer (PBSTx containing 500 mM α-methyl-D-mannopyranoside). Then, the LCA(−) fraction was incubated with 50 μL of AAL-agarose at 4 °C for 16 h, and the supernatant (LCA(−)/AAL(−) fraction) was recovered. The LCA(−)/AAL(+) fraction was recovered with 500 mM L-fucose.

Western Blot Analysis

Protein samples including an equivalent volume of serum were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA). The membranes were blocked and incubated with each antibody. Finally, the treated membrane was reacted with the Western Lightning chemiluminescence reagent (Perkin-Elmer, Boston, MA) at room temperature and exposed to Hyperfilm ECL (GE Healthcare) for 60 or 300 s.

RESULTS

Selection of Lectins for Capturing Glycopeptides Carrying HCC-Related Glycans

A tissue-specific protein carrying a cancer-associated glycan is suitable as a biomarker for the cancer. To develop such glycoprotein biomarkers, we designed an integrated strategy as described previously.[12] In accordance with this strategy (Figure

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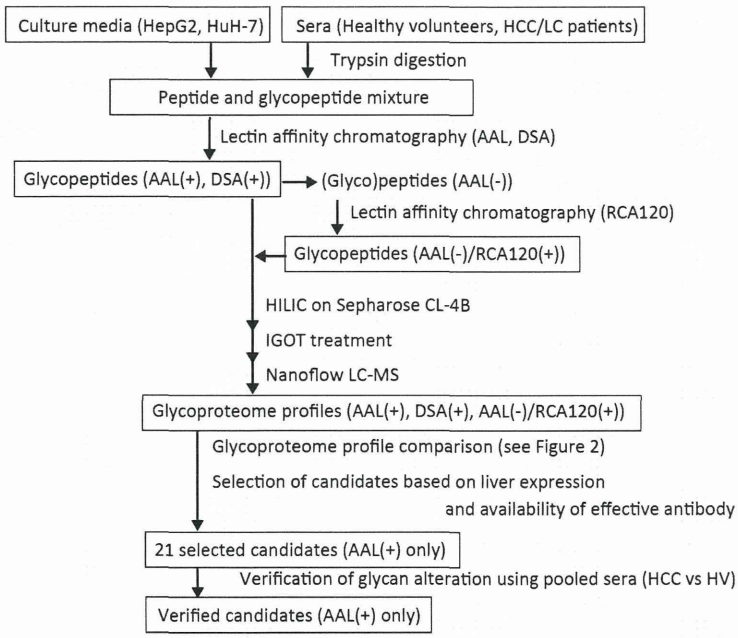

Figure 1. Experimental workflow of the discovery of serum glyco-biomarker candidates for liver disease. Glycopeptide fractions were captured with AAL- or DSA-affinity columns from tryptic digests of culture media (HepG2 and HuH-7) and serum taken from healthy volunteers and HCC patients. RCA-bound glycopeptides were recovered from pass fractions of the AAL-column for serum samples. After purification by HILIC, glycopeptides were identified by the IGOT-LC–MS method. From the AAL(+) glycoproteins, 21 glycoproteins were selected for further verification of HCC-associated glycan alteration (enhanced fucosylation). See Methods for further details.

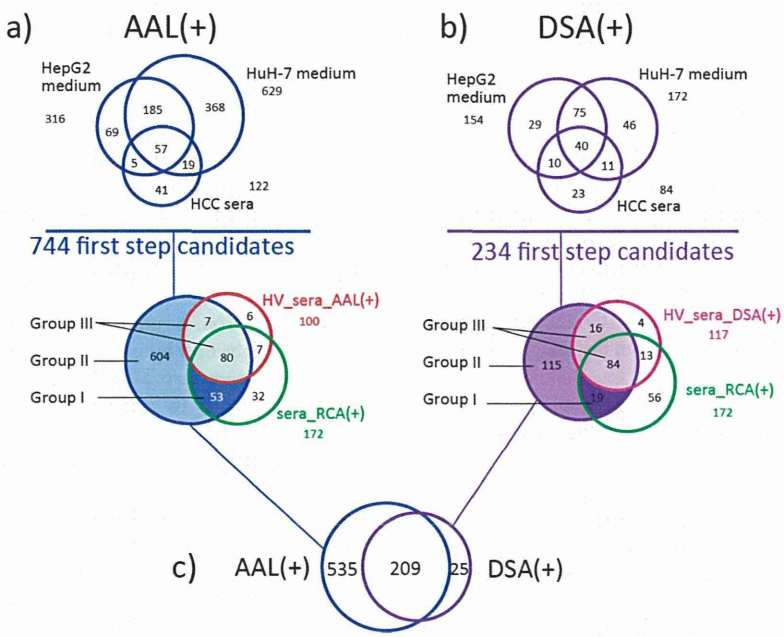

Figure 2. Venn diagram presentation for glycoprotein profile comparison. Lectin-bound glycopeptides were prepared from culture media (HepG2 and HuH-7) and sera of HCC patients and then identified by the IGOT-LC–MS method. In all, (a) 744 AAL(+) and (b) 234 DSA(+) glycoproteins were identified. AAL(+) and DSA(+) glycoproteins were identified from the sera of healthy volunteers (HV; red circle), and RCA120(+) glycoproteins were identified from the sera of HV and HCC patients (green circle). By comparing the glycoprotein profiles, HCC_AAL(+) proteins were classified into 3 groups. (c) Glycoproteins of HCC_AAL(+) and DSA(+) glycoproteins were compared. Most DSA(+) glycoproteins also possess fucosylated glycans.

1), we analyzed the glycan profiles of culture media of HCC cell lines (HepG2 and HuH-7)[13] and found that the lectin array signals of AAL and DSA as well as RCA120 were significant.

AAL has specificity for fucosylated glycans[18] and DSA for branched asialoglycans (tri- or tetra-antennary *N*-glycans).[19] It is known that the levels of these glycan motifs are increased by

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Table 1. Biomarker Candidates Selected for Further Verification of HCC-Related Glycan Alteration (Fucosylation)[a]

				applicability of antibody		results of verification of the candidatens			
								serial lectin fractionation and WB	
candidate no. at verification step	group in Venn diagram (Figure 2)	gene symbols	description	WB (for sera)	IP (from sera)	IP and Lectin array (AAL)	AAL fractionation and WB	LCA(+)	LCA(−) AAL(+)
1	I	CPB2	plasma carboxypeptidase B2	√	√	ND	↑	→	→
2	I	CPN2	carboxypeptidase N, polypeptide 2, 83 kD	√	√	↑	NT	NT	NT
3	I	CSF1R	colony stimulating factor 1 receptor	√	√	↑	↑	↑	↑
4	I	FN1	fibronectin 1 isoform 7	√	−	NT	NT	→	↑
5	I	LAMP2	lysosomal-associated membrane protein 2	√	√	ND	NT	↑	→
6	I	SERPINA7	serine (or cysteine) proteinase inhibitor, clade A, member 7	√	−	NT	NT	→	↑
7	I	SHBG	sex hormone-binding globulin	√	−	NT	↑	↑	↑
8	II	ICAM2	intercellular adhesion molecule 2	√	−	NT	↑	→	→
9	II	SPARCL1	SPARC-like 1	√	√	→	↑	→	↑
10	III	AFM	afamin	√	−	NT	NT	↑	↑
11	III	AHSG	alpha-2-HS-glycoprotein	√	√	NT	NT	↑	→
12	III	APOD	apolipoprotein D	√	−	NT	NT	↑	→
13	III	AZGP1	alpha-2-glycoprotein 1, zinc	√	−	NT	NT	↑	↑
14	III	ICOSLG	inducible T-cell costimulator ligand	√	√	↑	↑	NT	NT
15	III	ORM1/2	orosomucoid 1/2 (= AGP)	√	√	↑[b]	NT	→	↑
16	III	PIGR	polymeric immunoglobulin receptor	√	√	↑	NT	↑	↑
17	III	PTGDS	prostaglandin H2 D-isomerase	√	−	NT	NT	↑	→
18	III	SEPP1	selenoprotein P isoform 1 precursor	√	−	NT	↑	↑	↑
19	III	SERPINA1	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	√	√	NT	NT	→	↑
20	III	SERPINA3	serpin peptidase inhibitor, clade A, member 3	√	√	NT	NT	→	↑
21	III	TF	transferrin	√	√	NT	NT	↑	→

[a]Symbols: √, applicable; −, not applicable; NT, not tested; ND, not detected;↑, enhanced in HCC; →, not enhanced in HCC. [b]See ref 32.

cancer-associated glycan alterations.[20−22] In addition, we showed that *N*-glycan fucosylation on AFP in the culture media was enhanced by comparison with that of placenta.[13] Therefore, we selected these lectins, AAL and DSA, to enrich HCC marker candidates. RCA120 recognizes Galβ1−4-GlcNAc-terminated glycans, which are present in the majority of serum glycoproteins after neuraminidase treatment.[23,24] Thus, RCA120 was used to comprehensively obtain glycopeptides from the samples in order to estimate their abundance.

Identification of Lectin-Captured Glycoproteins in the Culture Media and Serum Samples Using the IGOT-LC−MS Method

First, we collected glycopeptide subsets by AAL affinity chromatography from tryptic digests of the culture media of HepG2 and HuH-7. In order to evaluate the capturing specificity of the AAL column under the conditions employed, glycopeptides derived from the serum of an HCC patient were separated using the AAL column, and *N*-glycans released from AAL(+) and AAL(−) glycopeptides were analyzed by MALDI-TOF MS (Supplementary Figure 1 and Supplementary Method). As a result, almost all signals of AAL(+) glycans were assigned to have one or more deoxyhexose (dHex) residue(s) and only one slight signal having no dHex was observed, suggesting that the glycopeptides having fucosylated glycans were highly enriched in the AAL(+) fraction and that AAL has enough selectivity to capture glycopeptides having fucosylated glycans. However, because this result could not ensure that all individual glycopeptides were fucosylated, we dealt the AAL(+) glycopeptides as putative fucosylated glycopeptides. After purification by HILIC, the AAL(+) glycopeptides were treated with PNGase F in $H_2{}^{18}O$ and identified by LC−MS analysis followed by Mascot search. In total, 316 and 629 glycoproteins were identified from HepG2 and HuH-7, respectively (Figure 2a, Supplementary Tables 3 and 4). Similarly, we identified 122 AAL(+) glycoproteins from serum samples of 5 HCC patients. In total, 744 AAL(+) glycoproteins were identified as first-step biomarker candidates. Exemplary MS/MS spectra are shown in Supplementary Figure 2 to support the reliability of glycopeptide identification by the IGOT-LC−MS method. Next, we analyzed AAL(+) glycopeptides from HV sera and identified 100 glycoproteins. Finally, we used RCA120 to obtain glycopeptides from HV and HCC sera in order to estimate their abundance in serum and the number of *N*-glycosylated sites on each glycoprotein. Using this procedure, we identified 172 glycoproteins as abundant glycoproteins (Figure 2a).

The same experimental protocol allowed us to identify 154 and 172 DSA(+) glycoproteins from the culture media of HepG2 and HuH-7, respectively (Figure 2b). In addition, 84 DSA(+) glycoproteins were identified from HCC sera; in total, 234 glycoproteins were identified in HCC samples. Furthermore, 117 DSA(+) glycoproteins were identified from HV sera (Figure 2b). Details of the identified glycopeptides are given in Supplementary Table 3. Identified proteins in each sample are listed in Supplementary Tables 4 (AAL) and 5 (DSA). These results were registered to the glycoprotein database, GlycoProtDB, which was constructed using our experimental-based information and is

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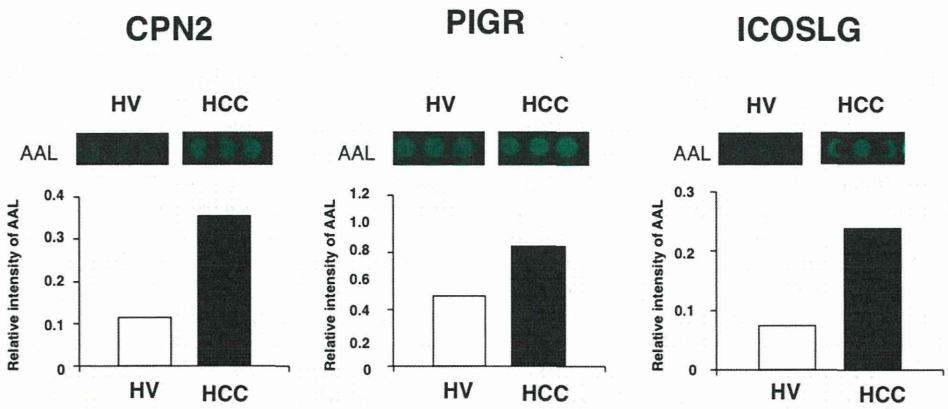

Figure 3. Detection of HCC-associated enhancement of fucosylation on the candidate glycoproteins by the immunoprecipitation-ALP method. Candidate glycoproteins were enriched from pooled sera of HV and HCC patients by immunoprecipitation with their antibodies and analyzed by lectin array (ALP method). Signal images for AAL spots (above, $n = 3$). Histogram indicating relative intensity of AAL to DSA (relative intensity of AAL signal against DSA signal).

currently available for mouse and *C. elegans* glycoproteins (http://jcggdb.jp/rcmg/gpdb/).[16] The overlap of AAL(+) and DSA(+) proteins was 209 (Figure 2c, Supplementary Table 6).

Selection of Biomarker Candidates for Further Verification by Glycoproteome Profiles and Availability of Their Antibodies

As described above, we identified 744 AAL(+) proteins in the media of HepG2 and HuH-7 and the serum samples of HCC patients. These proteins correspond to 398 gene symbols. Among them, 80 glycoproteins (gene symbols) are previously reported to have core-fucose in part or to bind with fucose-recognizing lectins.[25−28] The remaining 318 proteins are newly identified in this study as AAL-reactive proteins. Considering the selectivity of the AAL column under the employed conditions, as shown in Supplementary Figure 1, these are putative fucosylated glycoproteins that had not been formerly identified as fucosylated. Next, as a reference to select candidates to be verified, we classified the first-step AAL(+) candidates obtained from HCC samples into 3 groups by comparing the glycoproteome profiles as follows (Figure 2a). First, we compared these AAL(+) proteins with those in pooled HV serum (n = 100), and found 87 proteins in common. These common proteins were classified into Group III, whose *N*-glycans are also fucosylated in the healthy state. The other proteins were then compared with RCA120(+) serum proteins. This comparison identified 53 common proteins, suggesting that they are abundant in the serum. These proteins were classified into Group I. The remaining 604 proteins were designated as Group II. These proteins were detected only in the culture media, suggesting that they were less abundant in serum, but concentrated in the media. At this point, we minimized the number of proteins by using the gene symbols to avoid redundant identifications, such as splice variants and family proteins.

Next, we further selected the candidates on the basis of the tissue expression level and specificity for liver, as well as the availability of antibodies, which is essential to enrich or detect each candidate protein for verification. Moreover, glycoproteins supposedly produced by cells other than hepatocytes (e.g., immunoglobulins) were deleted from the list. We also eliminated some types of glycoproteins, such as complement factors and coagulation factors, as these were considered to be inappropriate for serological tests, because it is presumed easily that the level of complement components is influenced by inflammation in subject patients and the major parts of coagulation factors and fibrinogen are removed from serum samples during blood clotting (in preparation of serum from blood). Consequently, a total of 21 glycoproteins (gene symbols) were selected for further verification (Table 1). These candidates included glycoproteins known to have fucosylated glycans.[29,30] Nonetheless, we verified the glycan alteration between HCC and HV to confirm the effectiveness of our strategy.

The DSA(+) glycoproteins were also classified into 3 groups (Figure 2b) in the same manner as for the AAL(+) proteins. However, we did not select these candidates for further verification for the following reasons. First, although Group I (19) included glypican 3 (GPC3), which was reported to be a useful tumor marker of HCC and some other cancers,[31] other proteins were mostly coagulation factors, complement components, and fibrinogens, which are deemed unsuitable as described above. Thus, we could not find any suitable candidates in Group I of DSA(+) except GPC3. The DSA(+) candidates belonging to Group II (115) were identified only in the culture media, which means low abundance in serum. Furthermore, many of the DSA(+) proteins in Group III (100) were the same as those selected from the AAL(+) proteins. A further technical difficulty was that DSA(+) proteins were barely released from DSA-agarose in the preliminary experiments. Thus, subsequent verification of HCC-associated glycan alteration was performed only for the AAL(+) candidates.

Verification of HCC/LC-Associated Glycan Alteration (Enhanced Fucosylation) on the Candidate Glycoproteins

We verified HCC/LC-associated glycan alteration of the selected candidates using pooled sera of HV and HCC patients (with LC as background) by one or more of the following methods: (1) immunoprecipitation followed by lectin array analysis, (2) AAL-protein fractionation followed by Western blotting, and (3) LCA/AAL-serial lectin fractionation followed by Western blotting. Because these analyses require appropriate antibody for each protein, we tested commercially available antibodies using a pooled serum of HV (data not shown). Antibodies applicable for our verification analyses are listed in Supplementary Table 2.

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1. Immunoprecipitation Followed by Lectin Array Analysis. Enhancement of fucosylation associated with HCC was confirmed by purification of the candidate proteins using immunoprecipitation followed by antibody-assisted lectin profiling (ALP) analysis[17] Among the 8 candidate proteins analyzed by this method, carboxypeptidase N polypeptide 2 (CPN2), polymeric immunoglobulin receptor (PIGR) and inducible T-cell costimulator ligand (ICOSLG) showed an apparent increase of AAL signal in the HCC sera compared to HV. As shown in Figure 3, the AAL signal of HCC sera was increased compared with the respective HV sera by the following factors: 3.1-fold for CPN2, 1.7-fold for PIGR, and 4.1-fold for ICOSLG. Results of the remaining candidates are shown in Supplementary Figure 3a and summarized in Table 1. The number of candidate proteins applicable to the ALP analysis is limited due to the lack of suitable antibodies. However, the ALP analysis also provided the reactivity information against other lectins, which is useful for reselecting the most appropriate lectin(s) for detection of glycan alteration associated with HCC/LC (fibrosis) as exemplified by α1-acid glycoprotein (AGP or ORM1/2).[32]

2. AAL-Fractionation Followed by Western Blotting Analysis. We fractionated pooled sera of HV and HCC patients into AAL(+) and AAL(−) fractions using an AAL column. To estimate the amount of candidates in each fraction, the protein fractions and untreated sera were subjected to SDS-PAGE, and then each candidate was visualized by Western blotting. The levels of all 7 candidates tested by this analysis increased in the AAL(+) fraction of HCC sera compared with HV, suggesting enhanced fucosylation associated with HCC/LC. Specifically, as shown in Figure 4, sex hormone binding globulin (SHBG: Group I), colony stimulating factor 1 receptor (CSF1R: Group I), intracellular adhesion molecule 2 (ICAM2: Group II), and selenoprotein P (SEPP1: Group III) indicated significantly increased signals in AAL(+) of HCC sera compared with that of HV sera. Results of other 3 candidates are shown in Supplementary Figure 3b.

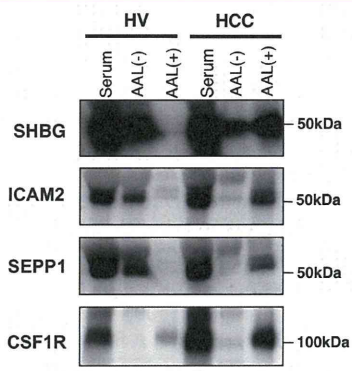

Figure 4. Detection of HCC-associated enhancement of fucosylation on the candidate glycoproteins by AAL-fractionation followed by Western blotting. Pooled sera of HV and HCC patients were fractionated with AAL-agarose. AAL(+), AAL(−), and untreated sera were separated by SDS-PAGE. The candidate glycoproteins were detected by Western blotting. Blot images of each candidate are shown.

3. LCA-AAL Serial Lectin Fractionation Followed by Western Blotting Analysis. As described above, branching and fucosylation of glycans are known to be enhanced in association with HCC.[20,22] Thus, measuring the glycoproteins that underwent both fucosylation and branching might be a clearer indication of glycan alteration by HCC compared to estimating the overall fucosylation level. In many cases, fucosylated glycoproteins are enriched by LCA or AAL; however, their specificities are different. LCA binds specifically to α1,6-fucosylated biantennary glycans, and AAL binds to fucosylated glycans regardless of branched structures.[33] Therefore, we separated the fucosylated candidate glycoproteins into 2 fractions by LCA-AAL serial lectin fractionation (refer to schematic shown in Figure 5a). First, a serum sample was applied to the LCA column to capture glycoproteins having α1,6-fucosylated biantennary glycans, and then the pass fraction (LCA(−)) was applied to the AAL column to recover the remaining glycoproteins having fucosylated glycans. Thus, by serial fractionation glycoproteins having both fucosylated and branched glycans are enriched in the LCA(−)/AAL(+) fraction. These fractions were separated by SDS-PAGE, and then each candidate protein was detected by Western blotting (Figure 5b). As a result, ORM1/2 (AGP), which is known to have highly branched glycans, was detected only in the LCA(−)/AAL(+) fraction (data not shown), indicating that the serial fractionation worked as intended. As summarized in Table 1, the ratio of the LCA(−)/AAL(+) fraction was higher in the HCC sera than HV sera for 12 of the 19 candidates tested (Figure 5b for SHBG, PIGR, CSF1R, and SEPP1; Supplementary Figure 3c for the remaining). These observations suggest that HCC/LC-associated increases of fucosylation and branching occur on the common glycans. In total, 16 of the 21 candidates were identified in both AAL(+) and DSA(+) fractions. Hence, glycans on a wide range of glycoproteins display both fucosylation and branching. However, the bands of proteins in the LCA(+) fraction also increased in HCC sera, suggesting that fucosylation associated with HCC/LC is independent of branching. Conversely, there were also proteins whose associated glycan was less highly branched or not branched at all. For 6 candidates, only the rate of the LCA(+) fraction was higher in the HCC sera (e.g., LAMP2 and SPARCL1). Thus, serial lectin fractionation may be useful for confirming the onset phase of fucosylation and branching individually in the course of chronic fibrosis, LC to HCC.

DISCUSSION

Currently, AFP, PIVKA-II (protein induced by vitamin K absence or antagonists-II, also known as des-γ-carboxy prothrombin [DCP]) and AFP-L3% (ratio of LCA-reactive AFP to total AFP) have been used for the diagnosis of HCC. However, the sensitivity and specificity of these markers are insufficient for diagnosis of early stage HCC.[34] Although many candidate serum biomarkers for HCC have been reported by proteomics- and/or glycoproteomics-based research, there is still a clinical need to develop more reliable markers for HCC.

However, it is fundamentally difficult to discover a serum biomarker for early stage cancer by comparing serum protein profiles between patients and healthy volunteers or disease controls. Empirically, we know that glycan alteration of particular cells is a good index for detecting a change of cell status such as differentiation and carcinogenesis. However, it is also difficult to detect early stage cancer by comparative profiling of the entire glycome of serum glycoproteins, i.e., by analyzing glycans released from serum glycoproteins *in vitro*. This is because the altered glycans emerge "ectopically" in association with carcinogenesis and thus do not have structures "specific to the cancerous cells". For example, sialyl Lewis X is known as a cancer-related antigen in colon,[35] non-small cell lung,[36] breast,[21] and pancreatic

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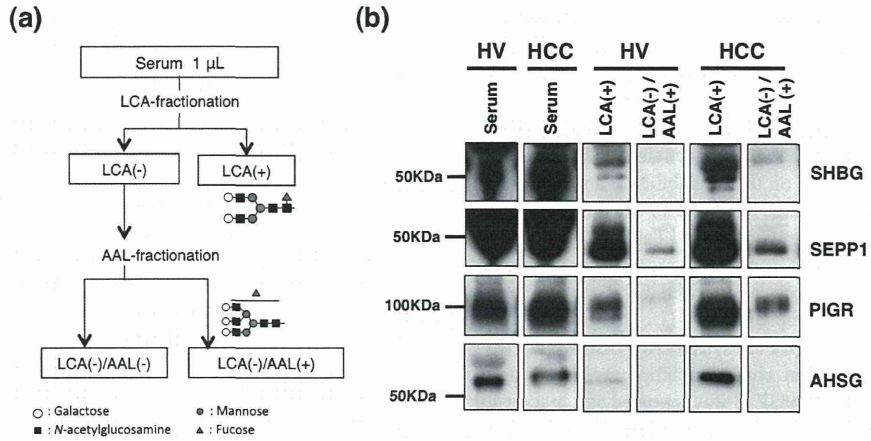

Figure 5. Detection of HCC-associated enhancement of fucosylation on the candidate glycoproteins by LCA/AAL-serial lectin chromatography followed by Western blotting. (a) Outline of the serial fractionation. Serum was fractionated with LCA-agarose. Glycoproteins in the LCA(+) fraction were presumed to have core-fucosylated biantennary glycans. Then, LCA(−) proteins were fractionated with AAL-agarose. Glycans of AAL(+) glycoproteins were presumed to be both fucosylated and branched. (b) Lectin-bound fractions were separated by SDS-PAGE, and then the candidate proteins in each fraction were detected by Western blotting.

cancers.[37] However, the glycan structure is also expressed by noncancerous cells such as endothelial cells of high endothelial venules (HEV) and lymphocytes associated with inflammation.[38] Similarly, although enhanced fucosylation is recognized as a glycan alteration related to HCC, fucosylation does not necessarily occur only in HCC, and fucosylated glycans are expressed ubiquitously. Therefore, it is important to detect glycan alteration on hepatic cell-specific proteins, especially when seeking a serum marker. In order to clarify glycan alteration associated with HCC, we first analyzed glycomes of the "culture media" of HCC cell lines (HepG2 and HuH-7) by lectin array as described previously.[13] Proteins in the culture media are undoubtedly secreted by HCC cells and carry glycans synthesized in the cancer cells. Thus, we used the culture media for the glycome analysis. In this sense, it is important to use a cell line that maintains *in vivo* characteristics of the cancer for the discovery procedure. In HCC, enhanced fucosylation and branching were reported in both cell lines and cancer tissues.[20,22] As expected, we found that the array signals of AAL and DSA in the media were very strong. Therefore, we selected the two lectins, AAL and DSA, to enrich glycoproteins carrying their cognate glycans, i.e., fucosylated and branched glycans, respectively. As exemplified by AFP-L3, which is AFP bearing α1,6-(core) fucosylated *N*-glycan, enhancement of fucosylation in HCC is a promising marker for cancer-associated glycan alterations.[39,40] Indeed, AFP-L3 is used as a U.S. Food and Drug Administration-approved HCC serum marker.

Neutral loss-triggered MS/MS (Electron Transfer Dissociation or Collision-Induced Dissociation) identification after partial deglycosylation with Endo F3 is a novel technology for assigning core fucose-bearing glycopeptides without employing lectin affinity. This method has been developed and used for marker discovery of HCC.[28,41] In general, however, glycoproteins having fucosylated *N*-glycans were enriched using fucose-recognizing lectins such as AAL, LCA, PSA, and VFA to capture not only the core fucose-bearing glycoproteins/peptides but also terminal α1,2/1,3-fucose-bearing molecules.[42]

Next, we identified AAL(+) and DSA(+) glycopeptides from the culture media of HCC cell lines to ensure they were actually produced by liver cancer cells. In total, 703 AAL(+) and 211 DSA(+) glycoproteins were identified from the media (Figure 2). These represent currently the largest data sets of human glycoproteins having a particular glycan motif (fucosylation or branch). It is well recognized that proteins obtained by affinity chromatography include those bound to the column nonspecifically or indirectly. For this reason, we captured glycopeptides, rather than glycoproteins, to minimize nonspecific identification. The IGOT method employed in this study also served to distinguish glycopeptides from non-*N*-glycosylated peptides with high precision.[15]

In addition, we also identified AAL(+) and DSA(+) glycopeptides from HCC sera. As the majority of serum glycoproteins are known to be derived from liver, serum samples are appropriate only for the marker discovery of hepatic diseases. We identified 703 and 122 AAL(+) glycoproteins from the culture media and the patients' sera, respectively. Roughly two-thirds of the AAL(+) glycoproteins from HCC sera (81/122) were also detected in the media, suggesting these glycoproteins were derived from liver cells. The remaining AAL(+) glycoproteins (41) were identified solely from the HCC sera, some of which are known to be primarily expressed in the liver. Thus, we selected 5 glycoproteins as candidates for further verification from the serum AAL(+) proteins.

Next, we compared the glycoproteome profiles as presented in Figure 2 to classify the candidates into three groups based on the following two factors. Due to the reasons outlined above, we confine our discussion to AAL(+) proteins only. The first factor is the degree of fucosylation under a physiological state. A preferable cancer marker is a glycoprotein having little or no fucosylated glycans in the healthy state. Thus, we divided the AAL(+) candidate proteins according to whether they were identified only in the HCC samples or in common with HV. Glycoproteins identified in common were classified into Group III (Figure 2). Another factor is the abundance of the core protein in serum. The RCA120(+) proteins identified from serum samples were thought to be highly abundant in serum. In addition, the number of RCA(+) peptides identified from each protein indicates the number of glycosylation sites on the target glycan. The more glycans the protein has, the easier the protein can be detected, for example, by sandwich ELISA using

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antibodies and lectins, because multivalent binding strengthens the affinity between glycans on the glycoprotein and lectin molecules. The AAL(+) proteins identified in HCC samples that were in common with RCA120(+) proteins were classified into Group I. The remaining proteins were designated into Group II as shown in Figure 2. From each group, we selected 21 candidates for further verification based on the availability of antibodies and their abundance in serum. These candidates were then verified by one or more of the subsequent methods (Table 1).

Among 7 candidates in Group I, 4 proteins were already known to have enhanced fucosylation in HCC by comparison to the healthy state.[25−28] Glycoproteins of Group I include AFP, GP73 (GOLM1), and GPC3. Fucosylated AFP, known as AFP-L3, is actually being used in clinical practice for diagnosis of HCC. Moreover, fucosylated GP73 was reported as a marker for HCC with higher sensitivity than AFP.[26,43] These results suggest our approach for identifying candidate markers has been successful. In this study, all selected candidates in Group I (including CPB2, CSF1R, LAMP2, and SHBG) were newly identified AAL(+) glycoproteins displaying enhanced levels of fucosylation (Table 1). Among them, we expected CSF1R and CPN2 to be promising candidate HCC markers. Specifically, in comparison with other candidates, CSF1R and CPN2 show lower fucosylation in HV and increased expression in the liver and have many potential *N*-glycosylation sites (7 in CPN2;[44] 9 in CSF1R[45]), which would facilitate detection by sandwich ELISA using their respective antibodies and AAL lectin.

Glycoproteins in Group III included many classical plasma proteins as represented by transferrin, hemopexin, alpha 2-macroglobulin, kininogen, ceruloplasmin, complement factors, and so on.[46] Among 11 selected candidates, all but ICOSLG were reported to show elevated fucosylation in HCC.[25] However, these proteins in Group III were suggested to have fucosylated glycans in healthy individuals. This also suggests that these Group III proteins might be biomarkers that reflect the status of the liver as a whole rather than a local change, i.e., carcinogenesis. Namely, if the ratio of fucosylation on a glycan of a candidate biomarker is 1% in the healthy state and enhanced to 100% in HCC, the amount of this protein having fucosylated glycan would increase only 2-fold at most in a patient with a small cancer, i.e., 1% by weight (1*100:1*99 + 100 = 1:2). Thus, as discussed above, these proteins may become surrogate fibrosis markers. These markers are also clinically desirable, because a quantitative index indicating the degree of fibrosis is important in identifying high-risk individuals, who should be referred for imaging and cytological diagnosis (biopsy), e.g., hepatitis patients infected by HBV or HCV. Moreover, such an approach will be useful for evaluating the benefit of treatment or medication in patients and for the development of new medicines for liver fibrosis.[47] In this sense, ORM1/2 (AGP), PIGR, SERPINA3, AZGP1, ICOSLG, PTGDS, and SEPP1 might be good markers for fibrosis. In fact, we found that glycan alteration on ORM1/2 correlated with progression of liver fibrosis, which can diagnose LC with an accuracy of over 90%.[5,48,49]

In Group II, the ratio of AAL(+) of 2 candidates, SPARCL1 and ICAM2, increased in HCC. Elevated expression of SPARCL1 is known to be associated with the progression of hepatic disease along with sinusoidal capillary transformation.[50] As the serum level of these candidates is low, improvement of detection sensitivity will be needed for practical use as biomarkers.

As demonstrated in this study, we could identify many glycoproteins carrying glycans with enhanced reactivity to AAL in HCC sera and hepatoma cell lines using our high-throughput strategy. Furthermore, this approach also enabled us to provide a large set of data on human glycoproteins having particular glycan motifs through our glycoprotein database, GlycoProtDB, http://jcggdb.jp/rcmg/gpdb/.

In this study, we also carried out a verification of glycan alteration between HCC patients and HV for many candidate proteins. The verification procedure involved three methods including the newly developed technique of serial lectin fractionation. Because we used sera of HCC patients having LC as samples for the discovery and verification, the glycan alteration is thought to be associated with HCC or LC. Therefore, for each marker candidate, it should be verified at which stage on the long-term pathological course toward HCC the enhancement of glycan alteration (fucosylation) occurs. Detailed comparison using sera of patients at various stages of the disease may allow us to identify stage-specific biomarkers. We have already verified that glycan alteration on ORM1/2 (AGP in Group III) can distinguish the fibrosis stage between F3 and F4 (LC) by immunopurification followed by a lectin array analysis using sera from over 100 patients.[32] Furthermore, subsequent validation studies of this marker using an automated bedside clinical chemistry analyzer in more than 1000 HBV/HCV-infected patients have demonstrated that the diagnostic accuracy for detecting LC is superior to that of other fibrosis indices or instruments.[5,48,49] Thus, we expect that a novel and clinically applicable glycobiomarker for early stage HCC can be selected using the stage-established samples in a future study. Furthermore, we believe that this approach is applicable to discover biomarkers not only for other cancers but also for fibrogenic diseases such as Alzheimer's disease and fibrosis of lung and kidney.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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